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Spinal Muscular Atrophy: A Deficiency in a Ubiquitous Protein; a Motor Neuron-Specific Disease

Review

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Spinal muscular atrophy (SMA) is a neurodegenerative disease in humans and the most common genetic cause of infant mortality. The disease results in motor neuron loss and skeletal muscle atrophy. Despite a range of disease phenotypes, SMA is caused by mutations in a single gene, the Survival of Motor Neuron 1 *(SMN1)* gene. Recent advances have shed light on functions of the protein product of this gene and the pathophysiology of the disease, yet, fundamental questions remain. This review attempts to highlight some of the recent advances made in the understanding of the disease and how loss of the ubiquitously expressed survival of motor neurons (SMN) protein results in the SMA phenotype. Answers to some of the questions raised may ultimately result in a viable treatment for SMA.

Introduction

One of the major challenges facing researchers engaged in the study of neurodegenerative and neurodevelopmental diseases is to explain why defects in ubiquitously expressed proteins have such a selective effect on the nervous system and its constituent cell types. Explaining the molecular mechanisms underlying the disease phenotypes has been hampered by their multifactorial nature and high incidence of sporadic cases. Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) are among the most common neurodegenerative diseases in humans and good examples of disorders that exemplify these challenges. Two less common neurological diseases which are monogenic in origin but are the result of defects in ubiquitously expressed proteins involved in housekeeping functions are Rett syndrome and 5q spinal muscular atrophy (SMA). The former is characterized by mutations in the gene encoding methyl – CpG binding protein 2 (MeCP2), which likely regulates gene expression and chromatin structure; the latter by a deficit of the survival of motor neurons (SMN) protein, whose best characterized function is in snRNP biogenesis and pre-mRNA splicing.

Yet, despite the apparent need for these proteins in all tissues, Rett syndrome, a prototypical neurodevelopmental disorder, is characterized by a specific defect in maintaining proper function of postmitotic neurons in the forebrain, hippocampus, and brainstem, while SMA has a particularly profound effect on lower motor neurons. This begs the question: do the proteins implicated in these diseases have functions in neurons other than their proposed housekeeping roles? Do they modulate downstream pathways specific to the tissues affected? Alternatively, are neuronal cells simply more susceptible than other tissues to genetic, biochemical, and environmental insults? Answering these questions using multifactorial diseases as paradigms poses an extra challenge precisely due to their complicated genetics. An attractive alternative would therefore be to address these issues in diseases with a simple Mendelian inheritance pattern. Spinal muscular atrophy provides researchers studying neurodegenerative diseases with this opportunity. This review highlights why (1) SMA might be considered a prototypical neurodegenerative disease in which to address certain general questions facing the field, and (2) why a thorough understanding of this disorder might shed light on the mechanisms that have a specific effect on the development, health, survival, and causes of motor neuron degeneration, which ultimately leads to a disease phenotype.

Proximal spinal muscular atrophy, commonly referred to as SMA, is a common autosomal recessive neuromuscular disease that affects the anterior horn cells of the spinal cord, resulting in atrophy of the proximal muscles of the limbs and trunk. There are numerous other forms of spinal muscular atrophy which share certain characteristics with proximal SMA; however, they are genetically distinct and often affect different subsets of neurons and muscle. They include autosomal dominant forms of the disease (Sambuughin et al., 1998; Van der Vleuten et al., 1998), X-linked forms (La Spada et al., 1991; Kobayashi et al., 1995), recessive forms that affect the distal muscles (Viollet et al., 2002), and a severe form of SMA (SMARD) with respiratory distress (Grohmann et al., 2001) (Table 1).

After cystic fibrosis, SMA is the most common autosomal recessive disorder in humans, with a carrier frequency of approximately 1 in 35 and therefore an incidence of 1 in 6000 in the human population. It is also the most common genetic cause of infant mortality. Despite the high incidence of the disease in the human population, SMA has gained relatively little attention among researchers studying neurodegenerative diseases. However, it has a fascinating biology, which includes two major players: the highly homologous *SMN1* and *SMN2* genes. A splicing defect in *SMN2* is a key factor in causing the disease phenotype, while the SMN protein, expressed by both genes, is very likely multifunctional. However, low levels of the protein have a particularly detrimental effect on one tissue type, the lower motor neurons. One of SMN's functions is essential to cell survival. These characteristics, coupled with a relatively simple Mendelian inheritance pattern, make a compelling case that study of this disorder in more detail will provide a prototype that might shed considerable light on motor neuron biology and disease. Such studies may eventually lead to a better understanding of SMA and other similar diseases and may accelerate progress toward rational therapeutics.

The Genetic Principles of SMA

Identifying the genes involved in SMA was complicated by the highly complex and unstable nature of the

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Table 1. The Different Forms of Human Spinal Atrophy

SMA Type	Mode of Inheritance	Gene/Chromosome Location	Phenotype/Disease Symptoms	Onset of Disease
Proximal SMAs				
1. Type I SMA	Autosomal recessive	SMN1;5q11.2-13.3	Proximal muscle weakness, patients never sit unaided; death < 2 years	< 6 months
2. Type II SMA	Autosomal recessive	SMN1;5q11.2-13.3	Proximal muscle weakness, patients sit unaided but become wheelchair bound, develop scoliosis of spine	6–18 months
3. Type III SMA	Autosomal recessive	SMN1;5q11.2-13.3	Proximal muscle weakness, patients walk unaided, normal lifespan	> 18 months
Distal SMA	Autosomal recessive	11q13	Distal muscle weakness, diaphragmatic involvement	2 months–20 years
SMARD	Autosomal recessive	IGHMBP2;11q13.2	Distal lower limb weakness, diaphragmatic weakness, sensory, autonomic neurons also affected	1–6 months
X-linked infantile SMA	X-linked	Xp11.3-q11.2	Arthrogryposis, respiratory insufficiency, scoliosis, chest deformities, loss of anterior horn cells	at birth
SBMA	X-linked	Androgen Receptor/ Xq11.2-12	Proximal muscle weakness, lower motor neuron loss, DRG neuron loss, bulbar involvement	30–50 years
Distal SMA IV	Autosomal dominant	7p15	Distal muscles affected, bilateral weakness in hands, atrophy of thenar eminence and peroneal muscle	12–36 years
Congenital SMA	Autosomal dominant	12q23-24	Arthrogryposis, nonprogressive weakness of distal muscles of lower limbs; several cases of affected pelvic girdle and truncal muscles	at birth

genome where they localize and by phenotypes that range in severity from the very severe (type I) to the intermediate (type II) to the very mild (some type IIIs and type IV) (Pearn, 1980). However, the genetics of SMA is actually relatively simple and unique.

