Molecular Mechanisms of Spinal Muscular Atrophy

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Significant strides have been made during the past decade in the understanding of the molecular mechanisms that lead to the autosomal recessive motor neuron disease spinal muscular atrophy. Genetic studies revealed that spinal muscular atrophy is caused by mutation of the telomeric copy of the survival motor neuron gene (SMN1), with all patients retaining at least one copy of the centromeric form of the gene, SMN2. SMN2 produces reduced amounts of full-length SMN messenger ribonucleic acid because of alternative splicing of SMN2-derived transcripts, a process that is governed by specific cis- and trans-acting factors. The resulting insufficient expression level of full-length SMN protein likely causes the disease manifestations of spinal muscular atrophy; however, the mechanism for the selective vulnerability of the motor unit to deficiency of this ubiquitously expressed protein remains unknown. It also remains unclear specifically when and where in the motor unit SMN is required. Despite the remaining questions, progress has been made in developing therapeutic strategies targeted to specific points along the pathogenetic pathway of spinal muscular atrophy. Histone deacetylase inhibitors will be discussed as an example.

Keywords: spinal muscular atrophy; motor neuron; survival motor neuron

As discussed in more detail by other authors in this issue of the Journal of Child Neurology, spinal muscular atrophy has several somewhat unusual clinical features that must be explained by any proposed model of molecular disease pathogenesis. First, spinal muscular atrophy has a stereotypical pattern of muscle weakness, which is more reminiscent of a myopathic disorder than a neurogenic one. The proximal muscles are more involved than the distal muscles, the legs are more involved than the arms, and the arms are more involved than the face and diaphragm. Second, there is a strikingly wide spectrum of phenotypic severity, with type 1 patients often dying before 2 years of age and some type 3 (or type 4) patients experiencing only modest weakness and living a normal life span. Third, spinal muscular atrophy has a particular disease course in those who survive beyond infancy. Rather than inexorable progression after onset, loss of muscle power in spinal muscular atrophy is often most evident at onset, after which residual muscle power may stabilize for months or years. In this article, I review some of what is understood about the molecular pathogenesis of spinal muscular atrophy. These insights provide some explanations for the peculiar clinical features of this disease, but also highlight unresolved questions. I also discuss how progress to date has led to the development of potential therapeutics.

Genetics of Spinal Muscular Atrophy

In 1990, the spinal muscular atrophy disease gene was mapped by linkage analysis to a complex region of chromosome 5q. Unlike in most organisms, in humans this region contains a large inverted duplication with at least 4 genes present in telomeric and centromeric copies. In 1995, Lefebvre et al reported that homozygous mutations in one of these genes, the telomeric form of the survival motor neuron gene (SMN1), cause spinal muscular atrophy. These mutations are most often large deletions, but may also be missense mutations (see review by Thomas Prior in this issue). Although 5%-10% of the normal population lacks the centromeric copy of SMN (SMN2), all patients with spinal muscular atrophy retain at least one copy of SMN2. The genomic sequences of SMN1 and SMN2 differ by only 5 nucleotides, but only one difference is functionally important: a translationally silent C→T transition located within an exonic splicing region of SMN2. Consequently, although SMN1 produces...
full-length transcripts, the majority of mature transcripts that arise from SMN2 lack exon 7. These transcripts encode a truncated protein (SMN∆7). This protein is presumably rapidly degraded, and the disease manifestations of spinal muscular atrophy likely result from a deficiency of full-length SMN protein (Figure 1). Several studies have shown that full-length SMN messenger ribonucleic acid (RNA) and protein levels are reduced in cell lines and tissues derived from patients with type 1 spinal muscular atrophy compared with controls.6-9

