Biochimica et Biophysica Acta 1852 (2015) 685–692

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Molecular mechanisms and animal models of spinal muscular atrophy ☆

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ARTICLE INFO

Article history:
Received 1 May 2014
Received in revised form 21 July 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
SMA
SMN
Animal disease models
C. elegans
Drosophila
Zebrafish

ABSTRACT

Spinal muscular atrophy (SMA), the leading genetic cause of infant mortality, is characterized by the degeneration of spinal motor neurons and muscle atrophy. Although the genetic cause of SMA has been mapped to the *Survival Motor Neuron1* (*SMN1*) gene, mechanisms underlying selective motor neuron degeneration in SMA remain largely unknown. Here we review the latest developments and our current understanding of the molecular mechanisms underlying SMA pathogenesis, focusing on the animal model systems that have been developed, as well as new diagnostic and treatment strategies that have been identified using these model systems. This article is part of a special issue entitled: Neuromuscular Diseases: Pathology and Molecular Pathogenesis.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disorder characterized by the loss of spinal motor neurons, which leads to muscle atrophy, paralysis, and ultimately death [12,54,68]. As the primary genetic cause of infant mortality and the second most common autosomal recessive genetic disorder, SMA affects one in every six thousand live births with a carrier frequency of one in forty people [72,74,84]. More than 98% of patients with SMA have a homozygous disruption of the *Survival Motor Neuron1* (*SMN1*) gene on chromosome 5q13 by deletion, rearrangement, or mutation [35,49]. In addition to the telomeric *SMN1*, humans also contain a centromeric *SMN2* produced by intrachromosomal duplication. *SMN2* differs from *SMN1* by only five nucleotides [13,49]. One silent single nucleotide change within exon 7 of *SMN2* disrupts its splicing [16,17,44]. Thus, *SMN2* produces only a small amount of full-length functional protein and mostly an unstable truncated isoform of SMN lacking exon 7 (SMNΔ7). Therefore, mutations of *SMN1* lead to reduced but not depleted levels of full-length SMN protein in SMA, which are sufficient to sustain the survival of most cell types with the exception of spinal motor neurons.

The expression level of SMN protein is inversely correlated with SMA disease severity. The pathological symptoms are highly variable. Patients can be classified into four categories (Table 1) according to the age of onset and maximum motor function achieved [71]. SMA type I, or Werdning–Hoffman disease, is the most common and severe type. Onset is usually before 6 months of age, and death occurs within the first 2 years of life. These infants have profound flaccid symmetrical weakness and hypotonia, and are unable to sit without support. Bulbar denervation results in tongue weakness and fasciculation with poor suckling and swallowing. SMA type II is of intermediate severity and characterized by onset of disease between 7 and 18 months of age. Patients are capable of sitting independently but do not achieve the ability to walk. Patients with SMA type III (Kugelberg–Welander disease) have onset of symptoms after 18 months and are able to achieve independent walking. Adult onset, or SMA type IV, starts around the second or third decade of life and is characterized by mild weakness without respiratory or nutritional problems [71]. Although the majority of patients (95%) have homozygous deletion of *SMN1* exon 7 or both exon 7 and 8, about 3–4% of patients are compound heterozygotes for the deletion of *SMN1* on one allele and a point mutation on the other. De novo mutations occur at a rate of 2% because of regional instability on chromosome 5 [56]. An updated list of *SMN1* gene mutations is available on Leiden Open Variation Database (http://www.dmd.nl/nmdb2/home.php?select_db=SMN). Mutations include nonsense, frame-shift, missense, deletions, inversions and splicing site changes. The Y272C and 813ins/dup11 mutations are reported to be the most common [96].

The main pathological feature of SMA is neuronal loss in the anterior horn of the spinal cord with chromatolysis, neurophagia and gliosis [3,46]. Neuromuscular junction ultrastructural abnormalities were also noted in humans with SMA [43]. Recently, neuromuscular junction

☆ This article is part of a special issue entitled: Neuromuscular Diseases: Pathology and Molecular Pathogenesis.

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http://dx.doi.org/10.1016/j.bbadis.2014.07.024
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Table 1
Classification of spinal muscular atrophy.

	Age of onset	Maximum motor function achieved	Age of death
Type I (severe, Werdnig–Hoffmann disease)	Before 6 months	Unable to sit	Less than 2 years
Type II (Intermediate)	7–18 months	Sit, never walk	More than 2 years
Type III (mild, Kugelberg–Welander disease)	After 18 months	Stand and walk independently	Adult
Type IV (very mild)	Second or third decade	Walking during adulthood	Adult

function was evaluated in SMA type II, III and IV patients using repetitive nerve stimulation. Pathological detrimental response was observed in about half the patients with SMA type II or III, but not in control patients or other motor neuron disease patients, suggesting specific neuromuscular junction dysfunction in SMA [94]. In addition, myotubes were found to be smaller in skeletal muscle tissue from severely affected SMA fetuses, indicating a delay in muscle growth and maturation [59]. Patients with severe SMA also develop congenital heart defect and arrhythmias, vascular abnormalities such as digital necrosis and mild hyperglycemia, suggesting pancreatic dysfunction. The first diagnostic test for a patient suspected to have spinal muscular atrophy should be *SMN1* gene deletion test. If this initial test is negative, further testing, including muscle creatine kinase level and nerve conduction study with repetitive nerve stimulation and electromyography, should be done. If electrophysiological studies suggest a motor neuron disease then sequences of both *SMN1* alleles should be determined.

