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Solving the Puzzle of Spinal Muscular Atrophy: What Are the Missing Pieces?

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Spinal muscular atrophy (SMA) is an autosomal recessive, lower motor neuron disease. Clinical heterogeneity is pervasive: three infantile (type I–III) and one adult-onset (type IV) forms are recognized. Type I SMA is the most common genetic cause of death in infancy and accounts for about 50% of all patients with SMA. Most forms of SMA are caused by mutations of the survival motor neuron (*SMN1*) gene. A second gene that is 99% identical to *SMN1* (*SMN2*) is located in the same region. The only functionally relevant difference between the two genes identified to date is a C → T transition in exon 7 of *SMN2*, which determines an alternative spliced isoform that predominantly excludes exon 7. Thus, *SMN2* genes do not produce sufficient full length SMN protein to prevent the onset of the disease. Since the identification of the causative mutation, biomedical research of SMA has progressed by leaps and bounds: from clues on the function of SMN protein, to the development of different models of the disease, to the identification of potential treatments, some of which are currently in human trials. The aim of this review is to elucidate the current state of knowledge, emphasizing how close we are to the solution of the puzzle that is SMA, and, more importantly, to highlight the missing pieces of this puzzle. Filling in these gaps in our knowledge will likely accelerate the development and delivery of efficient treatments for SMA patients and be a prerequisite towards achieving our final goal, the cure of SMA.

Key words: spinal muscular atrophy; SMN

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INTRODUCTION

Spinal muscular atrophy (SMA) is a lower motor neuron disease that predominantly affects spinal cord anterior horn cells and brain stem nuclei [Dubowitz, 1995; reviewed in Hamilton and Gillingwater, 2013]. Alpha-motor neuron cell degeneration results in progressive, symmetrical skeletal muscle atrophy of limbs and trunk, spreading proximal to distal [Crawford and Pardo, 1996]. Clinical heterogeneity is pervasive: based on the age of onset and on the maximum motor achievement of patients, three infantile (type I–III) and one adult-onset (type IV) forms are commonly recognized. Classification criteria are summarized in Table I. However, this rigid classification does not accurately depict the range of SMA clinical phenotypes, which display a more continuous spectrum, ranging from prenatal onset to almost asymptomatic patients. SMA is a common autosomal recessive disorder (incidence is ~1 in 6,000 in most ethnicities). Type I SMA is the most common genetic cause of death in infancy and accounts for about 50% of all patients with SMA [Crawford and Pardo, 1996; Prior, 2010].

Most forms of SMA are caused by mutations in the survival motor neuron (*SMN*) gene, which was isolated in 1995 in chromosome 5q13 harboring a segmental duplication [Lefebvre et al., 1995]. Mutations in *SMN1* are responsible for the four forms of SMA [Wirth, 2000; Alías et al., 2009]. A second gene that is 99% identical to *SMN1*, the *SMN2* gene, is located in the same region [Lefebvre et al., 1995]. The *SMN2* gene is unique to the *Hominidae* lineage [Rochette et al., 2001; Courseaux et al., 2003]. The only functionally relevant difference between the *SMN1* and *SMN2* genes identified to date is a C → T transition at position +6 of exon 7 of *SMN2* [Monani et al., 1999; Lorson et al., 1999] which disrupts interactions of the pre-mRNA with splicing enhancer and silencer proteins such that *SMN2* transcripts predominantly exclude exon 7 (SMNΔ7) [Lorson et al., 1999; Cartegni and Krainer, 2002; Hofmann and Wirth, 2002; Kashima and Manley, 2003; Singh et al., 2006; Chen et al., 2008; Gladman and Chandler, 2009]. *SMN2* genes do not produce sufficient full length SMN protein to prevent the onset of the disease but, on the other

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TABLE I. Clinical Classification of Spinal Muscular Atrophy