The SMN2 copy number varies in the population, and in patients with spinal muscular atrophy this variation has an important modifying effect on spinal muscular atrophy clinical disease severity.10 Most patients with the most severe form of the disease, spinal muscular atrophy type 1, have 1 or 2 SMN2 copies; most patients with type 2 have 3 SMN2 copies; and most patients with spinal muscular atrophy type 3 have 3 or 4 SMN2 copies.11 It appears that some individuals with 5 or 6 copies of SMN2 are very modestly affected or can be completely protected against developing disease manifestations.12,13 This dose relationship between SMN2 copy number and disease severity has been dramatically recapitulated in spinal muscular atrophy model mice. Mice lacking the endogenous mouse Smn gene but expressing 2 copies of the human SMN2 gene develop severe spinal muscular atrophy and die within 1 week of age; however, mice that express 8 copies of SMN2 do not develop disease.14 It has been demonstrated in cell lines isolated from patients and in spinal muscular atrophy mouse tissues that increased SMN2 gene copy number correlates with increased levels of full-length SMN transcript and protein,6-8 suggesting that variations of full-length SMN transcript and protein levels determine much of the variation in spinal muscular atrophy clinical disease severity. Interestingly, this relationship has not been borne out in recent studies looking at SMN levels in peripheral blood cells collected from spinal muscular atrophy patients,15,16 but it is not yet clear whether SMN levels in blood cells are representative of what is happening in motor neurons. In addition to SMN2 copy number, other genes likely influence the phenotypic variability of spinal muscular atrophy. There have been rare reports of families in which markedly different degrees of disease severity are present in siblings with the same SMN2 copy number.17 There are ongoing efforts to identify other disease-modifying factors in such kindreds.
Splicing and Spinal Muscular Atrophy

A fundamental step in the molecular pathogenesis of spinal muscular atrophy is the altered splicing of SMN2-derived transcripts, compared with SMN1-derived transcripts. In general, splicing is carried out by a complex macromolecular machine known as the spliceosome, which recognizes sequences at exon/intron junctions called the 5’ and 3’ splice sites. In addition to these splice sites, splicing is guided by auxiliary cis-elements known as exonic and intronic splicing enhancers and silencers. These enhancers and silencers are recognized by transacting factors such as serine-arginine-rich proteins and heterogeneous nuclear ribonucleoproteins. Full-length SMN transcript is encoded by 9 exons (1, 2a, 2b, 3-8), and exons 1-7 are translated into SMN protein. Two models have been proposed to explain the inhibitory effect of the C→T transition in SMN2 on exon 7 inclusion.\textsuperscript{18-20} According to the exonic splice enhancer model, SMN1 exon 7 contains a heptamer sequence motif that recruits the splicing factor SF2/ASF, which promotes exon 7 inclusion. When this motif is interrupted by the C→T (U in messenger RNA) transition that is present in SMN2 transcripts, the SF2/ASF factor is not recruited, the 3’ splice site is not recognized, and exon 7 is excluded.\textsuperscript{21,22} The silencer model, in contrast, proposes that the C→U transition creates an exon silencer element that interacts with heterogeneous nuclear ribonucleoprotein A1, a splicing factor that represses exon 7 inclusion.\textsuperscript{20} These models are not necessarily mutually exclusive, and it may be that both mechanisms are at play simultaneously (Figure 2). It has also been shown that exon 7 skipping can be partially overcome by a complex of splicing factors that binds an AG-rich exonic splicing enhancer region downstream of the heptamer motif. Htra2-β1 facilitates exon 7 inclusion\textsuperscript{21} and the function of Htra2-β1 is enhanced by interaction with heterogeneous nuclear ribonucleoprotein-G.\textsuperscript{24} Srp30c also

\textbf{Figure 2.} Splicing and spinal muscular atrophy. The splicing of SMN-derived transcripts is likely to be determined by multiple cis- and trans-acting elements. Within the sequences of SMN1 and SMN2 transcripts there are exonic and intronic splicing enhancers that are recognized by trans-acting factors. In SMN1-derived transcripts, there is a C at position 6 of the exon 7 sequence. This C is part of an exonic splicing enhancer that recruits the splicing factor SF2/ASF, which promotes exon 7 inclusion. Htra2-β1 binds another exonic splicing enhancer downstream, and together with heterogeneous nuclear ribonucleoprotein-G (hnRNP-G) and Srp30c also promotes exon 7 inclusion. In contrast, in SMN2-derived transcripts there is a U at position 6 of exon 7. This change interrupts the exonic splicing enhancer and also create an exonic splicing silencer that recruits the splicing factor hnRNP A1, that acts to exclude exon 7.
The SMN Protein