SMN has been implicated in several functional processes, including pre-mRNA splicing, mRNA transport, and axon growth [1,63,64,76,80]. Although SMN is ubiquitously expressed in all tissues, spinal motor neurons are particularly vulnerable to diminished levels [68]. The underlying mechanisms of spinal motor neuron susceptibility remain largely unknown. There are, however, a number of prevailing and not mutually exclusive hypotheses based on the data yielded from disease models that may begin to explain. That the wild-type SMN protein has an established function in small nuclear ribonucleoprotein (snRNP) assembly suggests a role for pre-mRNA splicing in SMA disease progression [76]. Alternatively, because motor neurons have highly specialized, far-extending axons, it has been postulated that the localization of mRNAs to these distal processes is affected in SMA, which may be a driver of the selectivity for motor neuron degeneration [80]. At present, no direct target of mRNA splicing defect has been identified that can explain SMA pathogenesis, and there is equally sparse evidence to support the hypothesis that defects of distal mRNA transport and localization lead to SMA. Despite these unanswered questions concerning disease development and progression, much has been gleaned by modeling SMA with laboratory model systems. Here, we review the animal model systems that have been developed, as well as new mechanistic insights, diagnostic and treatment strategies that have been identified using these models.

1.1. Caenorhabditis elegans

The nematode *Caenorhabditis elegans* has been an efficient model for studying various disease-related gene functions. Genetic mutations in *C. elegans* can be induced by exposing worms to mutagens, including ethyl methanesulfonate (EMS) and gamma irradiation. RNA interference (RNAi)-mediated knockdown of gene expression can be easily achieved by feeding worms with small interfering RNA (siRNA) libraries. The *C. elegans* genome contains a single *SMN* ortholog, *smn-1*, that encodes an SMN protein 36% identical to the human ortholog (Fig. 1) [6]. Reducing the expression of *smn-1* by RNAi causes larval lethality, suggesting that *smn-1* is essential for survival of *C. elegans* animals [67]. A null mutation of *smn-1*, *smn-1*(*ok355*), that deletes most of the *smn-1* coding region, causes developmental arrest, reduced lifespan and progressive loss of motor functions [10]. Neuronal expression of an *smn-1* transgene partially rescues the developmental arrest and motor defects, while muscle-specific expression of the transgene does not, suggesting that the *C. elegans smn-1* primarily functions in neurons [10].

Extensive genetic screens have been performed to identify genes capable of modifying the deleterious phenotypes of *smn-1*-deficient *C. elegans* mutants [26]. Among these genes, the small conductance Ca^{2+}-activated K^+ channel (SK channel) was identified and manipulated pharmacologically to seek new modifiers of SMN functions for potential SMA therapy. Activating the SK channel by the neuroprotective drug Riluzole improved the motor functions of the *C. elegans smn-1*(*ok355*) null mutant and restored axon outgrowth in *Smn*-deficient rat hippocampal neurons [25], suggesting that genes identified in these screens could be potential targets for treating *SMN*-related defects. A caveat of using the *smn-1*(*ok355*) null mutant for screens is that severe defects of the mutant make identification of modifiers of the phenotype very demanding. To overcome this drawback, a point mutation in *smn-1* that mimics a human SMA disease mutation was isolated, which causes weak motor defects and a slightly reduced lifespan [88]. This mutant was used to screen a library of chemicals for potential drugs that could ameliorate the mild defects. Six chemicals were identified for further analysis [88]. The most effective ones include two FDA-approved drugs, 4-AP (a potassium channel blocker) and gaboxadol hydrochloride (a $GABA_A$ receptor agonist), and one novel compound Neu5Ac (a monosaccharide) [88]. With this, the *C. elegans smn-1* mutants represent an efficient discovery tool for performing large-scale screen for modifiers of SMN function.

1.2. Drosophila

The *Drosophila* genome contains a single copy of *SMN* ortholog, *Smn*, with 41% sequence homology to human *SMN1* (Fig. 1) [66]. Ectopic expression of SMA disease-related human SMN1 or truncated forms of *Drosophila* SMN causes pupal lethality and developmental arrest in a dominant-negative manner [66], suggesting that *Smn* is essential for *Drosophila* survival. Consistent with this finding, the *Drosophila* ortholog of Gemin 3, an SMN-interacting protein, is required for larva motor functions and animal survival [18,87]. *Drosophila* models carrying different *Smn* mutations have been developed and extensively studied. Mutant animals carrying an *Smn* point mutation similar to that in human SMA patients exhibit reduced excitatory post-synaptic currents, disorganized motor neuron boutons, loss of glutamate receptors at the neuromuscular junctions and compromised motor abilities [19]. Hypomorphic *Drosophila Smn* mutants isolated by Rajendra et al display defective axonal arborization in motor neurons and a failure to form thin filaments in muscles [78]. Altogether, these studies establish an essential role for *Smn* in regulating motor neuron and neuromuscular functions in *Drosophila*.

