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VERSITA

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C. ELEGANS MODELS OF NEUROMUSCULAR DISEASES EXPEDITE TRANSLATIONAL RESEARCH

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Abstract

The nematode *Caenorhabditis elegans* is a genetic model organism and the only animal with a complete nervous system wiring diagram. With only 302 neurons and 95 striated muscle cells, a rich array of mutants with defective locomotion and the facility for individual targeted gene knockdown by RNA interference, it lends itself to the exploration of gene function at nerve muscle junctions. With approximately 60% of human disease genes having a *C. elegans* homologue, there is growing interest in the deployment of low-cost, high-throughput, drug screens of nematode transgenic and mutant strains mimicking aspects of the pathology of devastating human neuromuscular disorders. Here we explore the contributions already made by *C. elegans* to our understanding of muscular dystrophies (Duchenne and Becker), spinal muscular atrophy, amyotrophic lateral sclerosis, Friedreich's ataxia, inclusion body myositis and the prospects for contributions to other neuromuscular disorders. A bottleneck to low-cost, *in vivo*, large-scale chemical library screening for new candidate therapies has been rapid, automated, behavioural phenotyping. Recent progress in quantifying simple swimming (thrashing) movements is making such screening possible and is expediting the translation of drug candidates towards the clinic.

Keywords

Caenorhabditis elegans • Neuromuscular disease • Drug screening • Duchenne muscular dystrophy
Spinal muscular atrophy • Amyotrophic lateral sclerosis

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
Aβ	amyloid-β peptide
BMD	Becker muscular dystrophy
DMD	Duchenne muscular dystrophy
DPC/DAPC	dystrophin-associated protein complex
FALS	familial ALS
FRDA	Friedreich's ataxia
FTX	frataxin
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
IBM	inclusion body myositis
RNAi	RNA interference
RRM	RNA-recognition motif
SMA	spinal muscular atrophy
SMN	survival motor neuron
SOD	superoxide dismutase
TDP/TARDBP	TAR DNA-binding protein

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1. Introduction

With a combined frequency of at least 1 in 3000, human neuromuscular disorders such as Duchenne and Becker muscular dystrophies (DMD and BMD), spinal muscular atrophy (SMA), and amyotrophic lateral sclerosis (ALS) pose a significant physical and emotional strain on patients, their families and society [1]. They often present abruptly with dramatic and progressive decline in motor function and lead to premature mortality. Despite improvements in diagnosis resulting from the identification of genes underlying neuromuscular disorders, physical therapy with palliative care is often the only available treatment. Animal models each with their own strengths and limitations have been developed, which permit testing of potential therapeutics before the translation of a strong candidate into human clinical trials. Owing to the prohibitive costs and longer time-scale involved in using the vertebrate models, some researchers have turned to the relatively simple invertebrate *Caenorhabditis elegans* as a rapid, low-cost, first-pass filter in the search for drugs to treat neuromuscular diseases.

Introduced as a genetic model organism in the 1960s, *C. elegans* is a small (1.0-1.5 mm), hermaphroditic nematode worm with an optically transparent body allowing visualisation of internal structures and biological processes throughout development [2]. Its invariant lineage of 959 cells, including 302 neurons and 95 body wall muscles, has been mapped [3]. It remains the only animal with a complete nervous system wiring diagram [4]. The complete pattern of synaptic connections (≈5000 chemical synapses, 2000 neuromuscular junctions and 600 gap junctions) has been reconstructed [4], and it was the first organism with a nervous system to have its entire genome sequenced [5]. In addition, it is highly amenable to genetic manipulation. Unbiased forward genetic screens can be used to identify phenotype-

modifying mutations and reverse screening using RNA interference (RNAi) can reduce target gene function, allowing the development of disease models and their subsequent genetic dissection [6]. Pivotal to its use as a genetic model, conservative estimates suggest that approximately 60% of human genes posses a *C. elegans* homologue [7, 8, 9]. This high level of genetic conservation in combination with significant preservation of protein function, as demonstrated by transgenic rescue of worm mutants with human genes [10], suggests that many biochemical pathways are also preserved. Of particular significance for modeling neuromuscular disorders, basic neuronal cellular functions, major neurotransmitter systems (*i.e.* cholinergic, gamma-aminobutyric acid (GABA)-ergic, glutamatergic, serotonergic, and dopaminergic) and muscle structure, composition and function are all highly conserved. This is important if drugs are to translate successfully to humans. Indeed, several drugs used in the clinic to treat neuromuscular and neurodegenerative diseases have proven effective in phenotypic rescue of *C. elegans*, helping to validate the utility of worm models [11, 12, 13]. Despite this, a major caveat of using the worm is its thick cuticle, which acts as a barrier to bioactive molecules leading to differences in pharmacokinetics between humans and worms [14]. Nevertheless, the compound hit rate in *C. elegans* has been identified as similar to that of the vertebrate *Danio rerio* (zebrafish) [15], and approaches are being developed to improve screening effectiveness. A number of *C. elegans* mutants possess a cuticle that is more permeable to drugs, which can be crossed into strains of interest in order to enhance compound uptake [16, 17, 18]. Additionally, a recently developed predictive structure-based drug accumulation model can be used to pre-select compounds with optimal bioavailability in order to improve screening efficiency [19].