SMN is a widely expressed protein with a molecular weight of 38 kilodaltons (kDa). It has been highly conserved through evolution. SMN is present in both the cytoplasm and the nucleus of the cell body. In the nucleus, it is concentrated in punctate structures called “gems” that overlap with or are closely apposed to Cajal bodies. SMN has also been shown to be present in granules in the axons of neurons, where it is rapidly transported bi-directionally. It is also enriched at the growth cone of motor neurons and perhaps at the postsynaptic apparatus of the neuromuscular junction in muscle. SMN protein levels are developmentally regulated, with high expression levels in the embryonic period that decrease in the early postnatal period. It is possible that the spinal muscular atrophy disease process starts when SMN levels fall below a critical threshold and that the severity of disease manifestations depends, in part, on when this decrease occurs during development.

The SMN protein has several identified motifs, including a lysine-rich basic region encoded by exon 2, a Tudor motif encoded by exon 3, a polyproline region encoded by exons 4 and 5, and a region with several tyrosine-glycine (Y-G) pairs encoded by exon 6. Missense mutations have been identified in several of these regions, suggesting that each of these domains may be functionally important. SMN has been shown to oligomerize and form stable multiprotein complex with Gemins 2-8 and urrip. SMN protein lacking exon 7 (SMNΔ7) is rarely detectable in cells or tissues derived from human patients or from spinal muscular atrophy animal models, despite robust expression levels of SMNΔ7 messenger RNA, indicating that the SMNΔ7 protein is highly unstable. This instability is likely because SMNΔ7 protein has an impaired ability to oligomerize and to associate with its binding partners. When expressed at high levels, however, the SMNΔ7 protein appears to retain some functionality and to provide some amelioration of the disease phenotype in spinal muscular atrophy model mice.

This SMN complex interacts with several other proteins, many of which are components of various ribonucleoprotein complexes that are involved in distinct aspects of RNA processing. The SMN complex may, therefore, play a role in diverse aspects of RNA metabolism, including pre-RNA splicing, transcription, and metabolism of ribosomal RNAs. Presently, the best-characterized function of the SMN complex is regulating the assembly of a specific class of RNA-protein complexes, the uridine-rich small nuclear ribonucleoproteins. The small nuclear ribonucleoproteins are a critical component of the spliceosome; a large RNA-protein complex that catalyzes premessenger RNA splicing (please see Stephen Kolb’s review in this issue for a more extensive review of SMN and its role in small nuclear ribonucleoprotein assembly). In addition to its activity in small nuclear ribonucleoprotein assembly, SMN may have other functions in motor neurons. In neuronal processes, the SMN protein binds heterogeneous nuclear ribonucleoprotein, which in turn binds to the 3′-untranslated region of β-actin messenger RNA. This interaction is required for the efficient transport of β-actin messenger RNA to the growth cones of motor neurons. Motor neurons isolated from mice deficient in SMN show shortened axons and small growth cones, which are deficient in β-actin messenger RNA and protein. β-actin messenger RNA and protein localization in the growth cone is known to be necessary for axonal outgrowth, as the actin cytoskeleton is the driving force for growth cone mobility. Zebrafish deficient in SMN protein show a specific deficit in motor neuron axonal outgrowth. Please see Christine Beattie’s review in this issue for further discussion.

Remaining Questions

The many remaining questions about spinal muscular atrophy disease pathogenesis have driven efforts to recapitulate spinal muscular atrophy in model organisms. Spinal muscular atrophy has now been successfully modeled in several organisms, including worms, flies, zebrafish, and mice, by using various strategies to decrease SMN levels (please see the reviews of Christine Beattie, Arthur Burghes, and Christine DiDonato for more extensive discussion). These models have provided invaluable tools for further dissecting the molecular pathogenesis of this disease and have created essential in vivo systems for identifying and validating the efficacy of potential therapeutics. Three significant and interrelated questions that may be answered by ongoing studies in these models are the following: (1) Why are motor neurons uniquely susceptible to SMN protein deficiency? (2) Is spinal muscular atrophy a motor neuron cell autonomous disease? (3) Is spinal muscular atrophy, at least in part, a developmental abnormality?