The analysis of mouse *Smn* mutants has identified defective expression of both major intron snRNAs and minor intron snRNAs, consistent with the role of *Smn* in snRNP assembly and pre-mRNA splicing [8,30,98]. Reduced snRNA expression was also observed in *smn*-deficient *S. pombe* [14]. Conversely, major intron snRNA expression in *Smn*-deficient *Drosophila* was not obviously affected [78], and reduced expression of minor intron snRNAs does not apparently affect the splicing of minor introns [77], suggesting that the effects of *Smn* on snRNA expression is species-dependent.

That the splicing of both major and minor introns was altered in *Smn* mouse mutants raises the question as to which intron type accounts for

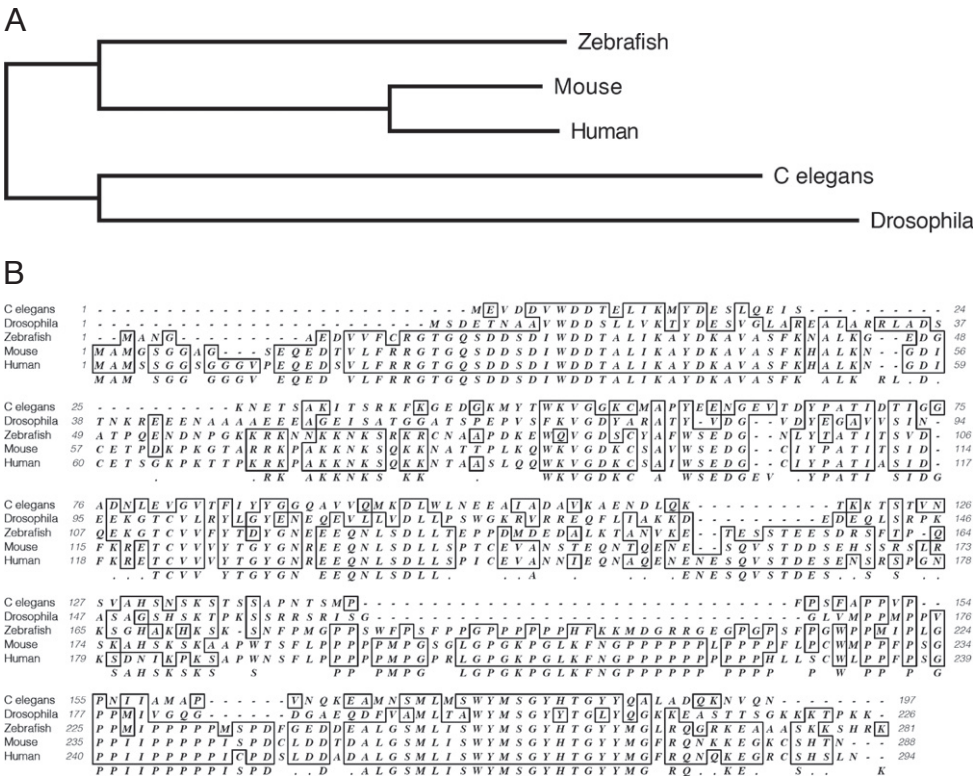

Fig. 1. SMN protein sequence and function are conserved across species during evolution. A. Phylogenetic tree based on SMN protein sequence showing the genetic divergence among SMN from different species using the neighbor joining method. B. Amino acid sequence alignment of SMN proteins from different organisms. Identical and similar amino acids are outlined.

the observed defects. Several U12 minor intron-containing genes were recently demonstrated to exhibit altered splicing in *Smn*-deficient *Drosophila* and mammalian cells [52], among which the defective splicing of the *Stasimon* gene appears to partially underlie the deficient neuromuscular function and improper muscle development [52]. However, studies from two other groups suggest that defects in minor intron splicing is an unlikely, if even possible, contributor to the mutant phenotypes of either *Smn*-deficient *Drosophila* [31] or mice [99]. Instead, these studies identified significant expression changes of genes involved in stress response [31] or altered splicing of genes involved in synaptogenesis [99]. Therefore it remains an open question as to how defective splicing of genes with major or minor introns contributes to *Smn* mutant phenotypes.

Drosophila Smn mutants have been used in screens for genes that interact with *Smn*, which identified members of the BMP signaling pathways [20] and genes involved in endocytosis and RNA processing [26]. Combining genetic screens and bioinformatics analyses, Sen et al recently identified hundreds of candidate *Smn*-interacting genes [85]. The most significant interactors identified in this study are a group of RNA splicing factors [85], which is consistent with our current knowledge that SMN primarily functions in regulating RNA splicing.