With its rapid life cycle (≈3 weeks), short generation time (≈3 days), considerable reproductive capacity (250-300 offspring), and economy of testing, *C. elegans* is an ideal organism with which to perform rapid, large-scale, *in vivo* chemical screens unfeasible in mammalian models (Figure 1). For instance, worms with reduced motility, a typical characteristic of neuromuscular disease, can be quickly dispensed in solution into microtitre plates containing chemical compounds and manually or automatically screened for amelioration of the swimming (thrashing) defect. Recent progress in automation of *C. elegans* behavior will likely facilitate the transition from medium- to high-throughput capacity by abolishing limitations on screening imposed by labor-intensive manual assays [20, 21, 22]. *In cellulo* screening commonly involves measuring the effects of compounds on disease gene transcript levels either directly or by using a phenotypic marker such as a fluorescence reporter. Unbiased, whole-organism behavioral screens using *C. elegans* possess two principal advantages over cell lines: (i) preliminary data on systemic toxicological profiles can be obtained; (ii) drugs targeting any disease-related pathway can be identified, *i.e.* not just those that result in the specific upregulation of disease gene transcription. This has the added benefit that novel disease pathways may also be established, which is particularly useful for the neuromuscular diseases as the mechanisms underlying pathophysiology are not always understood. *C. elegans* is emerging as a useful, low-cost option for triaging compounds from large libraries consisting of novel and pre-approved drugs, prior to validation in vertebrate models.

Complementary to its role in drug discovery, *C. elegans* can also be used to identify drug targets using chemistry-to-gene screening and to highlight genetic pathways pertinent to disease, two topics beyond the scope of this review and that have been recently covered elsewhere [23, 24]. Here, we will review *C. elegans* models of human neuromuscular diseases including DMD, SMA and ALS

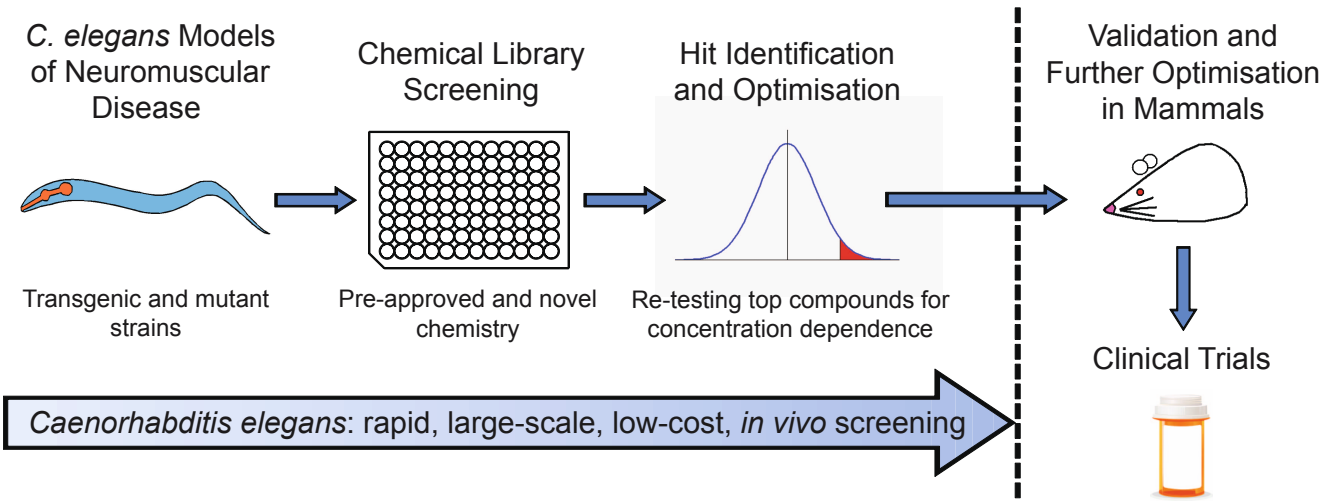

Figure 1. *C. elegans* models of neuromuscular disease allow rapid, low-cost, *in vivo* screening of large chemical libraries. Worm disease models can be used to screen libraries containing novel compounds and pre-approved drugs for their capacity to ameliorate phenotypic defects, such as reduced motility, prior to validation and optimisation in mammalian models.

(Table 1), highlighting their use and possible future application to identify and test potential human therapeutics.

2. Duchenne and Becker Muscular Dystrophies

Resulting from disruption of the dystrophin gene (*DMD*) and affecting approximately 1 in 3500 male births, Duchenne muscular dystrophy (DMD) and the milder Becker muscular dystrophy (BMD) are recessive, X-linked progressive myopathies that cause premature death in the late teens/early twenties and in the forties/fifties, respectively [1, 25, 26]. DMD usually manifests in early childhood with patients requiring the use of a wheelchair by age 12, which is the average age of onset for BMD. *DMD* encodes the 427-kDa cytoskeletal protein dystrophin and is the largest gene in the human genome, spanning approximately 2.5 Mbp encompassing 79 exons [27, 28, 29]. Typically, dystrophin is absent in DMD patients, but of abnormal size or quantity in BMD [30]. Predominantly expressed in skeletal and cardiac muscle, dystrophin is a constituent of a large, multi-protein complex called the dystrophin-associated protein complex (DPC or DAPC), which localises to the cytoplasmic side of the plasma membrane [31]. Proteins identified in the DPC include dystrobrevins, syntrophins, sarcoglycans and a dystroglycan [31]; however, the exact composition of the DPC can differ dependent on location, making assignment of a general function difficult [32]. Nevertheless, it is clear that dystrophin dysfunction causes destabilisation of the DPC, resulting in contraction-induced damage to muscle fibres and their subsequent degeneration.