Regardless of disease severity, 95% of all patients are deleted for a gene containing nine exons that localizes to chromosome 5q11.2-13.3, termed the telomeric survival of motor neuron gene *(SMN^T or SMN1)*. The remaining 5% carry small mutations in the gene. *SMN1* lies within an inverted duplication, the centromeric half of which contains an almost identical copy gene, *(SMN^C or SMN2)* (Lefebvre et al., 1995). The genes display an unprecedented level of homology that includes intronic and promoter sequences (Monani et al., 1999). SMA patients lack *SMN1*, but they always carry at least one copy of *SMN2*, which is only partially functional and therefore unable to compensate for the lack of the former. A critical, translationally silent single nucleotide C → T transition 6bp inside *SMN2* exon 7 that profoundly influences splicing (Lorson et al., 1999; Monani et al., 1999) is responsible for the difference in expression between the two genes. The single nucleotide transition also serves to make a genetic diagnosis of SMA in the vast majority of patients relatively straightforward, based on a simple PCR reaction (van der Steege et al., 1995). The PCR reaction distinguishes the nucleotides from one another and, thus, *SMN1* from *SMN2*. SMA, therefore, unlike other diseases with simple Mendelian inheritance patterns, e.g., Duchenne muscular dystrophy, cystic fibrosis, and Rett syndrome, is relatively easy to diagnose genetically. As a consequence of the nucleotide difference, the major transcript from *SMN1* is full length (FL-SMN) and includes all nine exons, while the major transcript from *SMN2*, *(SMN Δ7),* lacks exon 7. *SMN2* does produce the FL-SMN transcript but at relatively low levels (Figure 1). Patients therefore express only low levels of the functional FL-SMN protein, the SMN Δ7 isoform being unstable and rapidly degraded (Coovert et al., 1997; Lefebvre et al., 1997). Low levels of the SMN protein are clearly insufficient for the survival of motor neurons and result in the disease phenotype. Due to the unstable nature of the genome that contains the *SMN* genes, patients can carry a varying number of *SMN2* genes. The greater the number of *SMN2* genes, the more the FL-SMN protein is expressed, and the milder the disease phenotype (McAndrew et al., 1997; Feldkotter et al., 2002). This explains the range of phenotypes seen in SMA and intuitively makes *SMN2* an attractive molecular target in therapeutic strategies.

The Alternative Splicing of the SMN2 Gene

There are currently two prevailing views that provide different explanations as to the mechanism by which the single nucleotide change between the *SMN* genes alters the splicing of exon 7 in the *SMN2* gene. Cartegni and Krainer (2002) provided evidence that the difference disrupts an exonic splicing enhancer (ESE) in exon 7 to which the splicing factor ASF/SF2 binds. The efficient binding of ASF/SF2 to *SMN1* exon 7 but not *SMN2* exon 7 causes the latter to be skipped, resulting in ~10% of the transcript from the *SMN2* gene to be full length. Kashima and Manley (2003) have provided an alternative explanation in which the same difference in *SMN2* acts to create an exonic splicing silencer (ESS) to which a splicing repressor, hnRNP A1, binds. Binding of the repressor to *SMN2* exon 7 but not *SMN1* exon 7 induces skipping of this exon from a majority of the transcripts from the former gene. Although the mechanisms differ, the result is the same; i.e., vastly reduced levels of the FL-SMN transcript from the *SMN2* gene. Presently, it is unclear which of the mechanisms best explains the alternative splicing. It is possible that both contribute to the skipping of exon 7 from the *SMN2* gene. The C → T transition in *SMN2* does not affect expression of this

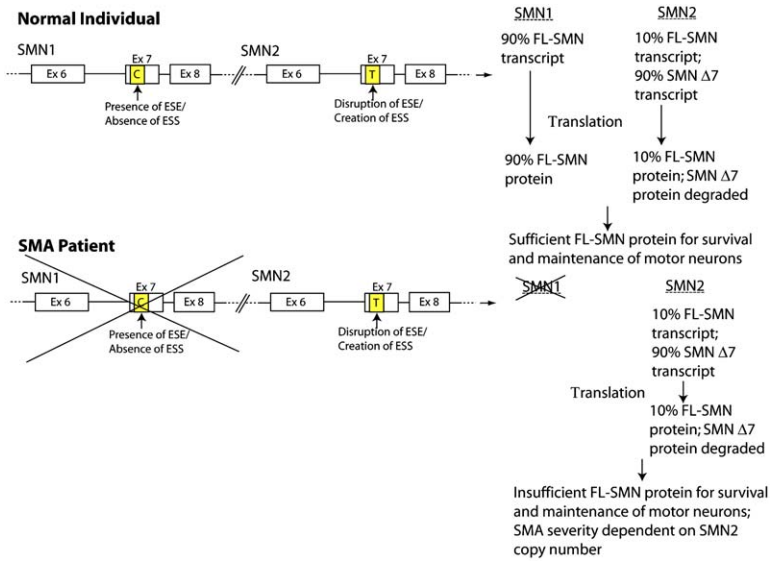

Figure 1. The Molecular Basis of Proximal Spinal Muscular Atrophy Depicting the Two Major Genes Involved in the Disease, *SMN1*, and the Modifier, *SMN2*

All SMA patients are either homozygously deleted for or contain point mutations in *SMN1* but always retain at least one copy of the *SMN2* gene. For simplicity, the genes are depicted in tandem repeat, although they can exist in a head-to-head or tail-to-tail configuration. Copy number of the genes can vary considerably in the population from 1 through 6. ESE, exonic splicing enhancer; ESS, exonic splicing silencer; FL-SMN, full-length SMN; yellow box in exon 7 denotes putative ESE/ESS.

gene at the transcriptional level, but the effect on the level of total SMN protein is profound. Studies have shown that the SMNΔ7 transcript is translated but is detected only at extremely low levels on Western blots, presumably because it is quickly degraded. *SMN2*, therefore, produces only a fraction of the SMN protein compared to its telomeric homolog, levels clearly insufficient for the health and survival of the motor neurons (Coovert et al., 1997; Lefebvre et al., 1997), and the loss of which constitutes a key characteristic of the human disease. In essence, then, SMA results from inadequate levels of the FL-SMN protein.