Studies in *Drosophila* have also uncovered new functions of *Smn*. *Drosophila Smn* is required for the integrity of the germline U body-P body pathway [48], the expression of FGF signaling components [86], stem cell division and differentiation [33], sensory–motor circuit function [42], and chronic glutamate receptor-dependent developmental homeostasis [93]. All of these directions warrant future study.

2. Zebrafish SMA models

The zebrafish (*Danio rerio*) is an excellent model organism for neurogenetics research because of its conserved yet simplified vertebrate nervous system, the ability to make transgenic animals with relative ease and to perform forward and reverse genetic studies [34]. Due to its well-characterized motor neuron circuits and relatively simple neuromuscular organization, zebrafish is well suited for analyzing neuromuscular defects and motor neuron diseases [23,63,79]. The optical transparency of zebrafish embryos offers accessibility of motor neurons, which can be manipulated in vivo for imaging, electrophysiology and motor behavioral studies [15,61–63]. The zebrafish spinal cord primary motor neurons are commonly used to study changes in axon morphology, protein aggregation, and neuromuscular junction formation, which are highly relevant to SMA pathogenesis [11,15,24,63]. Many techniques such as transgenesis and gene knockdown/knockout can be achieved in zebrafish, enhancing the power of this model for genetic studies. Zebrafish DNA has been adapted by deliberate mutation at targeted locations using transcription activator-like effector nucleases (TALENs) [40] or CRISPR-Cas [41] mediated gene knockout. Morpholino antisense oligos or in vitro transcribed RNA can be injected into zebrafish embryos to knockdown or over-express target genes, respectively. The resulting effects can be thoroughly assessed in the transparent embryo at the level of the entire organism, providing an excellent tool for assessment of gene function. Transgenic zebrafish expressing disease-related human proteins can serve as models for specific human diseases. With the zebrafish genome project nearly complete and innovative assays being developed, this model organism will become an increasingly important tool for dissecting motor neuron functions in physiological and pathological conditions.

Initial experiments aimed at characterizing the effects of inhibiting Smn protein in zebrafish were carried out via *smn*-targeted morpholino antisense oligo injection into one to four cell stage embryos [63]. This is a common method used to yield transient knockdown by way of binding mRNA or pre-mRNA, thus interfering with its translation or splicing, respectively. Antisense morpholino (MO) knockdown of *smn* in zebrafish recapitulates many aspects of motor neuron defects in SMA disease, including truncation and ectopic branching of motor axons [63]. These motor neuron defects can be rescued by the expression of wild type *smn* but not mutated *Δ7smn* identified in human patients [63].

Characterization of the spinal motor neurons and their innervated muscle segments in morpholino-injected zebrafish with ~60% Smn reduction revealed prominent early deficits of axonal branching and truncation [63]. An important finding from the characterization of this in vivo model was that motor axon development is the first phenotypic consequence of reduced Smn protein, preceding apoptosis. Moreover, that muscle development proceeds as normal in the morphant indicates that in zebrafish motor neuron-specific developmental deficits drive denervation and the skeletal muscle phenotypes characteristic of SMA.

Recently, a large-scale reverse genetics screen yielded three promising *smn* mutants: two stop mutations (*smnY267stop* and *smnL265stop*), and one exon 7 missense mutation (*smnG264D*) which was found to correspond to a human mutation (*SMNG279V*) known to cause SMA [7]. All of these mutations manifested in Smn protein reduction and/or instability, and homozygous embryos had a reduced body axis length and overall size. Characterization of these mutants revealed a selective decrease in the synaptic vesicle protein SV2, which was rescued by the motor neuron-specific expression of human *SMN1*. These results may suggest an important role for Smn in promoting presynaptic integrity [7]. Building on these studies, new zebrafish SMA models were generated by expressing a human *SMN2* transgene in zebrafish carrying endogenous *smn* mutations to more closely resemble the genetic characteristics of human SMA [37]. Characterization of this model revealed that *SMN2* is similarly spliced in fish as in humans, producing low levels of full-length SMN protein and comparatively high levels of exon 7 excluded protein. Furthermore, the manipulation of *SMN2* transcripts via an antisense oligonucleotide sequence directed against an intronic splicing silencer site in intron 7 was sufficient to promote exon 7 inclusion, thus increasing full-length SMN levels [37]. As the organism exhibits rapid ex utero development and ease of genetic and molecular manipulation, this model will prove invaluable in building the knowledge base of SMA disease development and progression, as well as serve an in vivo entry point into drug testing and therapy development.