The Ségalat group at the Unversité Lyon in France has been working for more than a decade on *C. elegans* models of muscular dystrophy. Over the years they have generated numerous mutant strains that have been used to elucidate properties of DPC-associated proteins and to screen for chemical compounds that may prove beneficial to DMD and BMD sufferers.

C. elegans possesses a single dystrophin-like gene called *dys-1*, which contains 46 exons over 31 kb and encodes a protein with conserved domain organisation and functional capacity [33]. Using constructs containing the *dys-1* promoter driving green fluorescent protein (GFP) expression, *dys-1* expression was seen solely in muscle cells (body wall, head, pharyngeal and vulval) from late embryonic stages into adulthood [33]. Five loss-of-function *dys-1* alleles isolated via EMS mutagenesis screening, which included deletion, substitution and frameshift mutants, were all shown to display hyperactive motility on plates [33]. They also exhibited an exaggerated sinusoidal bending of the head region when moving forward, coupled with hypercontraction when reversing. Despite the obvious behavioral phenotype, *dys-1* mutants showed no defects in muscle structure, which could perhaps be explained by the short lifespan of *C. elegans* [33]. Given its expression profile, it is not surprising that muscle- and not neuron-specific expression of *dys-1* could rescue the phenotype. The *dys-1* mutants were hypersensitive to aldicarb (acetylcholinesterase inhibitor) and acetylcholine as evidenced by paralysis assays and pharyngeal muscle electrophysiological recordings, respectively, suggesting that DYS-1 plays a role in cholinergic synaptic transmission [33]. In a later study, *dys-1* alleles were shown to have reduced acetylcholinesterase activity, which may serve to explain the locomotor dysfunction [34].

Interestingly, mutations in another *C. elegans* gene *dyb-1*, which encodes a dystrobrevin-like protein, result in similar phenotypes to *dys-1* worms including hyperactivity and acetylcholine hypersensitivity; although *dys-1;dyb-1* double mutants are no more severe than the respective single mutations [35, 36]. The dystrobrevin family of proteins show sequence homology with the C-terminus of dystrophin [37], co-localise with dystrophin at the sarcolemma [38], and interact with dystrophin [39]. *C. elegans* DYB-1 is 38% identical to human and mouse dystrobrevin *α*1 proteins, and retains the ability to bind to DYS-1 *in vitro*, suggesting potential conservation of function [35, 40]. DYB-1 is expressed from late embryogenesis to adulthood in most neurons and the same muscles as DYS-1, with additional weaker expression in intestinal muscle [36].

Table 1. *C. elegans* models of human neuromuscular disorders.

Human Disease	Human Gene	*C. elegans* Gene	Notes	Refs
Amyotrophic Lateral Sclerosis	*SOD1*	*sod-1*	-Transgenic expression of mutant forms of human *SOD1* causes various defects including reduced locomotion.	[88-91]
	TARDBP (TDP43)	*tdp-1*	-Transgenic expression of wild type human TDP43 in neurons results in toxicity, and an uncoordinated phenotype.	[104]
Duchenne Muscular Dystrophy	*DMD* (dystrophin)	*dys-1*	-Mutants display hyperactivity and hypercontraction of muscles. -*dys-1* mutants with a mild *MyoD* mutation (*hlh-1*) show progressive muscle degeneration leading to paralysis.	[33, 34, 43]
Friedreich's Ataxia	*FXN*	*frh-1*	-Knocking down *frh-1* using RNAi results in numerous phenotypes including dose- and time-dependent effects on lifespan.	[119, 122, 124, 125]
Inclusion Body Myositis	*APP*	*apl-1*	-Constitutive and inducible transgenic expression of Aβ protein in wild type muscle results in paralysis and intracellular amyloid deposits.	[143, 144, 147]
Spinal Muscular Atrophy	*SMN*	*smn-1*	-Genetically balanced null mutants show neuromuscular defects leading to larval lethality.	[68, 70, 72]

mdx mice in which the dystrophin homologue is mutated display relatively mild muscle wasting [41]; however when the muscle-specific transcription factor MyoD is also dysfunctional, myopathy is much more severe [42]. In an attempt to generate a *dys-1* worm in which muscle degeneration could be observed similar to the *mdx:MyoD* mice, Ségalat and colleagues crossed a null *dys-1* allele with a mild *MyoD* mutant (*hlh-1*) [43]. Reduced HLH-1 expression had no observable effect on locomotion in 95% of mutants; the remainder failed to elongate properly displaying a short, dumpy phenotype. The *dys-1;hlh-1* double mutant, however, displayed a progressive impairment of motility manifesting at the late larval/early adult stage. Correlating with this, a severe disorganisation of the body wall muscles was seen with approximately 50% of muscle cells having an abnormal or absent F-actin pattern. *dyb-1;hlh-1* were also generated and shown to phenocopy the *dys-1* double, with less severely affected musculature [36].

In order to assess whether a complex analogous to the DPC is present in *C. elegans*, significantly conserved homologues of dystroglycan, δ/γ-sarcoglycan and syntrophin were targeted for knockdown via RNAi [44]. Reduced function of all three genes in wild type worms resulted in similar defects to those seen in the *dys-1* and *dyb-1* mutants [44]. Similar to the *dys-1;dyb-1* double mutant, knockdown of the DPC homologues in the *dys-1* background had no obvious effect on phenotype. Reduced function of all three homologues in a *MyoD* mutant background exacerbated muscle structure defects in adults. A *β*1-syntrophin knockout mutant (*stn-1*) was subsequently used to confirm the RNAi results [45]. This work suggests that a dystrophin-associated complex may indeed be present in the worm, which serves to further validating the model for studying the function of dystrophin. *snf-6* (acetylcholine transporter), *slo-1* (potassium channel), *ctn-1* (cytoskeletal protein) and *dyc-1* (homologous to a rat nitric oxide synthase-binding protein, CAPON) mutants also phenocopy the *dys-1* allele, suggesting that their protein products may interact with or play a similar biological role as DYS-1 [43, 46-49].