The Biochemistry of the SMN protein in SMA

The FL-SMN protein consists of 294 amino acids and does not exhibit homology to any previously identified protein. It migrates as a 38 kDa band on an SDS gel and is ubiquitously expressed. Particularly high levels of the protein are found in the spinal motor neurons, apparently the most profoundly affected cells in SMA patients. Individuals lacking the *SMN1* gene express vastly reduced levels of the protein, but since there is a direct correlation between *SMN2* copy number and SMN protein levels/disease phenotype, milder patients generally produce higher levels of the protein than do severely affected ones.

In 1996, a serendipitous observation noted that the SMN protein is capable of binding heterogeneous nuclear ribonucleoproteins (hnRNPs) and localizes to dot-like structures within HeLa cell nuclei (Liu et al., 1997). These structures were often found to colocalize with coiled bodies and were therefore termed gems (gemini of coiled bodies). Since coiled bodies (Cajal bodies) are known to be rich in factors involved in the transcription and processing of many types of nuclear RNAs, small nuclear ribonucleoproteins (snRNPs), and small nucleolar ribonucleoproteins (snoRNPs), it was concluded that SMN probably plays a role in RNA metabolism. This was later confirmed in a series of papers published by the Dreyfuss and Fischer laboratories which showed that SMN exists in the cell as part of a large complex consisting of at least six interacting proteins termed gemins 2-7 (Reviewed in Gubitz et al., 2004). This complex has been postulated to function as an "assemblysome" that promotes high fidelity and nonpromiscuous interactions between RNA binding proteins and their target sequences. In this capacity, the complex is thought to scrutinize RNAs for specific features that define them as snRNAs and, thus, allow the assembly of only the appropriate Sm core proteins on them in a process called snRNP biogenesis. In a series of fairly well-defined steps (Yong et al., 2004), the formation of the SMN-bound snRNP particle in the cytoplasm is followed by the particle's translocation into the nucleus, where it dissociates with the SMN complex and concentrates in Cajal bodies. The SMN complex then engages in pre-mRNA splicing.

The role of SMN in snRNP biogenesis and pre-mRNA splicing (Pellizzoni et al., 1998; Meister et al., 2000; Hannus et al., 2000) has been most extensively documented. However, numerous other SMN binding partners have been identified, and SMN has been found in various cellular compartments. It is therefore likely that SMN has other functions. Many of the SMN binding partners were identified in yeast two-hybrid screens and include the snoRNP proteins, fibrillarin (Jones et al., 2001) and GAR-1; the hnRNPs U, R, and Q (Rossoll et al., 2002); RNA helicase A; a zinc finger protein, ZPR1 (Gangwani et al., 2001); the FUSE binding protein (Williams et al., 2000); profilins (Giesemann et al., 1999); RNA polymerase II (Pellizzoni et al., 2001); p53 (Young et al., 2002a); the Epstein Barr virus nuclear antigen 2 (Barth et al., 2003); coilin; and the NS1 protein of minute virus in mice (Young et al., 2002b). The exact implications of these interactions remain to be determined. However, they may shed light on why reduced SMN levels have relatively little impact on cells other than motor neurons in the human disease.

An interesting characteristic of the SMN protein and one that has provided an explanation as to why mutations result in reduced levels, is a tendency to form multimeric structures. Studies have shown that there are two domains responsible for self-association – a minor one encoded by exon 2 and a major one encoded by

exon 6 (Young et al., 2000; Lorson et al., 1998). The ability of SMN monomers to oligomerize and form a complex that includes the integral components of the complex, gemins 2-7, stabilizes the entire structure (Lorson et al., 1998; Paushkin et al., 2002). Mutations that disrupt self-association and the formation of multimeric complexes presumably expose mutant monomers to cellular degradation, most likely by a ubiquitin ligase-mediated pathway (Chang et al., 2004). Perhaps it is not surprising that most mutations in the *SMN1* gene that have been found in SMA patients cluster in close proximity to the self-association domain in exon 6. It may also not be surprising that mutations in type I patients have the most severe defects in self-association, while those in type III patients have a much less profound effect on the ability of mutant SMN molecules to bind each other.

In addition to being able to self-associate, SMN has been shown to directly bind Sm proteins, a critical step in snRNP biogenesis (Liu et al., 1997; Buhler et al., 1999). Most mutations in the SMN protein disrupt this interaction, suggesting that the disease is a direct consequence of an inability to form and regenerate snRNP particles. However, there is at least one point mutation, E134K, and perhaps others (Cusco et al., 2004) that lie in exons 3 or 4 that may suggest otherwise. Experiments have shown that the Sm proteins bind the tudor domain of SMN, a region that spans part of exons 3 and 4 (Selenko et al., 2001), but there is lack of clear agreement as to whether this is indeed the case. An alternative theory proposes that the Sm proteins bind exon 6 of SMN (Liu et al., 1997). What is certain is that the E134K mutation in the SMN tudor domain causes a type I SMA phenotype. It is critical, therefore, to conclusively and unambiguously determine whether this mutation disrupts binding of the Sm proteins. If, as suggested, the Sm proteins bind exon 6 and E134K does not affect binding of these proteins to SMN, it could well be that the SMA phenotype is not necessarily a reflection of a defect in snRNP biogenesis.