Type	Age of onset	Maximum motor achievement
Infantile		
I (Werdnig–Hoffmann disease)	0–6 months	Never sits unsupported
II (intermediate)	6–18 months	Never stand
III (Wohlfahrt-Kugelberg-Welander disease)	>18 months	Stand and walk
Adult		
IV	>18 years	Walk unaided

hand, because each *SMN2* gene can produce a small amount of full length SMN transcripts, no patient is devoid of SMN protein and disease severity is dependent on the amount of residual SMN protein [reviewed in Burghes and Beattie, 2009]. Deletions, most often associated with type I SMA, result in a drastic reduction in SMN protein [Lefebvre et al., 1995; Coovert et al., 1997]. Patients with Type II–IV SMA can have *SMN2*-like genes at the *SMN1* locus and thus often possess three or four copies of *SMN2* genes [Hahnen et al., 1996; van der Steege et al., 1996; Velasco et al., 1996; Burghes, 1997; DiDonato et al., 1997] capable of contributing variable amounts of functional and semi-functional SMN complexes [Lefebvre et al., 1995; Coovert et al., 1997; Burghes and Beattie, 2009] and modify disease presentation. Not surprisingly, disease severity correlates with *SMN2* gene copy number, although this correlation is not absolute [Burghes, 1997; Vitali et al., 1999; Feldkotter et al., 2002; Tiziano et al., 2007].

Since the identification of the genetic cause of SMA, biomedical research of this condition has progressed by leaps and bounds: from clues on the function of SMN protein, to the development of cellular and animal models of the disease, to the identification of potential treatments, some of which are currently in human trials. These advances have been the subject of several published monothematic reviews and will not be re-reviewed in this article; instead our aim is to not only elucidate the current state of knowledge, emphasizing how close we are to the solution of the puzzle that is SMA but to also, and more importantly, highlight the missing pieces of this puzzle. Filling in these gaps in our knowledge will likely accelerate the development and delivery of efficient treatments for patients with SMA and be a prerequisite toward achieving our final goal, which is to cure SMA.

SMN: HOW MANY FUNCTIONS CAN A SINGLE PROTEIN POSSESS?

Since the identification of the *SMN1* gene as being mutated in patients with SMA, a number of functions have been attributed to the SMN protein. So far, we know that SMN is ubiquitous, conserved across species, highly expressed during early development, and that SMN levels are higher in spinal cord and brain, but significantly down-regulated after birth [Battaglia et al., 1997; La Bella et al., 1998; Williams et al., 1999]. The SMN protein is one member of a large, highly stable macromolecular complex that localizes in both the nuclear and cytoplasmic compartments of a cell [Liu and Dreyfuss, 1996]. While we know that SMN protein produced by the *SMN1* gene is fully functional, the stability of SMNΔ7 isoform is still debated. Several lines of experimental evidence suggest that SMNΔ7 protein is rapidly degraded [Vitte et al., 2007; Cho and Dreyfuss, 2010]. The SMN C-terminal domain is highly conserved and responsible for oligomerization, a process that is indispensable for its inclusion into the SMN complex. It has been hypothesized that the inability of SMNΔ7 protein to oligomerize, coupled with the resulting reduction in interactions with its own partners, might be responsible for the instability of this isoform [Lorson et al., 1998]. However, because SMN is part of a very large multimeric complex, it is possible that SMNΔ7 is stabilized in vivo due to its association with other proteins in the complex [Burghes and Beattie, 2009]. In favor of this hypothesis is the observation that expression of the SMNΔ7 isoform in a $Smn^{-/-}$; $SMN2^{+/+}$ background dramatically improves the survival of a severe mouse model of the disease [Le et al., 2005].

The SMN complex is comprised of several proteins (many still unidentified), including Gemin2–8 and UNRIP (UNR-interacting protein, or STRAP) [Charroux et al., 1999; Charroux et al., 2000; Baccon et al., 2002; Gubitz et al., 2002; Pellizzoni et al., 2002; Carissimi et al., 2006]. The best characterized function of the complex is in the assembly of small nuclear ribonucleoproteins (snRNPs), which are involved in several aspects of RNA metabolism [see Workman et al., 2012 for a review]. These findings have led some authors to include SMA in the RNopathies, a group of diseases caused by various defects in RNA metabolism [Ibrahim et al., 2012], although the link from SMN-snRNP biogenesis to SMA pathology remains unclear.