Since overexpression or down regulation of multiple genes has previously been shown to ameliorate the muscle degeneration seen in the *dys-1;hlh-1* worm [43, 50, 51, 52], it is possible that a chemical could have similar effects. A small-scale screen of 100 compounds was subsequently performed for drugs that counteract the absence of DYS-1 [11]. The drug with the greatest effect was prednisone, which is a corticosteroid currently clinically prescribed for DMD in some countries due to its ability to delay disease progression [53]. Prednisone did not ameliorate the locomotor defect, but did reduce cell degeneration in *dys-1;hlh-1* worms at multiple concentrations, which was shown to be specific to the *dys-1* mutation [11]. This first pilot screen served as a proof-of-principle study suggesting that drugs used to treat patients with dystrophin-associated disease can be highlighted in a *C. elegans* model. A second slightly larger screen of bioactive molecules highlighted serotonin as an even more potent blocker of *dys-1*-associated muscle degeneration than prednisone and serotonin also partially rescued motility [54]. Similarly, serotonin re-uptake blockers, which can cause synaptic serotonin buildup, also significantly reduced degeneration, albeit to a lesser extent that serotonin itself. In an extension of the previous small-scale screens, the speed and efficiency of *C. elegans* screening was coupled with validation experiments in the more physiologically relevant *mdx* mouse model [55]. Approximately 1000 compounds were tested in the worm, identifying methazolamide and dichlorphenamide, sulfonamides known to inhibit carbonic anhydrases, which are involved in numerous homeostatic and respiratory processes [56]. Both drugs improved *C. elegans* motility and reduced muscle wasting, by acting on the carbonic anhydrase CAH-4. When tested in the dystrophin mouse model, both compounds displayed a modest ability to improve the dystrophic phenotype, including increasing tetanic muscle force.

The work using dystrophin-deficient worms to highlight drugs with the capacity to ameliorate muscle degeneration shows the utility of *C. elegans* for screening and identifying drug candidates with potential for treatment of neuromuscular disorders.

3. Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by reduced function of the widely and constitutively expressed survival motor neuron (SMN) protein [57]. Affecting 1 in 6-10,000 and with a carrier frequency of approximately 1 in 40, SMA is one of the leading genetic causes of infant mortality [1, 58, 59]. Loss of anterior horn lower motor neurons, the classic hallmark of SMA, leads to proximal muscle weakness, with subsequent paralysis and premature death in more severe cases. Two almost identical human genes encode SMN; *SMN1* (telomeric *SMN*) predominantly produces a full-length functional protein, whereas a critical mutation in *SMN2* (centromeric *SMN*) causes aberrant splicing of the majority of transcripts, leading to the production of a truncated and rapidly degraded protein [57]. SMN plays a role in pre-mRNA splicing [60], axonal transport [61, 62], transcription [63], and small nucleolar ribonucleoprotein biogenesis [64], however its function in small nuclear ribonucleoprotein assembly is the best characterised [65, 66].

C. elegans possesses a single gene (*smn-1*) of five exons and four small introns homologous to human SMN, which was first identified due to high sequence similarity with N- and C-terminal regions of human and fission yeast versions of the gene [67]. SMN-1 is a 207 amino acid protein that is 36% identical to its human counterpart and it retains the *in vitro* capacity to bind to RNA and SMI-1 (Gemin2 homologue) [68, 69, 70]. SMN-1 has also been shown to interact with worm fibrillarin [68], a component of small nucleolar ribonucleoproteins involved in processing pre-mRNAs [64], and self-oligomerise via its C-terminal region [68], defects in which correlate with disease severity in humans [71]. The conservation of gene/protein sequence and binding capabilities suggest that *C. elegans* SMN-1 plays a similar role to human SMN. Analogous to the situation seen in humans, *smn-1* transcripts can be detected at all stages of development [68]. Using a *smn-1::GFP* fusion construct, SMN-1 was shown to be expressed in most adult cell nuclei including those of neurons and body wall muscles, and in the cytoplasm of neuronal

processes and excretory cells. Depletion of SMN-1 by dsRNA injection ultimately results in embryonic lethality, similar to the case in humans, with a few surviving progeny displaying lack of muscle tone, paralysis and sterility. Intriguingly, SMN-1 overexpression in all adult tissues had a similar effect.

The *smn-1(ok355)* allele provided the first *C. elegans* model for SMA. Here a deletion removing the vast majority of the gene leaving just 87 bp at the 3' end accounts for the phenotype [72]. The mutant displays pleiotropic defects that resemble those seen when *smn-1* is targeted for knockdown by RNAi. Since the complete absence of SMN-1 is homozygous lethal, the null allele must be maintained by a balancer chromosome that also expresses GFP permitting a simple distinction between genotypes (homozygosity for the balancer is lethal). Loading of SMN-1 protein and mRNA into oocytes by heterozygous parent worms permits development of the homozygous *smn*-1-deleted progeny to reach early larval stages [68, 72]. However, when the maternal contribution becomes depleted, the *smn-1* homozygotes become discernible from wild type larvae, displaying reduced size and length with a rapidly progressive decline in motor function (motility and pharyngeal pumping) followed by larval arrest. Interestingly, the mutant does not display a reduction in the number of cholinergic motor neurons [72]. *smn-1(ok355)* was also used to show that pan-neuronal SMN-1 expression can partially rescue the phenotype of the mutant, whereas muscle-directed expression has little effect; similar to the situation seen in mice [73].