The role of SMN in SMA: Two Contrasting Views

Given the important housekeeping role of SMN in snRNP biogenesis and pre-mRNA splicing, coupled with the very selective motor neuron disease phenotype in SMA, two schools of thought have emerged about the role of this protein in motor neuron degeneration and muscle atrophy. These divergent views, described below, stem from an expectation that a function as universal as snRNP biogenesis and pre-mRNA splicing would affect all tissues equally, and the presentation of the disease phenotype in SMA patients and animal models, which clearly affects only a specific subset of tissues – the motor neurons and muscle. An interesting parallel involves the super-oxide dismutase *(SOD-1)* gene, the product of which is also ubiquitously expressed, and the motor neuron disease amyotrophic lateral sclerosis.

(1) SMA Is a Direct Consequence of a Defect in snRNP Biogenesis and Pre-mRNA Splicing

The first school of thought postulates that SMA and the motor neuron phenotype are a direct consequence of a disruption in SMN's housekeeping role in snRNP biogenesis and pre-mRNA splicing. Moreover, reduced SMN (Wan et al., 2005) or a mutation in *SMN1* (Buhler et al., 1999) found in SMA patients compromises snRNP assembly and, therefore, spliceosomal activity. One study that supports the hypothesis that the SMA phenotype is due to a specific defect in snRNP biogenesis found that motor axon defects caused by injecting antisense morpholinos against SMN, gemin 2, and the protein pICln into zebrafish embryos can be rescued by coinjecting purified U snRNPs (Winkler et al., 2005). All three proteins have been implicated in snRNP assembly (Reviewed in Gubitz et al., 2004; Meister et al., 2001). The data may be construed as strong evidence linking the SMA phenotype to inefficient snRNP biogenesis. However, the extent of rescue varies significantly between SMN-injected and gemin 2-injected fish. One possible explanation is that restoring snRNP biogenesis alone cannot rescue the motor neurons and that SMN has a second critical function that prevents these cells from degenerating. Although snRNP assembly is compromised when SMN mutations from patients are used to study this process in vitro, attributing the SMA phenotype to SMN's role in pre-mRNA splicing is somewhat less clear. In part, this is because conclusions about the SMA phenotype being a defect in pre-mRNA splicing are based (1) on the use of a dominant-negative mutation that has never been found in patients but which does inhibit splicing in vitro; (2) the absence of such an effect when an antibody to the C terminus of SMN, presumed to disrupt oligomerization, is used in the splicing assays; and (3) lack of an adverse effect on pre-mRNA splicing using mutations commonly found in SMA patients (Pellizzoni et al., 1998). In order to explain the tissue-specific nature of SMA, this view proposes that affected motor neurons, being large, high-energy-requiring cells, simply have a lower tolerance for depleted SMN levels and are, therefore, uniquely sensitive to loss of the *SMN1* gene, compromised snRNP assembly, and inefficient pre-mRNA splicing. However, a lack of an adverse effect on other large, high-energy-requiring cells, such as cortical motor neurons and sensory neurons in SMA, makes this a questionable explanation. It is possible that the unique sensitivity of affected motor neurons in SMA is a result of aberrant splicing of one or more RNAs critical to the appropriate functioning of these cells. However, such RNAs have yet to be identified.

(2) SMA Is a Consequence of a Motor Neuron-Specific Function of the SMN Protein

A second school of thought emerged from observations demonstrating the accumulation of the SMN protein in the axons and growth cones of neuron-like cells in vitro (Fan and Simard, 2002) and anterior horn cells in vivo (Tizzano et al., 1998). These observations have been bolstered by experiments identifying the presence of SMN-containing granules within the neurites of chick cortical neurons and rat spinal motor neurons (Figure 2) that associate with microtubules and exhibit bidirectional movement between the cell body and the growth cone (Zhang et al., 2003). These particles are RNP particles and suggest a specific role for the protein in neuronal cells and, perhaps, an even more specific one in motor neurons. It is now fairly well-established that SMN can bind RNA (Lorson and Androphy, 1998; Bertrandy et al., 1999) and ribonucleoprotein particles (Jones et al., 2001; Liu and Dreyfuss, 1996; Mourelatos et al., 2001). In addition to being a constituent of snRNPs,

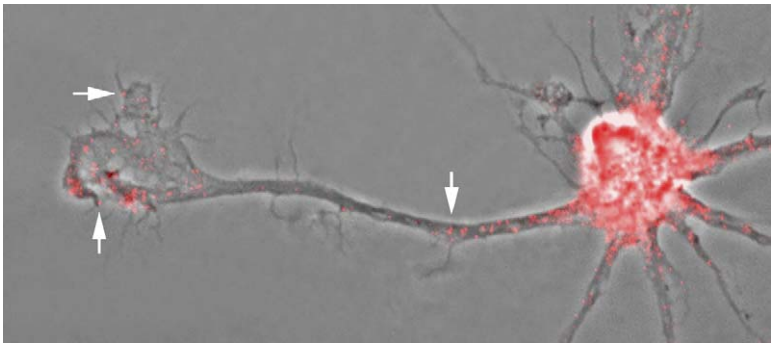

Figure 2. A chick cortical neuron stained with an antibody against SMN showing abundant nucleocytoplasmic staining as well as the presence of granules (arrows) within neurites and the growth cone. The granules, RNP particles, are found associated with microtubules, indicating movement along the axon, and supports the idea of a motor neuron specific function of the SMN protein (also see Zhang et al., 2003)

SMN may well be a constituent of a different RNP complex that in motor axons is capable of transporting specific RNAs to the growth cone in response to local cues during development. Data indicating a specific interaction between SMN and β-actin mRNA mediated by the protein hnRNP-R lends support to this idea (Rossoll et al., 2003). Reduced growth cone size, β-actin levels, and shorter neurites in primary motor neurons from SMA mice adds further weight to this line of thought. The idea of proteins involved not just in mRNA localization but also in regulating translation in neurites is not new. Both FMRP, the defective protein in Fragile X mental retardation syndrome, and the cytoplasmic polyA element binding protein have been implicated in these roles (Bassell and Kelic, 2004). SMN may have a similar role in motor neurons, thus, explaining the specific defect characteristic of the disease phenotype. Further work on the identification of specific RNAs associated with SMN granules in growth cones as well as studies examining whether SMN associates with poly-ribosomes in axons will be required to test this hypothesis.