3. Mouse SMA models

Similar to other subprimates and unlike humans, mice contain only one survival motor neuron gene, *Smn1*. Homozygous knockout of *Smn1* in mice leads to massive cell death during early embryonic blastocyst formation resulting in lethality [84]. This suggests essential biological functions of SMN protein. To test the tissue-specific requirement of *Smn1* expression, conditional alleles have been generated. Neuronal tissue-specific knockout of *Smn1* leads to progressive loss of motor axons and motor neurons [29], while muscle-restricted *Smn1* mutant mice display ongoing muscle necrosis with a dystrophic phenotype leading to paralysis and death. The dystrophic phenotype is associated with elevated levels of creatine kinase activity and reduction of dystrophin [22]. The observations from muscle-specific *Smn1* mutant mice suggest a primary involvement of skeletal muscle in human SMA, which may contribute to motor defects in addition to motor neuron degeneration.

To recapitulate the genetic foundation of human SMA, mouse models have been generated by expressing different copies of human *SMN2* through gene targeting or transgenesis on the background of homozygous disruption of *Smn1* gene. Phenotypic manifestations of severe (type I), intermediate (type II), and mild (type III) forms of SMA in different mouse models correlate directly with SMN expression levels [73,84], which is consistent with clinical observations in human patients. Among the most widely used SMA mouse models are the $Smn1^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (the Δ7 SMA mice or Jackson Laboratory stock #005025) [47] and the $Smn1^{hung\ -/-}$;SMN2Hung$^{tg/tg}$ (the Hung-Li SMA mice or Jackson laboratory stock #005058) [38]. Both models have an average lifespan of approximately 13 days and exhibit symptoms and neuropathology similar to patients afflicted with intermediate type II SMA [38,47]. Dr. Hung Li's laboratory established one of the first intermediate type II SMA modes by building upon an $Smn1^{hung}$ null mutant created independently by his group with the introduction of a transgene encoding a full-length *SMN2* (*SMN2Hung*) [38]. Intercrossing of animals hemizygous for the *SMN2Hung* transgene and heterozygous for the *Smn1* targeted mutation yields progeny that are hemizygous, homozygous or wild type for the transgene. As expected, mice carrying varying copies of the transgene exhibited differing levels of disease severity, thus this model has the ability to yield severe, intermediate and mild SMA phenotypes all within the same litter [38]. Mice from this model that are hemizygous for the transgene and homozygous for the targeted mutation ($Smn1^{hung\ -/-}$; $SMN2Hung^{tg/-}$) have very similar phenotypes as the intermediate type II mice generated from the most widely used Δ7 SMA mice described below [32,38]. Because this SMA model only has two genetically modified alleles, it is particularly useful for crossing with other mice carrying potential disease modifying targeted mutations or transgenes to generate compound mutant mice.

On the basis of the of *Smn1* knockout mice generated in Dr. Michael Sendtner's laboratory [84], Monani et al. from Dr. Arthur Burghes' laboratory generated the $Smn1^{-/-}$;$SMN2^{tg/tg}$ (Jackson Laboratory stock #005024) mice that express a transgene encoding the full-length SMN2 [70]. These mice are born with normal numbers of spinal motor neuron, but die around postnatal day 5 with a 40% loss of motor neurons and represent severe type I SMA [70]. This effect motivated the same group to add another transgene encoding a human *SMN2* cDNA (*SMN* Δ7) lacking exon 7 under the control of the human *SMN2* promoter. The resulting $Smn1^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (the Δ7 SMA mice or Jackson Laboratory stock #005025) mice, serving as a model for type II intermediate SMA, have been the work-horse of the research field. The beneficial effects of SMNΔ7 product demonstrate that up-regulation of *SMN2* can positively modify the SMA pathology. The Δ7 SMA mice die around 13.3 days after birth, with the loss of approximately 50% of spinal cord α-motor neurons (Fig. 2). As in *Drosophila*, loss of motor neurons in the Δ7 SMA mice occurs subsequent to the neuromuscular junction defects that affect both the pre-synaptic nerve terminal and the post-synaptic muscle fibers. Structural and functional abnormalities at neuromuscular junctions have been shown to occur before the onset of weakness, as low SMN levels arrest the postnatal development of neuromuscular junctions and impair the maturation of acetylcholine receptor clustering [43,50,58]. In addition to defects in peripheral synapses, glutamatergic excitatory synapse formation on spinal motor neurons is also compromised, potentially contributing to excitotoxicity in motor neurons affected by SMA [51,65]. Inhibitors of histone deacetylase have been used to increase SMN expression and to correct pathological defects in Δ7 SMA mice [4,60,89].

Phenotypic analysis also demonstrates that genetic background plays a key role in the survival of the Δ7 SMA mice. When backcrossed to complete congenic C57BL/6 J genetic background, the Δ7 SMA mice exhibit more severe phenotypes, with only 8% of the expected 25% survival rate to birth, suggesting that each mouse strain has unique background alleles that may interact with and modify the expression of a mutation or transgene. More recently, when the *SMNΔ7* transgene was replaced with an *SMN1 A2G* missense mutation, a mouse model ($Smn1^{-/-}$;$SMN2^{tg/tg}$;*SMN1A2G*, stock #005026) was generated [69]. These mice exhibit mild type III SMA phenotypes characterized by motor axon sprouting and loss, muscle atrophy and abnormal EMG patterns [69]. In addition, when a high copy number *SMN2* transgene *SMN2*(*566*) was incorporated into the *Smn1* knockout mice, the resulting mouse strain ($Smn1^{-/-}$;*SMN2*(*566*) or Jackson laboratory stock #008206) expresses 16 copies of *SMN2* transgene when made homozygous [70]. The increased copies of *SMN2* rescue these mice from overt SMA phenotypes except that mice homozygous for both *Smn1* targeted mutation and *SMN2*(*566*) transgene show a shorter and thicker tail [70]. A list of the most widely used SMA mouse models, including their genetic underpinning, phenotypes and ordering information from Jackson Laboratory is shown in Table 2.