To date no drug screening has been performed in this *C. elegans* model of SMA reflecting technical difficulties associated with partition of *smn-1* homozygotes. However, developments in large-scale phenotype selection and high-throughput automation of phenotyping may aid in this endeavour. The Complex Object Parametric Analyzer and Sorter (COPAS) called the 'BioSorter', which can rapidly sort up to 100,000 worms per hour based on size, optical density and fluorescence, can be used to separate *smn-1* mutant worms, based on their lack of GFP, for subsequent screening [20]. Alternatively, the BioSorter can also be used to ascertain data on the length and genotype of *smn-1(ok355)* animals. Very recently, this method was successfully used to screen for genetic modifiers of the SMA model in a genome-wide RNAi screen [6]. In a similar manner, this approach could be adapted to screen compound libraries for drugs that ameliorate *smn-1* loss-of-function defects. However, since *smn-1(ok355)* is a severe mutant, the neuromuscular defects manifest suddenly and rapidly progress, thus making screening of the locomotor phenotype difficult. A less severe and fertile mutant with a robust defect in swimming rate would greatly facilitate drug screening for compounds that improve motor function. We have therefore begun to explore a novel *smn-1* point mutation as a possible alternative for drug screening.

4. Amyotrophic Lateral Sclerosis

4.1 SOD1

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease in the UK, is the most common late-onset motor neuron disease with a lifetime risk of approximately 1 in 470 for women and 1 in 350 for men when adjusted for competing causes of mortality [74]. The disease affects both the upper and lower motor neurons causing muscles to weaken and atrophy, with death occurring between 1 to 5 years after symptom onset. The vast majority (90-95%) of ALS cases is sporadic; however, a small percentage is familial in origin, exhibiting an autosomal dominant mode of inheritance [75, 76]. The most common source of familial ALS (FALS) has been identified as a disruption of the copper/zinc superoxide dismutase (*SOD1*) gene, causing approximately 20% of cases [77, 78]. As a result, the majority of ALS research has focused on the role of SOD1 mutations in disease pathogenesis. A widely expressed homodimeric, cytosolic enzyme, SOD1 catalyses the conversion of unstable superoxide radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2), providing an important antioxidant defense [79, 80]. Since overexpression of mutant human SOD1 in mice causes a motor neuronopathy similar to ALS [81], whereas knockout of endogenous murine *SOD1* has no such effect [82], it is hypothesised that SOD1 mutations resulting in FALS are due to a toxic gain of function; however the exact mechanism(s) remain elusive.

The *C. elegans* homologue of SOD-1 (*sod*-1) is 56% identical to its human counterpart [83], is widely expressed in most adult tissues [84], and its activity in long-lived mutant strains is upregulated [83, 85]. Major *sod-1* deletion has no effect on average lifespan under *ad libitum* and dietary restricted conditions at 25°C; however, at 16°C mutants display significantly reduced longevity compared to wild type [86]. Concordant with this trend, a study in the same year reported that the two *sod-1* deletion alleles used by Yen *et al.* have a significantly reduced lifespan when assayed at 20°C [84]. This is recapitulated when *sod-1* is knocked down using RNAi [87]. *sod-1* deletion mutants display increased sensitivity to paraquat (confirmed by RNAi [87]), which is enhanced at lower temperatures [86]. Targeting *sod-1* using RNAi has no effect on post-embryonic rate of development or levels of embryonic lethality, although it does result in diminished fertility and elevated levels of oxidative damage [87].

Since deletion of *sod-1* has relatively little effect on the worm and the resulting phenotypes do not appear to mimic neuromuscular aspects of ALS, various research groups have tried expressing in *C. elegans* mutant forms of human SOD1 found in FALS patients. The first model was generated by driving expression of wild type or three different forms of FALS-associated human *SOD1* (A4V, G37R and G93A) using a muscle-specific (*myo-3*) or a universal heat-shock inducible (*hsp-16.2*) promoter [88]. Under standard conditions, there was no detectable altered phenotype in worms expressing mutant SOD1 under the general promoter; however, they became more sensitive to paraquat-induced oxidative stress, similar to *sod-1* deletion and RNAi knockdown studies [86, 87]. Wild type SOD1 expression had no effect on paraquat sensitivity. Pan-muscular expression of A4V SOD1 resulted in discrete aggregates, which contained the mutant protein in body wall muscles in the presence of paraquat.

A second transgenic model was generated expressing dimeric forms of wild type and misfolding mutant (G85R) human SOD1 behind a pan-neuronal promoter (*sng-1*) [89].

L4 larvae expressing mutant G85R SOD1 homodimers displayed a marginal reduction in locomotion, while lifespan in the presence of paraquat was no different to control. Contrastingly, mutant heterodimer-expressing (with wild type SOD1) worms showed reduced cellular aggregation, a highly significant decrease in motility and a shortened lifespan when exposed to paraquat.

Another well-characterised transgenic ALS model expressing human G85R SOD1 driven by the *snb-1* pan-neuronal promoter, displayed severe locomotor defects roughly correlating with cytosolic neuronal SOD1 protein aggregation, visualised using SOD1-YFP fusions [90]. These worms showed a reduction in the number of ventral nerve cord processes, which were reduced in diameter with diminished numbers of mitochondria and vesicles. Furthermore, pre-synaptic vesicle density was reduced and mutants were resistant to aldicarb, suggestive of a pre-synaptic defect. In contrast, body wall muscles appeared relatively spared and mutants were equally susceptible to the nicotinic acetylcholine receptor agonist levamisole, indicating that the post-synapse was less affected.