Animal Models of SMA

Animal models can shed considerable light on the pathogenesis and mechanisms of their respective human diseases. This has been the case in SMA. Since the SMN genes were first identified, numerous organisms have been manipulated in order to generate animal models of the disease (Table 2). However, given the fact that humans are the only species with an *SMN2* gene and that the SMN protein is involved in a housekeeping function in cell survival, this has not been a particularly straightforward endeavor.

Perhaps the most pertinent of the animal models of SMA and those most likely to provide answers to the many remaining questions facing researchers studying the human disease are the murine models. Several mouse models of SMA have been generated in the last five years. These have involved basically two approaches, both dictated by the presence of only one *SMN* gene in mice, murine *Smn*, which is the equivalent of human *SMN1* and results in an embryonic lethal phenotype when homozygously deleted (Schrank et al., 1997).

To circumvent this problem, the Melki laboratory selectively and completely deleted FL-SMN in all neurons expressing a Cre recombinase under the neuron-specific enolase promoter. In what is described as the "neuronal" mutant, mice displayed motor abnormalities and signs of skeletal muscle denervation and succumbed to the disease at a mean age of 25 days (Frugier et al., 2000). These mice display a significantly larger loss of motor axons than of cell bodies, exhibit a massive accumulation of neurofilaments in terminal axons, and fail to show signs of axonal sprouting (Cifuentes-Diaz et al., 2002). The pathology described was construed to indicate a dying back axonopathy in SMA. Since muscle atrophy is a key characteristic of human spinal muscular atrophy, the Melki lab also selectively and completely deleted Smn in mature myofibers, resulting in what they termed a severe "muscular" mutant (Cifuentes-Diaz et al., 2001). Although the dystrophic phenotype including necrotic muscle elevated creatine kinase and a mean lifespan of 33 days was unexpected, in retrospect it is not completely surprising, given Smn's housekeeping function in snRNP biogenesis and pre-mRNA splicing. In fact, knocking out SMN in any cell would be detrimental to its survival, as was demonstrated subsequently in mice in which the protein was selectively deleted in liver (Vitte et al., 2004). In an extension of the muscle study, an increase in SMN levels by 50% in muscle satellite cells resulted in a much milder phenotype, termed a "mild" muscular phenotype (Nicole et al., 2003). These mice live considerably longer (~8 months), reportedly due to the regenerative capacity of satellite cells, homozygously intact for murine *Smn*, to form new muscle. These studies, in addition to in vitro work involving nerve-muscle cocultures (Guettier-Sigrist et al., 1998), have argued for a role for SMN in muscle in preventing the SMA phenotype and, thus, for targeting muscle as a therapeutic strategy in SMA.

The second approach that was used to create mouse models of SMA involved introducing the *SMN2* gene onto the $Smn^{-/-}$ genetic background either alone (Monani et al., 2000) or in the form of a BAC clone (Hsieh-Li et al., 2000) containing *SMN2*, the *SERF1* gene, and part of a neighboring gene, the neuronal apoptosis inhibitory protein *(NAIP).* Both strategies showed that the phenotype depends on the *SMN2* transgene copy number and closely mimics the human disease condition wherein all patients carry at least one *SMN2* gene. Expressing SMNΔ7, the major product of the *SMN2* gene, on a type I SMA genetic background alleviates the disease phenotype, arguing for limited ability of the mutant protein to function. An A2G missense mutation expressed in type I SMA mice generates a mild mouse model of SMA. Severe SMA *(SMN2*;$Smn^{-/-}$*)* mice carrying two copies of the *SMN2* transgene are phenotypically indistinguishable from their littermates at P0. By P3 they are visibly smaller and weaker, stop suckling

Table 2. Organisms Manipulated to Mimic Human Spinal Muscular Atrophy and Phenotypic Characteristics Observed

Organism Manipulated	Type of Manipulation	Phenotypic Characteristics of Disease
S. pombe	Deletion (null allele)	Cells not viable
	Overexpression of wild-type allele	Increase in growth rate; viability not compromised
	Overexpression of deletion alleles	Dominant-negative phenotype; growth/viability compromised; missense mutations cause mislocalization of protein to cytoplasm
C. elegans	Knockdown of wild-type allele with RNAi	Variable phenotype in progeny ranging from embryonic lethality to multiple, severe early developmental defects
	Over-expression	Embryonic viability compromised in progeny
D. melanogaster	Spontaneously occurring missense mutation	Late larval lethality; mutant larvae display loss of mobility, excitatory post-synaptic currents reduced; abnormal clustering of GluR subunits at NMJ
D. rerio	Knockdown of fish smn using anti-sense morpholinos	Embryonic lethality between late gastrulation and early somitogenesis; motor axon outgrowth and pathfinding defects
M. musculus	Homozygous knockout of Smn	$Smn^{-/-}$ → Embryonic lethality before implantation
	Cre-loxP deletion of Smn in neurons	$Smn^{\Delta 7/F7}$;*NSE-Cre* → Profound motor axon but not cell body loss; muscle atrophy of neurogenic origin; death at 4 weeks
	Cre-loxP deletion of Smn in muscle	$Smn^{\Delta 7/F7}$;*HSA-Cre* → Muscular dystrophy; death at ~33 days in "severe" muscular mutants; similar pathology but increased survival in "mild" muscular mutants ($Smn^{F7/F7}$; *HSA-Cre*) to ~8 months
	Complementation of mouse *Smn* knockout with varying copies of human *SMN2* and mutant *SMN* transgenes	1–2 copy *SMN2*;$Smn^{-/-}$ mice → type I SMA phenotype death between 1 and 8 days
		$SMN2^{+/+}$;$\Delta 7^{+/+}$;$Smn^{-/-}$ mice → severe type II phenotype death between 10 and 16 days
		$SMN2^{+/-}$;*SMN A2G*$^{+/-}$;$Smn^{-/-}$ → type III SMA phenotype death ~6 months
		8-16 *SMN2*;$Smn^{-/-}$ mice → complete phenotypic rescue
		All SMA mice display motor neuron degeneration, muscle weakness and have reduced SMN protein levels