Two additional ubiquitous functions for the SMN protein relevant to SMA pathogenicity include its involvement in the modulation of apoptosis and a novel role in translational regulation. Studies detailing SMN's anti-apoptotic role will not be discussed here as this has been reviewed recently: SMN is involved in the modulation of apoptosis not only by blocking the activation of several caspases, but also by modulating other key regulators of cell survival [see Anderton et al., 2013]. The role of SMN in translational regulation has been uncovered recently by the demonstration that SMN is associated with polysomes; this association results in the repression of translation in vitro [Sanchez et al., 2013].

Several studies have evaluated the role of SMN protein in the two cell types that are more likely the specific targets of the disease: motor neurons and skeletal muscle. In neurons, SMN protein is localized in axonal and dendritic processes, in the cytosol mainly bound to microsomes and in the cytoplasmic side of nuclear pores [Cisterni et al., 2001]. In motor neurons, SMN is localized in growth cones, along the axon and in the pre- and post-synaptic sides of the neuromuscular junctions (NMJs) [Francis et al., 1998; Broccolini et al., 1999; La Bella et al., 2000; Pagliardini et al., 2000; Fan and

Simard, 2002; Rossoll et al., 2002] where it forms a macromolecular complex distinct from the nuclear SMN complex [Zhang et al., 2006]. The SMN protein is subject to cytoskeletal-based, bidirectional transport between the soma and growth cones suggesting that SMN may have a cytoplasmic function related to neuronal transport of proteins and mRNA required at the distal tips of axons [Zhang et al., 2003; Rossoll et al., 2003; Jablonka et al., 2004; Fallini et al., 2012].

Hints regarding neuronal- and muscle-specific SMN functions were recognized through the identification of interacting proteins in the SMN-complex. These can be divided into two groups: proteins associated with cytoskeletal dynamics and mRNA binding proteins (mRBPs) involved in the regulation of translation, transport, stabilization and localization of mRNAs [Giesemann et al., 1999; Sharma et al., 2005; Bowerman et al., 2007; Wen et al., 2010]. In keeping with these findings, SMN protein deficiency could lead to the disruption of axonal transport and localization of several mRNAs, and/or of the assembly of specific snRNPs involved in transport and translation of a subset of axonal mRNAs: these defects would be responsible for the pathogenesis of SMA [see Fallini et al., 2012 for a review].

Despite these significant advances, there are several questions that remain to be answered regarding SMN function(s). While it is quite clear that SMN protein has a ubiquitous function in snRNP assembly and mRNA splicing, does this function initiate SMA pathogenicity in all forms of the disease? It is possible that this ubiquitous function might have tissue-specific effects related to a limited subset of motor neuron- and/or muscle-specific mRNAs whose splicing could be altered in the face of reduced SMN levels. However, there is no evidence of splicing defects in in vitro models unless SMN levels are severely reduced [Lotti et al., 2012]. Whereas splicing defects have been reported in motor neurons of SMA mouse models, it is unclear whether these abnormalities are directly related to the disease or a late consequence of a disturbance in motor neuron function [Zhang et al., 2008; Bäumer et al., 2009]. An alternative model might be that cells specifically targeted for SMA disease pathology might be more sensitive to changes in SMN protein levels (an SMN protein dosage effect). Arguing against this hypothesis is the observation of Gabanella et al. [2005] who reported that snRNP assembly activity is independent of SMN levels.

Also widely debated are the specific target cell types of the disease: motor neurons alone, or skeletal muscle as well? Several lines of experimental evidence suggest that skeletal muscle is not an innocent spectator but that SMN deficiency in muscle could actively contribute to SMA disease pathology [Bowerman et al., 2007, 2009, 2010, 2012]. Indeed, current evidence indicates that SMN likely plays a role in both the pre- and post-synaptic sides of the NMJ. Consequently, while SMA has classically been thought to be a purely neurogenic disease, the NMJ represents a unique functional unit that interfaces both cell types. There are other examples of proteins, such as agrin, which have a dual function in the proper formation and maintenance of the NMJ [see Zong and Jin, 2013 for a review]. As proposed in a recent review, other cell types may also be involved in SMA pathology [Hamilton and Gillingwater, 2013]. These authors argue that SMA is a multi-system disorder, especially in the case of the most severe patients. Should this model be proven correct, it is likely that systemic delivery of therapeutic medicines would be more successful in treating SMA compared to local administration in the spinal cord alone [see below and Hua et al., 2011].