The final transgenic SOD1 *C. elegans* model of ALS expresses mutant SOD1-YFP fusion proteins (G85R, G93A and 127X) in body wall muscles using the *unc-54* promoter [91]. The mutant SOD1 proteins all formed morphologically heterogeneous aggregates in muscles throughout development, consistent with the neuronal aggregation seen by Wang *et al.* [90]. Mutant SOD1 expression caused little toxic effect on muscle cells; however, phenotypic defects and cellular toxicity were exacerbated in various unrelated mutants with mildly destabilised polymorphic proteins (*i.e.* temperature-sensitive alleles), which have previously been shown to hasten polyglutamine aggregation [92]. This phenomenon seen in two models of disease involving protein misfolding and aggregation perhaps suggests that the toxicity threshold of a protein prone to aggregate is dependent on the availability of the protein folding machinery [91].

This toolkit of *C. elegans* strains stably expressing FALS-associated forms of SOD1 under a range of promoters provides a useful platform with which to screen compounds for amelioration of the phenotypes. Lifespan and paraquat-sensitivity are technically difficult to assay on a large-scale. Nevertheless, the motility defects displayed by a number of the models could easily be screened for improvement [22]. The protein aggregation seen in worms expressing mutant SOD1-YFP in neurons or muscles could also be screened for compounds affecting fluorescence pattern and intensity [90, 91]. An RNAi-sensitive strain expressing G85R SOD1 fused to YFP has previously been used in this manner to screen a whole-genome RNAi library, serving as a proof-of-concept for compound screening [90].

4.2 TDP-43

Recently the highly conserved 43-kDa TAR DNA-binding protein (TDP-43) was identified as a major component of nuclear and cytoplasmic inclusions in both spinal cord neurons and glial cells of patients with ALS [93, 94]. Interestingly, these inclusions are found in most ALS patients, with the remarkable exception of patients with SOD1-associated FALS [95]. Encoded by *TARDBP* (TAR DNA-binding protein), TDP-43 is a widely expressed RNA/DNA-binding protein with two RNA-recognition motifs (RRM1 and RRM2) and a glycine-rich C-terminus that likely mediates protein interactions. Significantly, the vast majority of identified *TARDBP* mutations reside in this carboxy terminal region [96]. TDP-43 predominantly localises to the nucleus, translocating to cytoplasmic inclusions under pathophysiological conditions [94], and has been implicated in a number of processes including repression of gene transcription [97], exon splicing [98, 99], and mRNA stabilisation [100]. Over 30 different mutations in TDP-43 have been identified in ALS patients [101], which in combination with the cytoplasmic aggregation seen in the majority of sufferers suggests a causal role in the disease. It remains unclear whether TDP-43 dysfunction results in motor neuron loss through protein overexpression, loss-of-function or toxic gain-of-function.

The *C. elegans* homologue of *TARDBP* (*tdp-1*) retains specific RNA and DNA binding capacity and displays high homology to RRM1 and RRM2; however the C-terminal glycine-rich region is absent [102, 103]. TDP-1 also retains the nuclear localisation signal and caspase cleavage sites of human TDP-43 [104], and has been shown to affect alternative splicing [104]. Transcript levels are at their highest in oocytes and during early larval stages [105]. Deletion of endogenous *tdp-1* has no observable effect on movement or GABAergic synapse formation, whereas *tdp-1* overexpression causes an uncoordinated phenotype with reduced thrashing rate, suggesting that TDP-43 neurotoxicity is a result of an overabundance of wild type human protein [104].

A *C. elegans* strain expressing human TDP-43 using the pan-neuronal *snb-1* promoter has now been developed [104]. These worms display an apoptotis- and caspase-independent uncoordinated phenotype that manifests at larval stages and persists until death. The defect was characterised by slow movement on plates, reduced swimming rate and disruption of wild type sinusoidal movement. Transgenic worms also responded inappropriately to mechanical stimulation, displayed a coiled body posture, and possessed ubiquitin-free TDP-43 inclusions in the nuclei of neurons. The number and distribution of GABAergic synapses were significantly affected, with occasional defasciculation of axons, although GABAergic motor neuron loss was not seen. Effects on cholinergic motor neuron number were not reported. Endogenous *tdp-1* deletion does not abrogate the uncoordination induced by human TDP-43 expression, suggesting that TDP-43 toxicity is independent of an interaction with TDP-1 [104]. Selective deletion of RRM1, RRM2 or the C-terminus of TDP-43 had no effect on worm coordination, but did alter its subnuclear distribution. Expression of the C-terminal TDP-43 fragment alone resulted in large cytoplasmic aggregates. Fusion of the TDP-1 C-terminal region to C-terminus-deleted human TDP-43 restored wild type nuclear distribution in both *C. elegans* and HeLa cells, restored protein exon splicing ability and impaired locomotion. Targeting TDP-43 to the cytoplasm by specific mutation of the nuclear localisation signal did not cause uncoordination despite similar levels of

protein expression, suggesting that nuclear TDP-43 is toxic to the worm.

This first *C. elegans* model used to study TDP-43 aggregation and toxicity, displays an uncoordinated phenotype with reduced motility, making it a possible candidate for drug screening similar to the SOD1 models.