Why Are Motor Neurons Uniquely Susceptible to SMN Protein Deficiency?

A minimal level of SMN protein appears to be essential for survival of all cells, consistent with the essential housekeeping functions of SMN. Nematode, fly, and mouse models that have no functional SMN protein have a uniformly early embryonic lethal phenotype. In contrast,
transgenic mice that express reduced levels of full-length SMN protein are rescued from embryonic lethality, but they develop motor neuron loss, atrophy of muscle fibers, muscle weakness, and early death.\textsuperscript{14,50,51} The mechanism of selective disruption of the motor neurons in mice and humans owing to reduced levels of SMN protein remains unknown. One possibility is that overall, motor neurons have a uniquely high demand for efficient small nuclear ribonucleoprotein assembly and messenger RNA processing. Alternatively, inefficient small nuclear ribonucleoprotein assembly could cause inappropriate splicing of one or more motor neuron-specific messages crucial for the survival of these cells. Supporting the hypothesis that deficiency of small nuclear ribonucleoprotein assembly causes spinal muscular atrophy are data showing that cell lines derived from spinal muscular atrophy patients have reduced small nuclear ribonucleoprotein assembly activity that correlates with reduced SMN protein levels,\textsuperscript{52} and that delivery of small nuclear ribonucleoproteins to zebrafish deficient in SMN ameliorates motor neuron abnormalities.\textsuperscript{53} Others argue, however, that SMN has unique functions in motor neurons, such as in axonal messenger RNA trafficking, that explains the specific susceptibility of the motor unit to deficiency of this widely expressed protein.\textsuperscript{33,34,54} One recent study has demonstrated that some forms of mutant SMN protein that retain the ability to oligomerize and bind Sm protein, functions required for normal small nuclear ribonucleoprotein biogenesis, are not able to rescue motor axon abnormalities in a zebrafish model of spinal muscular atrophy.\textsuperscript{54} This result suggests that SMN function in small nuclear ribonucleoprotein biogenesis may be dissociated from its function in motor axons and that the specific susceptibility of motor neurons to SMN deficiency relates to some other specialized function of SMN in these cells.

**Is Spinal Muscular Atrophy a Motor Neuron Cell Autonomous Disease?**

As mentioned in the introduction to this review, the pattern of predominantly proximal weakness in spinal muscular atrophy is more reminiscent of a myopathic disorder rather than a neuropathic one. The neuromuscular pathology of severe spinal muscular atrophy is also distinct from other motor neuron diseases. In addition to loss of large-diameter anterior horn cells in the spinal cord, autopsy studies of type 1 spinal muscular atrophy patients have documented very large areas of rounded, small myofibers rather than angulated, atrophic myofibers typical of denervation.\textsuperscript{55} These myofibers ultrastructurally appear to be arrested in development,\textsuperscript{56} and they express abnormal amounts of developmental isoforms of myosin.\textsuperscript{57} These pathological observations have raised the hypothesis that spinal muscular atrophy is not a cell autonomous disease. Perhaps SMN deficiency results in primary abnormalities in both motor neurons and muscle that lead to a failure of communication between these 2 components of the motor unit. Evidence from several in vitro and animal studies may support this hypothesis. Spinal muscular atrophy patient-derived myoblasts and Smn-deficient C2C12 myoblasts show impairments of myofiber proliferation and fusion.\textsuperscript{58,59} and coculture of satellite cells derived from spinal muscular atrophy patients with normal motor neurons causes premature motor neuron disorganization and death.\textsuperscript{60} In mice, removal of SMN exon 7 specifically from muscle causes dystrophic changes\textsuperscript{42} and satellite cell abnormalities\textsuperscript{61} leading to paralysis and death. Drosophila deficient in SMN show significant disorganization of both the presynaptic terminus and the postsynaptic neurotransmitter receptors, and replacement of SMN is required in both the motor neuron and muscle compartments in this model to provide a phenotypic rescue.\textsuperscript{62} Skeletal muscle is known to be a source of signals that influence motor neuronal survival, growth, and maintenance,\textsuperscript{63} and it is possible that a primary abnormality of muscle contributes to the pathogenesis of spinal muscular atrophy. If this is the case, it raises the possibility that therapy delivered directly and solely to muscle, a more accessible tissue than the spinal cord, could be beneficial for spinal muscular atrophy patients. Studies are ongoing in a mouse model of spinal muscular atrophy to test this hypothesis.