Finally, independent of the SMA-related SMN function, SMN's role in snRNP biogenesis is critical, absence of which leads to cell death. We can speculate that in motor neurons, which require high SMN levels, the cell must make a choice between maintaining SMN's housekeeping function and thus survival, and carrying out SMN's cell-specific function. Thus, depending on how much functional protein is available and the strength of protein–protein interactions, there might be low to higher amounts of complexed protein available for nuclear, cytoplasmic and axonal roles. In type 1 SMA, it is more likely that most SMN complexes are sequestered to the nucleus to ensure cell body viability.

ARE SMN1 AND SMN2 AS IDENTICAL AS THEY APPEAR TO BE?

The SMN protein is subject to both temporal and spatial regulation: the highest SMN levels have been reported in brain, spinal cord, kidney and heart [Lefebvre et al., 1997; Coovert et al., 1997; Burlet et al., 1998] and SMN is especially abundant throughout embryonic development [Battaglia et al., 1997; Burlet et al., 1998; La Bella et al., 1998]. The levels of SMN protein diminish during the early postnatal period; however, the timing of this repression varies among tissues [Kernochan et al., 2005]. While SMN expression diminishes after birth, it remains high in spinal motor neurons even into adult life [Tizzano et al., 1998; Bechade et al., 1999; Pagliardini et al., 2000; Giavazzi et al., 2006]. Except for humans, expression patterns relate to the *SMN1* gene; in humans, it is difficult to associate SMN expression with one or the other copy gene so it is unclear whether regulation of the *SMN1* and *SMN2* genes is identical.

The 35.5 kb *SMN2* transgene recapitulates normal *SMN* expression patterns in SMA mice suggesting that the ~4.1 kb sequence upstream of the translation initiation site is sufficient for normal expression in vivo [Monani et al., 2000]. Both the human and mouse promoters have been systematically interrogated in a variety of cell types; however, most of this work has concentrated on the core promoter region [Echaniz-Laguna et al., 1999, Monani et al., 1999; Germain-Desprez et al., 2001; Rouget et al., 2005]. Three transcription start sites have been mapped upstream of the initiating methionine in exon 1 [Echaniz-Laguna et al., 1999; Monani et al., 1999; Germain-Desprez et al., 2001] and all three sites are used by both copy genes in a temporal and tissue-specific manner [Monani et al., 1999; Germain-Desprez et al., 2001]. The consequence of differential transcription start site usage remains unclear. A small number of transcription factors regulating the core SMN promoter have been identified including IFNγ and IFNβ [Baron-Delage et al., 2000], the CRE binding protein CREB-1 [Majumder et al., 2004] as well Sp and Ets family members [Rouget et al., 2005]. Finally, core SMN promoter activity can be modulated by a number of upstream enhancer and silencer elements [Echaniz-Laguna et al., 1999; Monani et al., 1999; Boda et al., 2004]; however, aside from their location, little else is known about their function.

The epigenetic state of DNA, histones and proteins involved in transcriptional regulation further contribute to the control of gene expression. A variety of HDAC inhibitors have been shown to up-regulate *SMN2* gene expression resulting in increased levels of SMN mRNA and protein in cultured cells and SMA mice [reviewed in Van Meerbeke and Sumner, 2011]. The role of acetylation in this response has been interrogated for the endogenous *Smn* and *SMN2* genes using NSC34 (mouse motor neuron neuroblastoma hybrid line) and fibroblast cell lines from patients with SMA [Kernochan et al., 2005]. The highest levels of acetylated H3 and H4 histones binding was mapped to the proximal promoter region, in a previously described region protected from DNase digestion [Rouget et al., 2005]. Treatment of NSC34 cells with either suberoylanilide hydroxamic acid (SAHA) or VPA resulted in hyperacetylation of the upstream promoter region [Kernochan et al., 2005]. Down-regulation of *Smn* gene expression during development was tightly associated with a decrease in acetylated H3 and H4 histones in the mouse *Smn* promoter region. In brain, HDAC1 and HDAC2 were specifically bound to the promoter and this interaction was enriched in adult compared to embryonic tissues. HDACs 3, 4, and 5 do not appear to interact with the *Smn* gene. Finally, DNA methylation was also shown to be important: distinct methylation signatures were significantly correlated with SMA disease severity [Hauke et al., 2009].