5. Friedreich's Ataxia

Friedreich's Ataxia (FRDA), the most prevalent recessive ataxia, is predominantly caused by a large GAA-trinucleotide expansion in the first exon of the frataxin (*FTX*) gene, resulting in reduced frataxin protein levels due to transcription inhibition [106-110]. The remaining patients show compound heterozygosity for the triplet expansion and single base pair changes in DNA encoding the *FTX* protein [111, 112]. Characterised by loss of large sensory neurons in dorsal root ganglia and spinocerebellar tracts, FRDA symptoms include a progressive gait and limb ataxia, dysarthria, lower limb areflexia, cardiomyopathy and premature death at an average age of 38 [106, 113]. Frataxin is expressed in the mitochondrial matrix and is involved in cellular iron metabolism [114, 115, 116]; however its precise role in iron homeostasis is unclear [113, 117]. Complete absence of frataxin is embryonic lethal in mice [118], and homozygous deletion of *FTX* has not been seen in humans, suggesting that frataxin is essential for survival [119].

C. elegans possess a single *FTX* homologue called *frh-1* [119], which is a constituent of a large eight gene operon with heterogeneous function [120, 121]. *frh-1* is expressed in sensory neurons in the head, pharyngeal muscles, intestine, body wall muscles and spermatheca, and has a complex regulatory pattern with numerous promoters [119, 121]. Pharyngeal *frh-1* expression co-localises with a mitochondrial marker [119].

Early studies showed that using RNAi to knock down *frh*-1 caused extension of lifespan by approximately 25% [122]. Worms in which *frh-1*was targeted by RNAi were small and pale yet fertile, albeit with an egg-laying defect and reduced total brood size. Interestingly, the fertility window was extended; at 20°C animals with reduced *frh-1* expression were capable of laying eggs for up to 17 days compared to 8 days for wild type. Furthermore, experimental worms were resistant to hydrogen peroxide but hypersensitive to the superoxide generator juglone (similar to paraquat). These results were mostly corroborated by an independent group, which showed that *frh-1* deficiency caused additional defects of slowed defecation rate and reduced, arrhythmic pharyngeal pumping in the presence of food, perhaps explaining the slow growth and pale appearance [119]. However, in stark contrast to previous findings [122, 123], this group reported a decrease in longevity under similar conditions [119]. A third group, also reported a decrease in lifespan, which correlated with respiration rate [124]. Caution should be exercised in the interpretation of the apparent conflict in results; in a subsequent study, incremental RNAi dilution used to knockdown genes encoding mitochondrial proteins, including *frh-1*, resulted in dose-dependent effects on lifespan when administered before adulthood [125]. Severe reduction in gene expression levels caused diminished lifespan, while intermediate knockdown led to increased longevity. The first group injected 5 day old adults, whereas the second and third groups injected L4 larvae and used RNAi by feeding from hatching, respectively; it is therefore likely that differences in experimental procedure explain the diversity of findings reported in lifespan experiments.

A knock out *frh-1* mutant is available, *frh-1(ok610)*, which must be maintained by a balancer chromosome due to homozygous lethality [122], similar to the null mutant used to model SMA, *smn-1(ok355)* [72]. *frh-1* mutants arrest in early larval stages corroborating evidence from the RNAi experiments that when *frh-1* expression is severely reduced, lifespan is diminished. Discrepancies in the extent of gene knockdown in *frh-1(RNAi)* worms could hinder drug identification in this FRDA model. Nevertheless, two cofactors called flavin adenine dinucleotide and flavin mononucleotide derived from riboflavin, which has shown capacity to treat some other mitochondrial disorders, are able to improve the *frh-1(RNAi)* phenotype [126]. Both compounds ameliorated the overall phenotype of the *frh-1* deficient worms; lifespan, fertility and defecation rate were also significantly improved. Riboflavin did not improve the growth-retarded phenotype but did extend lifespan. In spite of being able to use the knock down model to highlight drugs with potential for FRDA treatment, manually screening compounds on a large scale in a similar manner would be demanding and time-consuming. As an alternative, the smaller size and retarded development of the null mutant could be used in a comparable way to the SMA worm model for drug screening using the COPAS BioSorter. Unfortunately, the *frh-1(ok610)* deletion also removes part of *cgt-3*, the upstream gene in the operon [119]. The defects associated with this deletion have not yet been shown to be specific to the removal of *frh-1*, which has hindered the further characterisation of this mutant [119].

6. Inclusion Body Myositis

Leading to progressive muscle weakness that descends from proximal to distal limb regions and later affecting respiratory muscles, sporadic inclusion body myositis (IBM) is the most common myopathy in the over 50 age group [127], but also occurs in younger people [128]. An autosomal dominant, familial form of IBM also exists but is rare [129]. The underlying cause of sporadic IBM is unknown and many fundamental questions remain about disease pathogenesis. Patients present with skeletal muscle inflammation [130], yet surprisingly antinflammatory/ immunosuppressive drugs show little or no ability to slow disease progression [131, 132, 133]. Numerous proteins have been identified as relevant to IBM, many of which have also been implicated in Alzheimer's disease (AD) [134]. Amyloidosis is thought to play a key pathogenic role in IBM; the amyloid precursor protein (APP) is proteolytically cleaved forming amyloid-β peptide (Aβ) [135], which form amyloid aggregates in patient muscle cells and may contribute to degeneration of muscle [136]. Several forms of Aβ exist, yet $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ appear to be the most relevant to IBM, with $A\beta_{1\text{-}42}$ being the more cytotoxic and prevalent [137]. The *APP* gene encodes a type I transmembrane glycoprotein, which contains

the Aβ sequence within the extracellular and membrane-spanning domains [138]. APP has been implicated in a number of processes, including synpatogenesis, cellular adhesion and axon growth [139].