**Is Spinal Muscular Atrophy, at Least in Part, a Developmental Abnormality?**

Unlike most motor neuron diseases in which there is relentless progression of muscle weakness after disease onset, a deterioration of muscle power may be clearly evident early in spinal muscular atrophy, but then strength may stabilize for many months or years. In addition, some children with spinal muscular atrophy do not appear ever to have a clear deterioration of muscle power; rather, they just never develop the motor skills of their peers. This disease course might suggest that there is abnormality in the development of the motor unit such that it cannot meet the increased demand necessary during the neonatal and childhood periods of rapid growth of muscle and increasing muscle strength, but once this developmental period has been completed, there is stabilization. Some studies support the idea that there could be abnormalities in the development of the motor unit in spinal muscular atrophy. Primary motor neurons isolated from a mouse model of spinal muscular atrophy have been shown to have morphological abnormalities of distal termini, with shortened axonal length and small growth cones.\textsuperscript{54} Zebrafish deficient in SMN show specific abnormalities of motor neuron outgrowth and branching.\textsuperscript{46} In addition, spinal muscular atrophy patient-derived myoblasts and Smn-deficient C2C12 myoblasts show impairment of myofiber proliferation and fusion.\textsuperscript{58,59} Further studies will be needed to define whether an abnormality of development of the motor unit contributes to the disease pathogenesis, but if it does, it could be critical in determining the timing of therapeutic intervention.
Molecular Therapeutic Targets for Spinal Muscular Atrophy

Despite many remaining questions about the molecular pathogenesis of spinal muscular atrophy, our current understanding has led to identification of specific targets for potential therapeutic intervention. In patients, spinal muscular atrophy disease severity correlates inversely with increased SMN2 gene copy number, and in transgenic mice lacking endogenous SMN, increasing SMN2 gene copy number from 2 to 8 prevents the spinal muscular atrophy disease phenotype. These observations strongly suggest that increasing SMN expression levels may be beneficial to spinal muscular atrophy patients.

Currently pursued therapeutic strategies for spinal muscular atrophy include induction of SMN2 gene expression, modulation of splicing of SMN2-derived transcripts, stabilization of SMN protein, neuroprotection of SMN deficit neurons, and SMN1 gene replacement (Figure 3). Early clinical trials of some candidate therapeutics are now ongoing in spinal muscular atrophy patients (please see Kathy Swoboda’s review in this issue). Below I provide an example of one class of drugs that has been evaluated in cell culture models, mice, and in early human clinical trials in spinal muscular atrophy patients.

**Histone Deacetylase Inhibitors and Spinal Muscular Atrophy**

Histone deacetylase inhibitors can increase expression of genes by modifying chromatin structure. The basic unit of chromatin, the nucleosome, consists of 146 base pairs of DNA wrapped around a core of histone proteins. Histones are small, basic proteins consisting of a globular domain and a more flexible and positively charged NH2-terminus (or “histone tail”) that protrudes from the nucleosome. After acetylation of lysine residues embedded in the histone tails, chromatin takes on a more relaxed conformation that is more transcriptionally active owing to increased accessibility of DNA to the transcriptional
The level of histone acetylation is determined by the balance of activities of histone acetyltransferases, which acetylate histones, and histone deacetylases, which deacetylate histones. There are at least 4 biochemical classes of histone deacetylase inhibitors: aliphatic acids, hydroxamic acids, benzamides, and cyclic peptides.