While we have made some important advances in our understanding of *SMN* gene regulation, many unanswered questions remain. What is the significance of multiple transcription start sites? Are the *SMN1* and *SMN2* genes regulated identically in all tissues and during every developmental stage given their >99% sequence identity [Monani et al., 1999] or do their unique chromosomal addresses provide distinct chromatin environments that are differentially regulated? As *SMN2* genes are not all identical, what distinguishes one from the other and how does this relate to SMA disease severity and response to therapeutic interventions? What are the signaling pathways, transcription factors, and epigenetic modifications governing temporal and spatial *SMN* gene regulation of the core promoter and distal regulatory elements? How do these elements communicate with the SMN promoter to enhance or dampen transcription and can this knowledge reveal new therapeutic targets or refine existing ones?

SMA TREATMENT: HOW, WHEN AND WHERE?

The unique hallmark of SMA is the existence of the *SMN2* gene in humans, an alternative endogenous target for the development of therapeutic strategies designed to increase the production of SMN from the copy gene. SMN dependent approaches aim to (1) increase SMN2 promoter activity, (2) reduce exon 7 exclusion, (3) protect SMNΔ7 protein from proteasomal degradation, (4) stabilize SMNΔ7 protein, or (5) introduce an exogenous *SMN1* gene. Alternative strategies independent of SMN induction are mainly aimed at preserving motor neurons through neuroprotection [see Tsai, 2012 for a review]. There have been several recent publications analyzing these strategies in depth [e.g., see Pruss, 2011], so these will not be reviewed here. Rather, we will highlight some critical studies that could fill the missing pieces required to drive future SMA therapeutics.

Since histone deacetylase inhibitors (HDAC-inhs) are the most studied compounds to date and have been tested in human trials, we will concentrate more on these molecules. HDAC-inhs not only increase *SMN2* levels, they also modify gene expression more globally. HDAC-inhs have been tested in vitro and in vivo: hydroxybutyric acid was the first compound shown to increase *SMN2* levels in lymphoblastoid cell lines from SMA patients and to also increase the lifespan of a SMA mice [Chang et al., 2001]. Since then, valproic acid, phenylbutyrate, trycostatin A, and SAHA have also been shown to increase SMN levels in vitro and/or to improve the survival of SMA models in pre-clinical studies [Brichta et al., 2003; Sumner et al., 2003; Andreassi et al., 2004; Hahnen et al., 2006; Avila et al., 2007; Narvel et al., 2008; Riessland et al., 2010]. Valproic acid and phenylbutyrate have been tested in patients with SMA but with discordant outcomes. Initial open label trials were quite encouraging both clinically and molecularly [Mercuri et al., 2004; Brahe et al., 2005; Tsai et al., 2007; Swoboda et al., 2009; Piepers et al., 2011; Kissel et al., 2011; Darbar et al., 2011]; however, these results were not confirmed in double blind placebo controlled studies [Mercuri et al., 2007; Swoboda et al., 2010; Kissel et al., 2013]. Nonetheless, these trials underscored a number of critical, as yet unanswered, issues.

SMN: How Much Is Enough?