Note: $Smn^{\Delta 7/F7}$ denotes mice carrying one deletion allele (Δ7) and one floxed exon 7 allele that can be deleted by expression of the Cre protein; NSE, neuron specific enolase; HSA, human α skeletal actin.

at ~P4, and death occurs on average between P5 and P6. Motor neuron cell body loss is not apparent in severe SMA mice at P1, but by P5 between 30% and 40% of the spinal and facial motor neurons have degenerated, the animals stop suckling, presumably due to weakness in muscles controlling swallowing and suckling, and are unable to right themselves when placed on their sides. A similar, albeit milder, phenotype is seen in mice carrying the SMN Δ7 transgene (Le et al., 2005). No abnormalities in the dystrophin-associated complex were seen in the muscle of these mice, suggesting that SMA does not involve a dystrophic phenotype. On the other hand, there is clear evidence of denervated NMJs and reduced acetylcholine receptor (AChR) clusters. Mice carrying an A2G missense mutation (Monani et al., 2003) display classic signs of type III SMA, including delayed motor neuron degeneration (Figure 3), EMG abnormalities, and axonal sprouting. Although it has been adequately demonstrated that these mice represent accurate models of the range of disease phenotype seen in human SMA, there remains a wealth of information to be gleaned from them about the details of the pathophysiology of this disease.

In addition to using the mouse to model human SMA, a number of other organisms including invertebrates have been utilized. These too lack the *SMN2* gene and, therefore, may be somewhat limited in the way they mimic the human disease. Indeed, in some cases, the phenotypes seen in the mutant animals are also rather confusing. In the nematode *C. elegans*, expression of mutant SMN protein or overexpressing the normal protein seems not to affect the particular animal in which the manipulation is carried out, but rather its offspring, in which it is generally embryonic lethal (Miguel-Aliaga et al., 1999). In *Drosophila*, a knockout of the SMN ortholog results in viable larvae but only due to a large maternal contribution of the protein, which consistently declines until death at late larval stages (Chan et al., 2003). Although these animals do exhibit a neuromuscular phenotype and suggest that SMN is important in muscle, it is unclear whether death ultimately occurs due to developmental arrest when maternal SMN is completely depleted and a failure of the imaginal discs to form adult structures.

In the zebrafish *Danio rerio*, the human disease condition was modeled by using anti-sense morpholino technology to reduce SMN levels to those presumably found in SMA patients (McWhorter et al., 2003). In a striking finding, it was observed that a knockdown of the SMN protein systemically caused pathfinding defects in motor axons. This involved truncations and premature branching at the growth cone. Furthermore, by knocking down SMN in motor neurons alone, the effect was found to be cell autonomous, having no effect on muscle development. This adds considerable support to a specific role for SMN in motor neurons and makes a strong argument for SMA being a neurodevelopmental defect. Although interesting, the finding is inconsistent with observations made in presymptomatic human SMA patients who have normal numbers of motor units, as assessed electrophysiologically by motor unit number estimation (MUNE) analysis (Bromberg and Swoboda, 2002). The latter observation argues for appropriate nerve-muscle connections to be made during development and instead suggests that SMA is not a defect in motor neuron development, but rather a problem

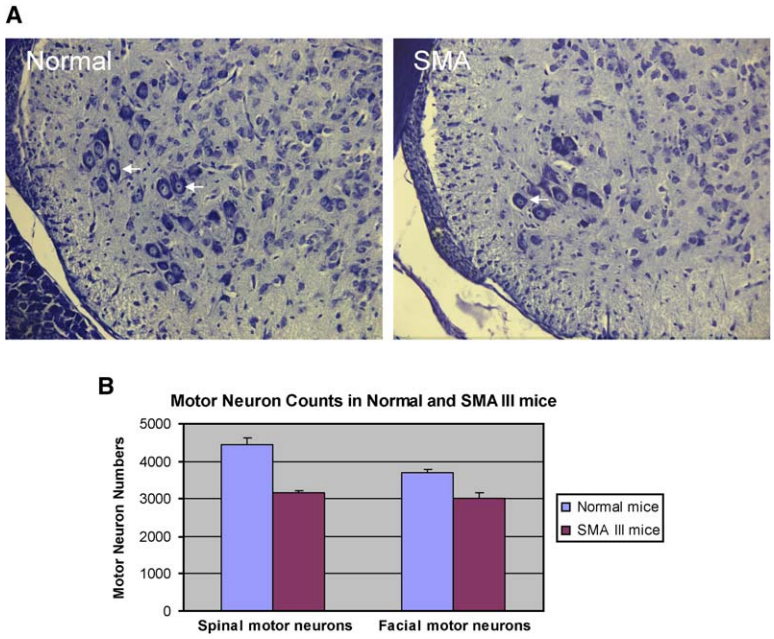

Figure 3. Motor Neuron Loss in Type III SMA Mice

(**A**) Cresyl-echt violet section of lumbar spinal motor neurons (arrows) in a 3-month-old type III SMA mouse and its normal littermate, showing reduced cells in the former.

(**B**) Quantification of lumbar spinal motor neurons and motor neurons of the facial nucleus in SMA III and normal mice (n = 3 in each case).

that may involve synaptic maintenance. At least two explanations could account for these apparently conflicting observations. First, the inability to exercise fine control of SMN levels using anti-sense morpholinos in fish resulting in correspondingly more or less severe phenotypes, and, second, a fundamental difference in neuronal development in fish and man. Findings in the fish will have to confirmed or countered in murine models of SMA.