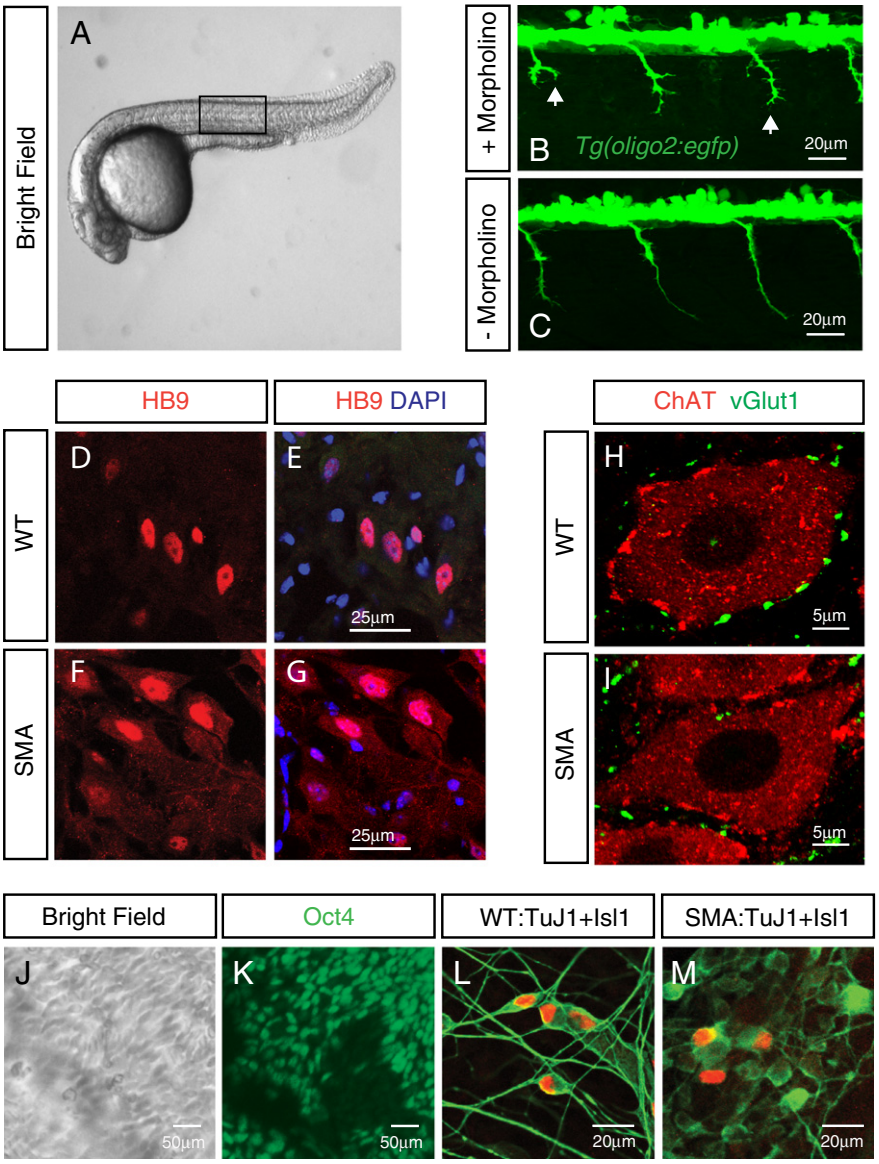

Fig. 2. Defects in SMA model systems represent different aspects of disease pathogenesis. A– C. Morpholino antisense oligonucleotide knockdown of *smn* in zebrafish leads to truncation or ectopic branching (white arrow heads in B) of motor axons in *Tg*(*olig2:egfp*) embryos 28 hours post fertilization. Boxed area in A is shown in B and C. D–G. The number of lumbar level spinal motor neurons recognized by the anti-HB9 antibody (red) is reduced by ~50% in SMA Δ7 mice compared to wild type littermates at postnatal day 9. Motor neurons affected by SMA show swollen morphology before dying. H–I. The number of glutamatergic excitatory synapses (green) on spinal motor neurons (red) is reduced by ~40% in SMA Δ7 mice at postnatal day 9. Glutamatergic synapses are shown by immunostaining with the anti-vGlut1 (vesicular glutamate transporters1) antibody, and spinal motor neurons are identified by immunostaining with the anti-ChAT (choline acetyltransferase) antibody. J-M. The number of motor neurons differentiated from SMA iPS cells is significantly less compared to wild type iPS cells. Human iPS cells generated from SMA patient skin fibroblasts differentiate into spinal motor neurons after 4–6 weeks of development. Brightfield microscopy (J) and immunofluorescence staining (K-M) are used to show undifferentiated iPS cells (J-K) expressing the pluripotency marker Oct4 (green in K), iPS cell-derived motor neurons (L-M) coexpressing neuronal marker TuJ1 (green in L, M) and the motor neuron marker Isl1 (red in L, M). The number of Isl1 positive motor neurons as a percentage of all TuJ1 positive neurons from SMA iPS cells (M) is significantly less than those from wild type (WT) iPS cells (L).