C. elegans possesses a single *APP*-like homologue called *apl-1*, which is expressed in various adult cells including several neurons and muscles [140, 141]. Deletion of *apl-1* resulted in molting and morphogenesis defects followed by larval lethality, whereas overexpression caused defects in brood size, motility and viability [141]. APL-1 lacks the internal Aβ sequence of human APP [140], so transgenic strains expressing human Aβ in body wall muscle have been developed to model both IBM and AD. Detailed reviews discussing the use of *C. elegans* to model IBM have recently been published [24, 134, 142], so we will only very briefly summarise the literature on Aβ models.

There are several transgenic *C. elegans* strains expressing the human $A\beta_{1-42}$ peptide in either muscles or neurons [143-145], which likely accumulates as truncated $A\beta_{3-42}$ in all models [142, 146]. Expression of the human $A\beta_{1-42}$ protein in *C. elegans* using the muscle-specific *unc-54* promoter causes progressive paralysis and intracellular accumulation of deposits with Aβ immunoreactivity in muscle cells [143]. These deposits co-localise with amyloid-specific dyes, and display a fibrillar ultrastructure [147, 148]. This model (strain CL2006) was used to show that specific point mutations in Aβ can affect the formation of the amyloid deposits [147, 149], that Aβ interacts with and induces the expression of the HSP-16 family of heat-shock proteins [150, 151], and that *daf-2(RNAi)* and dietary restriction can both reduce Aβ toxicity [152, 153].

A second, temperature-inducible allele (Strain CL4176) expressing human $A\beta_{1-42}$ driven by the muscle-specific *myo-3* promoter was used to identify genes differentially expressed in the transgenic model, highlighting AIP-1 (arsenite-inducible protein) as a moderator of Aβ toxicity [144, 154]. In addition to affecting gene expression, Aβ production in this strain has been shown to increase oxidative stress, which precedes Aβ deposition [155]. Worms with the transgene are essentially wild type until the temperature is upshifted inducing Aβ production, which leads to a rapid paralysis within about 24 h. This strain was used to show that HSP16.2 overexpression slows the rate of paralysis without affecting the amount of Aβ produced, perhaps suggesting that chaperone proteins are able to reduce the toxicity of Aβ [156]. In a follow up study, heat shock treatment of the worms was revealed to cause upregulation of HSP16.2 expression, a reduction in the rate of paralysis, and a decline in Aβ oligomerisation [157]. CL4176 was also used to demonstrate that the production of Aβ leads to autophagosome accumulation and that impairment of insulin signalling can partially relieve this, slowing the rate of paralysis [158].

Expression of Aβ in the neurons (Strain CL2355) causes intracellular peptide accumulation, serotonin hypersensitivity and learning deficiency [145, 159]. The strains expressing Aβ in muscle (CL2006 and CL4176) are perhaps a better model of IBM due to the intramuscular Aβ accumulation seen in IBM patients [142], whereas the neuronal strain (CL2355) better mimics AD. Nevertheless, due to parallels between the two diseases, it is likely that insights into IBM and AD can be made using all of the *C. elegans* models.

The Aβ-induced progressive paralysis seen in the muscle-specific strains provides a valuable phenotype with which to identify compounds that can ameliorate the defect. Indeed, *Ginkgo biloba* leaf extract EGb 761 and one of its components, ginkgolide A, have been shown to do just that [145]. Significantly, EGb 761 has shown promise in the treatment of dementia, serving as a proof-of-principle that drugs with efficacy against diseases associated with Aβ deposition can be identified in the worm [160, 161, 162]. Reserpine, an antihypertensive drug, shows a similar ability to delay worm paralysis and increase lifespan [163]. Manual screening could thus be performed on a small library of compounds in order to identify further potential therapeutics. This assay could feasibly be automated, which would greatly improve its potential for drug discovery.

7. Conclusions and Future Perspectives

C. elegans possesses a great number of advantages for tackling significant biological challenges, which has resulted in it becoming one of the major animal models in modern science. Virtues of speed, economy and genetic tractability, coupled with a surprisingly high degree of biochemical conservation, have led to many researchers now including the worm in their experimental arsenal. *C. elegans* mutants mimicking particular aspects of incurable neuromuscular disorders can be used to answer fundamental questions about disease etiology and pathogenesis. In addition, these models can be used to screen compound libraries for drugs with potential to treat these devastating diseases, before further experimentation in mammalian models. An improvement on less physiologically relevant *in cellulo* screening, drug discovery in *C. elegans* nicely complements that of zebrafish embryos. Recent advances in automated phenotyping will likely be pivotal to the implementation of available and future models in large-scale chemical compound screens. The high level of genetic conservation seen in *C. elegans* will allow the development of new models of human neuromuscular disease that could lend themselves to the identification of potential drugs. Congenital myasthenic syndrome (fast and slow channel forms), congenital myotonia, and Machado-Joseph disease are just a few neuromuscular disorders that have potential to be modeled in *C. elegans* [164, 165, 166]. Due to evolutionary divergence between worm and human sequences, chemical optimisation is very likely required to improve drug pharmacokinetics in humans. Nevertheless, the utility of the worm in potential drug identification is evident. In this review, we have illustrated progress to date on several *C. elegans* models of neuromuscular disorders and highlighted how they have been and can be used for expediting translational drug research.

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