Identifying the minimal threshold of SMN protein required for cell viability and normal function is crucial. Meeting this requirement is challenged by the fact that the extent of SMN deficiency in target tissues of SMA patients, compared to normal, is largely unknown. Also unclear is what role is played by the SMNΔ7 protein, if it is stabilized by the SMN complex in vivo. The studies of SMN expression in human tissues have been performed using semi-quantitative approaches only [Lefebvre et al., 1997; Coovert et al., 1997; Soler-Botija et al., 2005]: Coovert et al. [1997] reported a 100-fold reduction in SMN protein levels in a spinal cord sample of a type I child while Soler-Botija et al. [2002] found about a 70% reduction in SMN protein in fetuses with type I SMA. Regarding quantitative approaches, the only available data is that obtained for peripheral blood mononuclear cells (PBMCs); these values probably do not reflect those in diseased target tissues. Additionally, while *SMN2* full length transcript levels are lower in patients compared to controls and are grossly related to phenotypic severity, this observation was not recapitulated for SMN protein, which is significantly reduced only in the most severely affected patients [Sumner et al., 2006; Tiziano et al., 2010a; Tiziano et al., 2010b; Piepers et al., 2011; Crawford et al., 2012; Tiziano et al., 2013]. This being said, studies in SMA mice suggest that as little as 70% of control SMN protein might be sufficient to revert disease pathology if introduced during the pre-symptomatic period [Foust et al., 2010].

Clinical Outcomes: How to Evaluate the Efficacy of a Treatment?

The wide phenotypic spectrum of the disease, coupled with the wide age range of patients, poses significant challenges as it is clear that the same outcome measures can hardly be used in children and adults with chronic SMA. The identification of unbiased primary

endpoints for children with type I SMA is even more challenging, also due to the lack of longitudinal natural history data. These issues will not be further discussed here since they have been recently reviewed [Mercuri et al., 2012]. A battery of relevant biomarkers that significantly correlate with clinical performance could partially mitigate the effect of some of these issues. So far, in addition to the quantification of *SMN2* levels in PBMCs, that is associated with the caveats mentioned above, a panel of 12 plasma markers (SMA-MAP) correlating with the clinical performance have been identified and made commercially available, but still need further validation studies [Kobayashi et al., 2013].

Treatment Schedule: What Is the Most Appropriate Regimen for SMA Children, Youth and Adults?

A significant flaw of past clinical trials relates to the fact that, for commercially available compounds such as phenylbutyrate and valproic acid, the administration schedules used in SMA trials were those imported from the existing conditions for which the molecule was registered [Williams et al., 2012]. Consequently, it remains unclear whether these trials failed because the drugs were truly ineffective, because they did not result in the production of the required minimal threshold levels of SMN, because the clinical outcome measures were not sensitive enough to detect clinical changes in patients, or because disease-specific pharmacodynamic studies directly related to *SMN2* gene modulation were lacking and thus drug regimens were inappropriate for the treatment of SMA.

Therapeutic Window: When Will Treatment Yield the Best Benefit?

Human SMA trials of valproic acid and phenylbutyrate provided data suggesting that younger patients might be more responsive to treatment compared to older patients [Mercuri et al., 2004; Swoboda et al., 2010]. Older patients might be refractive to treatment because they have been living with their disease for longer periods of time or because critical cells/tissues are irreversibly damaged. In other words, is there a therapeutic window outside which any treatment would be ineffective or at least non-curative? The issue of a "therapeutic window" is widely discussed in the scientific community since data arising from pre-clinical studies are complex and data from similar human studies are nonexistent. Some pre-clinical studies support the existence of a critical therapeutic window, since increases in SMN levels at post-natal day 1 (P1) rescue the majority of SMA mice, but this rescue is absent if SMN levels are restored later [Foust et al., 2010; Le et al., 2011]. However, other studies did not confirm these findings [Lutz et al., 2011]. Arguing against the existence of a therapeutic window is the observation that the loss of MNs is a late and end-stage event in SMA mouse models [Monani et al., 2000; Cifuentes Diaz et al., 2002]. If suitably stimulated, and if the dying back process has not reached the point of no return (but what this point would be is not known so far), surviving MNs could hopefully still be able to provide appropriate innervation to muscle fibers. In humans, the only data that have been published so far are from post-mortem tissues and thus inform on final stages of the disease only [Ito et al., 2011] or from SMA fetuses [Fidziańska and Rafalowska, 2002; Soler-Botija et al., 2002]. Soler-Botija et al. [2002] observed an increase in apoptotic motor neurons in fetuses with SMA compared to controls, although this increase did not result in a significant reduction in the number of anterior horn cells. Ito et al. [2011] found a reduction in the number of motor neurons in infants with type I SMA post-mortem, but not in those with type II and III disease. Motor unit number estimation and compound motor unit potential data demonstrate a rapid decline in motor neuron cells early in the disease trajectory [Swoboda et al., 2005]. However, the latter findings likely relate to denervation and may not necessarily reflect motor neuron loss. We can speculate that the surviving motor neurons may represent a therapeutic reservoir which could potentially expand the size of their motor unit by sprouting, an event that commonly occurs in chronic SMA, but is unlikely in patients with type I disease due to their short survival. Nonetheless, earlier treatment should also prevent long term complications that are very common in patients with SMA; thus, the expectation is that the earlier the therapeutic intervention, the better the expected outcome. The demonstration of the existence of a therapeutic window in SMA might propel changes in our current guidelines regarding neonatal screening for SMA, a very controversial topic. While some researchers support population-based neonatal screening [Prior et al., 2010; Prior, 2010; Swoboda, 2010], the main concerns of others are related to the lack of efficacious treatments for the condition and the poor prognostic value of *SMN2* copy number assessment.