4. Discussion

SMN is expressed ubiquitously in all somatic cells; however, reduced SMN levels make spinal motor neurons particularly vulnerable to degeneration. The underlying reasons are not understood, though some hypotheses regarding SMA disease mechanism may begin to explain. In accordance with SMN's role in pre-mRNA splicing [75], it has been proposed that transcripts encoding proteins vital for motor neuron function and survival may depend on SMN. This hypothesis has been supported by the identification of genes with altered splicing in SMA mouse models [5,98,99]. Utilizing the newly developed deep sequencing approach to identify splicing defects in spinal motor neurons affected by SMA will likely lead to more candidate splicing target genes for verification. Another attractive, and not mutually exclusive, potential explanation for the motor neuron-specific vulnerability lies in the mislocalization of RNAs in the distal segments of motor axons in SMA mice [80]. Given that these axons are particularly long, subcellular RNA localization presents a daunting challenge for these cells. A demonstrated defect in transportation of the related transcripts or RNA processing machinery would be expected to explain the cellular specificity of SMA. However, much vital evidence in accordance with this hypothesis is lacking. A definitive mechanism linking reduced SMN levels with axonal transport deficiency resulting in motor neuron dysfunction and atrophy in an SMA model will be required. Importantly, despite the utility of SMA models, there are several considerations that must be made with their use and interpretation of the data they yield. For example, although SMA is a disease affecting primarily the nervous and motor systems, our understanding of these systems' defects might

Table 2
Widely used spinal muscular atrophy mouse models.

Common name	Jackson Stock #	SMA type	Mortality	Genotype	Phenotype
Smn1 targeted mutation	006214	Severe (type I)	Embryonic lethal	*Smn1*$^{-/-}$	Created in the laboratory of Dr. Michael Sendtner. Exon 2 of the mouse *Smn1* gene was disrupted by a neomycin cassette and a *lacZ* gene fused to the first 40 nucleotides of the disrupted exon 2.
Burghes' Severe Model	005024	Severe (type I)	Stillborn or 4–6 days	*Smn1*$^{-/-}$;*SMN2*$^{tg/tg}$	Low birth weight, decreased suckling and movement, tremoring limbs, and labored breathing.
Δ7 mouse	005025	Intermediate (type II)	Mean survival of 13 days	*Smn1*$^{-/-}$; *SMN2*$^{tg/tg}$;*SMN*Δ*7*$^{tg/tg}$	The work-horse and the most widely used model in the field. By postnatal day 5 (P5), signs of muscle weakness appear and become progressively more pronounced with an abnormal gait, shakiness in the hind limbs and a tendency to fall. Severe NMJ defects and loss of over 50% of spinal motor neurons by P9.
Hung-Li mouse	005058	Intermediate (type II)	Mean survival of 13 days	*Smn1*$^{hung\,-/-}$; *SMN2Hung*$^{tg/-}$	Mice hemizygous for the transgene and homozygous for the targeted mutation exhibit phenotypes similar to the Δ7 SMA mice. Because only contains two genetically modified alleles, this model is particularly useful for crossing with other mice carrying potential disease modifying targeted mutations or transgenes.
A2G mouse	005026	Mild (type III)	Shortened lifespan of less than 1 year	*Smn1*$^{-/-}$; *SMN2*$^{tg/tg}$;*SMN1A2G*	Lower body weight, diminished activity, muscle weakness, motor axon sprouting and loss, muscle atrophy and abnormal EMG patterns. The Jackson Laboratory notes a milder phenotype than the original publication.
	008206	Very mild (type IV)	None	*Smn1*$^{-/-}$;*SMN2(566)*	Homozygous mice have 16 copies of *SMN2*. No overt features of SMA. Shorter and thicker tail.

be obscured by the multi-system morbidity of the mouse models. It is entirely possible that the motor defects observed are not direct, but rather indirect consequences of SMN mutations.