While data from the animal models described above have certainly provided us with important clues about spinal muscular atrophy, they have also raised a number of additional questions. Answers to these will be critical in translating basic science findings into therapeutic strategies. The most pressing of these include: why does a ubiquitously expressed protein cause a motor neuron disease, and why do defects in the SMN protein fail to have such profound effect on other tissues? A related question is what is the primary target of the disease and is there is a specific compartment of the cell type most affected in SMA, where a critical level of SMN protein is required to prevent the disease phenotype? Assuming motor neurons are the primary target of the disease, one strategy to address the latter half of the question would be to separate SMN's role in snRNP biogenesis/pre-mRNA splicing from other presumed functions of the protein by selectively targeting SMN to axons or the cell body. Although the tissues most affected are the motor neurons and muscle, it is feasible that reduced SMN levels contribute to the disease phenotype by also affecting peripheral schwann cells (PSCs) or glia. In this respect, it is worth noting the important role PSCs play in the development and maintenance of NMJs and in nerve sprouting/regeneration (Koirala et al., 2003). In light of this and important insights into the pathogenesis of ALS from chimeric mice carrying a mixture of wild-type and SOD-1 expressing neuronal and nonneuronal cells (Clement et al., 2003), it may be interesting to determine the effect of normal nonneuronal cells on SMA motor neurons. Second, why are certain motor neurons and therefore certain muscles affected in this disease more profoundly than others? This implies a differential effect of low SMN levels on a particular subset of motor neurons. It may well be that these motor neurons simply constitute a larger motor unit than those that innervate fewer muscle fibers. Motor neurons that form large motor units may be selectively vulnerable to decreased SMN levels, particularly if the critical function of the protein in these cells requires it to be transported to the growth cones. In this regard, it may be worth noting that it is not even clear whether distal muscles are really spared in SMA. MUNE studies in humans carried out primarily on the ulnar nerve, which innervates the hypothenar muscle group, suggest that SMA is not restricted to atrophy of the proximal muscles (Bromberg and Swoboda, 2002). Third, is SMA a neurodevelopmental disease and, if so, how early during development are the motor neurons/muscle affected? One possibility is that nerves do make contact with muscle but the connections are simply not functional. This would likely require a detailed study of the NMJ including electrophysiological experiments. Equally important is to determine whether the disease affects only the anterior horn cells or whether it involves a defect in synaptogenesis/synaptic maintenance between Ia afferents and these cells. 5) Finally, it would be of interest, from a therapeutics standpoint, how early during the course of the disease normal SMN levels would have to be restored to affected motor neurons before they are irreversibly damaged. These experiments may be extended to answer a related question, i.e., do high levels of the SMN protein need to be maintained at all times in the relevant critical tissues or is there a window of time during development when there is an increased requirement for SMN?

An answer to the last question may already be available based on the expression profile of the SMN genes during development. Numerous studies have indicated

that SMN levels are highest during embryonic development and drop sharply by postnatal day 7. It is quite likely that this period defines when a minimum level of SMN must be maintained in order to ensure the health and normal development of motor neurons. A subsequent decrease in SMN levels, particularly during adult life, may be quite compatible with a disease-free phenotype despite a significant loss of motor neurons. A case in point is 12-month-old *Smn*$^{+/-}$ mice that display no overt motor neuron disease phenotype despite a 50% loss of their spinal motor neurons (Jablonka et al., 2000).

Possible Therapeutic Strategies in SMA and Future Prospects

Since the *SMN* genes were first cloned, much has been learned about the biology of SMA. Based on the collective findings of researchers in the field, one can begin to explore ways of treating this disease. Currently the most promising, although by no means only, strategies are as follows.

(1) Targeting the SMN2 Gene

The correlation between SMA phenotype and *SMN2* copy number in SMA patients and the demonstration that sufficient SMN protein from *SMN2* in transgenic mice can completely ameliorate the disease (Monani et al., 2000) has made this gene an obvious target that can be modulated in therapeutic strategies. In the last four years, a number of compounds, many of them histone deacetylase (HDAC) inhibitors, have been shown to upregulate SMN protein from the *SMN2* gene by activating its promoter (Chang et al., 2001; Sumner et al., 2003; Brichta et al., 2003; Grzeschik et al., 2005). One such compound, phenylbutyrate, has already been tested in humans and shown to increase FL-SMN RNA from *SMN2*, although whether this translates into increased SMN protein remains to be determined (Brahe et al., 2005). HDACs are often nonspecific and known to affect expression of ~2% of all known genes by opening up the chromatin structure of DNA and, thus, making it accessible to the transcriptional machinery of the cell. One report provides a mechanistic explanation for the effect of HDAC inhibitors on the *SMN2* gene. Treatment with one such compound, valproic acid (VPA), resulted in an increase in acetylated histones in the upstream promoter region of the *SMN2* gene that correlated with a 2-fold increase in promoter activity (Kernochan et al., 2005).

An increase in SMN protein from *SMN2* can also be achieved by altering its splicing to increase levels of the FL-SMN transcript. Small molecules (Andreassi et al., 2001) as well as the use of reagents such as peptide nucleic acids (PNAs) have been shown to effect this increase in cell culture (Cartegni and Krainer, 2003; Skordis et al., 2003). Precise mechanisms of action of these compounds have yet to be defined, but it is possible that they act indirectly by altering the activity of serine-arginine (SR) proteins which influence the inclusion/exclusion of exons into gene transcripts.

A third approach stems from the fact that the major product of the *SMN2* gene is the *SMNΔ7* isoform. The SMNΔ7 protein is unstable but is clearly at least partially functional, since it is able to increase survival when overexpressed in severe SMA *SMN2;Smn*$^{-/-}$ mice. Studies suggest that the SMNΔ7 isoform can be stabilized by using the aminoglycosides tobramycin and amikacin, which are thought to suppress recognition of the first naturally occurring translational stop codon in exon 8 of the SMN mRNA and force read through. In tissue culture assays, the modified protein resulted in increased SMN levels in fibroblasts from SMA patients, providing proof of principle of such a strategy (Wolstencroft et al., 2005). The study will have to be validated in whole organisms, but it is similar in concept to an approach used to treat a mouse model of Duchenne muscular dystrophy that harbors a nonsense mutation in the dystrophin gene with the aminoglycoside gentamycin (Barton-Davis et al., 1999).