The possibility that histone deacetylase inhibitors might be useful in the treatment of spinal muscular atrophy was first raised when an aliphatic acid, sodium butyrate, was identified as a hit in a cell-based drug screen to identify compounds that increase SMN levels in patient-derived lymphoblastoid cell lines. Histone deacetylase inhibitors from different biochemical classes have now been shown to activate the SMN2 promoter, likely through direct modification of the acetylation state of histones at the promoter. The aliphatic acids valproic acid, sodium butyrate, and phenylbutyrate increase full-length SMN messenger RNA and protein in cell lines derived from spinal muscular atrophy patients, in part by activating the SMN2 promoter and in part by promoting exon 7 inclusion in SMN transcript by increasing expression of the SR protein HtraB1. Sodium butyrate has also been shown to improve the survival of spinal muscular atrophy mice when delivered to pregnant mothers. Recently, we have shown that the potent and specific histone deacetylase inhibitor trichostatin A, when delivered after onset of weight loss and motor deficit, significantly improves motor performance, attenuates weight loss, and increases survival in spinal muscular atrophy model mice (Figure 4). In addition, trichostatin A improves the pathology of the motor unit in spinal muscular atrophy model mice. In particular, there is improvement in the number and size of myofibers (Figure 5). These improvements are also associated with increases in both SMN2-derived transcript and SMN protein levels in neural tissues and muscle, and an improvement in small nuclear ribonucleoprotein assembly. This study demonstrates that postsymptomatic delivery of therapeutics in spinal muscular atrophy can be effective. This finding is particularly relevant for patients with spinal muscular atrophy, as the vast majority are not diagnosed until after they display overt weakness. Based on these in vitro and in vivo studies, there has been an effort to quickly move to clinical trials in spinal muscular atrophy patients with this class of compounds.
Figure 5. Trichostatin A increases myofiber size and number in a mouse model of spinal muscular atrophy. (A) H&E stained cross-sections of the tibialis anterior muscle. Myofibers are diffusely small in vehicle-treated spinal muscular atrophy mice compared with unaffected heterozygous littermates, but trichostatin A results in increased myofiber size. Bar = 10 µm. (B) Histograms of myofiber diameters quantified in 3 spinal muscular atrophy mice treated with vehicle, 4 spinal muscular atrophy mice treated with trichostatin A, and 3 heterozygous littermates at 13 days of life. (C) Median tibialis anterior muscle cross-sectional area is reduced in spinal muscular atrophy mice compared with heterozygous littermates (P = .05) and increased with trichostatin-A treatment (P = .03). The bar within the box depicts the median, the box depicts all values between the 75th percentile and 25th percentile, the whiskers depict all values that are 1.5 times the interquartile range, and the dots are values outside this range. (D) Median myofiber diameter in the tibialis anterior muscle is reduced in spinal muscular atrophy mice compared with heterozygous littermates (P < .001) and increased in spinal muscular atrophy mice treated with trichostatin-A (P < .001). (E) Median tibialis anterior muscle total myofiber number is reduced in spinal muscular atrophy mice compared with heterozygous littermates (P = .05) and increased by trichostatin A treatment (P = .08).

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in patients with spinal muscular atrophy. In one open-label study of valproic acid in type 3/4 patients, there was improved strength as mentioned by hand-held dynamometry. In the only published randomized, double-blind, placebo-controlled trial, phenylbutyrate was well tolerated but did not show efficacy in improving motor performance in spinal muscular atrophy patients. Both valproic acid and phenylbutyrate are weak histone deacetylase inhibitors and have multiple other off-target biological effects that may minimize their effectiveness. Newer and more potent histone deacetylase inhibitors are being actively developed by the pharmaceutical industry and some compounds are now in phase 1, 2, and 3 clinical trials and showing reasonable tolerability. These compounds may prove to be more effective for the treatment of spinal muscular atrophy.

Summary

Our understanding of the molecular mechanisms that cause spinal muscular atrophy have enabled us to develop powerful in vivo models of the disease, to identify some initial targets for therapeutic intervention, and to begin to test potential therapeutics. The availability of animal models as well as drugs that modify the spinal muscular atrophy disease course may enable us to address such fundamental questions as why the motor unit is selectively vulnerable to SMN deficiency and precisely where and when SMN is required within the motor unit. These insights, in turn, will likely explain the peculiar clinical features of this disease and lead to more effective treatments for this as-yet untreatable and often fatal disease.

References


43. Le TT, Pham LT, Butchbach ME, et al. SMNDelta7, the major product of the centrmeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet*. 2005;14:845-857.


64. Sumner CJ. Therapeutics development for spinal muscular atrophy. NeuroRx. 2006;3:235-245.