Spinal muscular atrophy has historically been considered a pure motor neuron disorder. However, recent studies have shown that in severe type 1 SMA, other organs can also be involved [36,57]. In one report, three of four patients with only one copy of *SMN2* had hemodynamically relevant atrial or ventricular septal defects. These patients required mechanical ventilation at birth and survived only a few months [82]. In another report, two unrelated patients with severe SMA type I and only one *SMN2* copy developed ulcerations and necroses of the fingers and toes. Sural nerve biopsy was normal in one patient, but skin biopsy showed thrombotic occlusion of the small vessels causing perfusion abnormalities and tissue necrosis, suggesting that SMN deficiency may present as a vasculopathy [83]. In a previous study, these same authors performed sural nerve biopsies on 19 patients with SMA I through III and noted significant sensory nerve pathology in severely affected patients with SMA type I. None of the patients with SMA type II or III had any evidence of clinical or pathological sensory nerve alterations [81]. These extra-neuronal manifestations seem to occur in severe SMA patients with only one copy of SMN2 and usually require supportive respiratory measure at birth.

Due to the lack of understanding of disease mechanism, there is currently no cure for SMA. As the number of clinical trials continues expanding, the availability of reliable biomarkers will be crucial for an objective measurement of disease progression, monitoring of response to treatment and shortening of trial duration. Electrophysiological biomarkers that assess motor units in vivo, including compound muscle action potential (CMAP) and motor unit number estimate (MUNE), have been tested in SMA clinical trials [92]. Although CMAP and MUNE showed some correlation with disease phenotype and *SMN2* copy number [91] and seemed to increase in response to valproic acid treatment, the trial was negative. SMN protein and transcript levels can be easily measured in peripheral blood. However these levels do not seem to correlate with disease severity [90]. More recently biomarkers from a large multicenter SMA study "BforSMA" used proteomic, metabolomic and transcriptomic approaches to identify candidate proteins, plasma and urine metabolite markers that correlated with motor scores in a wide range of SMA patients [27]. Using these data sets, a commercial biomarker panel (SMA-MAP) for plasma proteins in SMA patients was developed. This panel includes 27 analytes and showed a significant association with motor functions and other measures of SMA disease activity [45]. An ongoing natural history study, NeuroNex, conducted by the Network for Excellence in Neuroscience Clinical Trials, is collecting motor function measures, molecular biomarkers (SMN protein and transcript levels as well as SMA-MAP) and electrophysiological markers (CMAP and electric impedance myography) in the immediate post natal period of afflicted infants in order to validate these measures and to facilitate the future conduct of clinical trials in these cohorts.

Although at present there is no cure for SMA, a number of promising advancements have been made in search of treatment. Current therapeutic strategies under development are almost exclusively based on trying to increase levels of SMN protein. These include expression of ectopic *SMN* by gene therapy [28], increasing inclusion of exon 7 in *SMN2* mRNA transcripts, upregulation of *SMN* transcription by promoter activation or correcting splicing defects [2,9,39,55], modulation of SMN protein translation [53,60,97], and prevention of SMN protein degradation [21,95]. Some histone deacetylase (HDAC) inhibitors have been demonstrated to increase the production of full-length SMN from SMN2 by promoting exon 7 inclusion, in addition to enhancing gene expression levels by promoting histone acetylation and open chromatin structure [2,4,9,89]. However, altered gene expression on a more global basis was also observed, suggesting undesired side effects of nonspecific HDAC inhibitors as therapeutic agents. Recently, significant progress has been made in developing gene therapy for SMA following the surprising finding that scAAV9 can mediate *SMN1* expression very specifically in spinal motor neurons [28]. In addition, antisense oligonucleotides targeted to *SMN2* pre-mRNA have been developed as a prototype for potential treatment. These oligos have been shown to effectively modulate the splicing of *SMN2*, resulting in the production of transcripts with exon 7 inclusion, thus elevating the level of functional SMN [39]. Recent rapid progress in stem cell research, including the development of induced pluripotent stem (iPS) cells from SMA patient fibroblasts [100], presented the possibility of using motor neurons derived from iPS cells, or other stem cell sources, to replace degenerated motor neurons in SMA patients, study disease pathogenesis and perform drug screening. Because iPS cell-derived motor neurons carry the

patient's genetic makeup, their clinical use will likely improve projected transplant outcome. Though the challenges facing the application of this approach in human are appreciable, it may be the only applicable therapy for individuals who've already experienced substantial motor neuron loss, since methods aimed at increasing full-length SMN levels can only act to promote survival in motor neurons that have not yet initiated apoptosis. Importantly, the divergence in prognoses given SMA type designation may warrant the development and application of multiple therapies.

Taken together, model systems have so far greatly advanced our understanding of the genetics and biology of SMA. However, a critical gap still exists in our knowledge about how SMN mutations lead to SMA pathogenesis: we have little information about the identities of genes, proteins and other biological factors that mediate the particular vulnerability of spinal motor neurons. More extensive studies using these model systems should provide crucial understanding of this causal effect and contribute to the development of efficacious therapies for SMA.

Acknowledgements

The authors would like to thank Drs. Nimrod Miller and Zhihua Feng for assistance with making figures. B.M.E. and Y.C.M. are supported by the Schweppe Foundation, Whitehall Foundation, Families of SMA and NIH grant AG043970.

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