Although the strategies described above are clearly capable of increasing SMN in cell culture assays, it is not clear how well the compounds tested will work in whole organisms. The next and most obvious step would therefore be to validate these strategies in SMA mice. These experiments are currently underway, although concrete results have yet to be made available. Despite the lack of this information, there is a pressing need to treat patients, and clinical trials using FDA-approved HDAC inhibitors have begun.

(2) Modulating Non-SMN Targets

While considerable attention has been focused on the *SMN2* gene, the identification of patients with similar 5q haplotypes but different disease phenotypes clearly points to modifiers outside the SMA locus (Prior et al., 2004). This provides the opportunity to identify additional targets that might be modulated in treating SMA. To this end, it would be useful to carry out whole genome suppressor/modifier screens in tractable organisms such as worms, flies, and fish deficient in SMN protein and displaying an SMA phenotype. This strategy may be particularly promising given a recent report that suggests that two of the members of the SMN complex, gemins 2 and 6, can modulate SMN complex activity and may therefore be candidates for modulation in SMA therapies (Feng et al., 2005). If one or both of these genes can be upregulated to increase SMN activity, particularly in the motor neurons, it is possible the effect will be of therapeutic value.

3) Gene Therapy in SMA

Although the use of small molecules to upregulate SMN protein in patients seems presently to be the most attractive approach as a therapeutic means to treat SMA, there are other opportunities as well that need to be explored. Gene therapy may be one alternative approach. In one report, SMN delivered to motor neurons using a lentiviral vector had a modestly beneficial effect in type II SMA mice (Azzouz et al., 2004). In combination with other molecules such as neurotrophic factors, the effect may be yet further enhanced, although the key challenge here will be the efficiency with which the vectors can deliver their cargo to their targets. One neurotrophic factor that has been tested is cardiotrophin-1, a member of the IL-6 cytokine family, that has a beneficial effect on the "neuronal" mouse model of SMA when administered intramuscularly in an adeno-associated vector (Lesbordes et al., 2003).

4) Stem Cell Therapy

Stem cells provide a fourth approach. Stem cell therapy has gained considerable attention for the treatment of Parkinson's and Alzheimer's diseases but poses

particular challenges if it is going to be used to replace lost cells in spinal motor neuron diseases such as ALS and SMA. A major problem is to get these cells to differentiate into the appropriate cell type and induce them to exit the spinal cord to make contact with muscle. A more feasible approach would be to use stem cells to provide trophic support and protect surviving motor neurons rather than to replace lost ones as has been demonstrated in a rat model of motor neuron degeneration (Kerr et al., 2003). Since muscle is also affected in SMA and the phenotype critically dependent on muscle atrophy, stem cells may be used to prevent muscle atrophy or replace lost muscle. A strong argument has been made for this approach based on work showing that intact satellite cells greatly improve survival in a "muscular" mouse model of SMA and nerve-muscle coculture studies indicating a deleterious effect of SMA muscle on the coculture (Guettier-Sigrist et al., 1998). Along these lines, arguments have also been made to prevent muscle atrophy by blocking ubiquitin ligases supposedly involved in this process and/or by causing hypertrophy of muscle using agents such as IGF-1 or myostatin delivered directly to muscle. However, one caveat associated with these strategies is that if SMA is a disease of muscle and nerve, attempting to rescue one without a concomitant rescue of the other will not provide long-term benefits. In fact, an unpublished study has already shown that expressing SMN in muscle tissue of type I SMA mice has no beneficial effect at all (A. Burghes and T. Gavrilina, personal communication).

Ultimately, an effective treatment for SMA will probably depend greatly on what we learn about the function of the SMN protein in motor neurons and the pathophysiology of the disease in animal models. Experiments to answer many of these questions are already underway and will, no doubt, contribute significantly to our understanding of the disease, not just from a basic science standpoint, but also in the quest to find a treatment for this devastating neurodegenerative disease.

Conclusions

In conclusion, it is obvious that significant challenges remain toward an eventual treatment for SMA. These stem from the many unanswered questions this review has raised. Readers may come away thinking that SMA research is still in its infancy. However, it should be noted that the tools to answer these questions, i.e., an excellent set of animal models and a basic understanding of the disease, already exist. It should also be reiterated that there are a number of compelling reasons investigators outside the field may find problems in SMA an attractive challenge to take on; for example, SMA is a neurodegenerative disease in which a defect in a ubiquitously expressed protein affects a very specific tissue type. The genetics of the disease are also relatively simple: SMA is autosomal recessive and involves a loss of function in an essential protein in certain cells. In essence, SMA results from an insufficient amount of the SMN protein. Furthermore, there is an almost perfect correlation between disease severity and SMN protein levels. A fascinating biology, not the least of which involves a splicing defect in the copy gene *SMN2*, is yet another reason to recruit new investigators into the field.

The presence of the *SMN2* gene in all patients immediately provides an obvious target that may be modulated in order to develop therapeutic strategies. In this respect, SMA is different from a number of other common Mendelian diseases such as Rett syndrome, cystic fibrosis, and Duchenne muscular dystrophy, wherein a loss of function due to mutations in a single gene often require the reintroduction of the native protein, a challenge that researchers have been confronted with for decades. In SMA, the problem of delivery may not exist if therapies involving the upregulation of the *SMN2* gene are formulated. Furthermore, the relatively high incidence of the disease and the unexpectedly low attention it has gained provides researchers studying related aspects of neurodegeneration with an opportune moment to apply their expertise to address some of the unanswered questions about SMA. One therefore finds it heartening that NIH has recently placed a special emphasis on finding a cure for SMA in the form of requests for applications under the aegis Project SMA dedicated to translating basic findings into a clinical treatment.

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