Multi-System Pathology: What Cell Types Are in Need of Rescue?

Two recently developed therapeutic approaches appear to be very promising and are in development for human trials: antisense oligonucleotides (SMN-ASOs) targeting alternative splicing of exon 7, and the restoration of *SMN1* by self-complementing adeno-associated virus (scAAV) delivery. The rationale behind the first approach is to reduce exclusion of exon 7 from *SMN2* transcripts by recruiting SF2/ASF, the main splicing factor promoting the inclusion of SMN exon 7 into mature mRNA [see Porensky and Burghes, 2013 for a review]. Several groups have demonstrated the in vitro efficiency and the pre-clinical therapeutic efficacy of SMN-ASOs [Singh et al., 2007; Hua et al., 2007, 2008, 2010; Passini et al., 2011] that have recently entered human trials: two safety open label studies have been completed (www.clinicaltrial.gov IDs: NCT01494701, and NCT01839656) and two further trials are currently recruiting (www.clinicaltrial.gov IDs: NCT01703988 and NCT01780246). In these studies, SMN-ASOs have been administered by intrathecal (IT) injection. This approach offers the invaluable advantage of modulating endogenous *SMN2* genes and sparing the use of non-SMN-targeted compounds. However, the choice of IT delivery excludes the potential beneficial effects afforded by systemic administration of the medicine; this may not be critical in the treatment of less severely affected SMA patients, but could be therapeutically relevant for type I SMA patients who may also display signs of a defect of SMN related to its housekeeping function. Recent reports suggest that, at least in

type I, SMA may be a multi-system disorder given the presence of associated pathologies that do not relate to the neuromuscular system. These co-morbidities [Bach, 2007], as well as autonomic dysfunctions [Hachiya et al., 2005], have been observed in type I patients that have survived for much longer times than usual. However, these pathologies cannot be unequivocally attributed to the peripheral defect of SMN, that is, involving tissues not directly associated with the pathogenesis of SMA.

SMN1 gene replacement by viral vector delivery is likely to be highly successful in the restoration of SMN levels. Several groups are currently evaluating the pre-clinical efficacy of scAAV which have been shown to efficiently infect motor neurons [Foust et al., 2010; Passini et al., 2010; Valori et al., 2010; Dominguez et al., 2011]. Two distinct routes of administration, intravenous (IV) and IT injections, have been tested in pre-clinical models: a better outcome, in terms of survival of the affected mice, has been achieved by IV compared to IT [Foust et al., 2010; Passini et al., 2010; Valori et al., 2010; Dominguez et al., 2011]. However, this therapeutic approach presents some issues: while IT administration could limit the spreading of scAAV infection outside the CNS, potentially reducing the risk of immunization against the virus, it precludes provision of SMN to peripheral tissues. Moreover, since scAAV does not integrate into the host genome but remains as an episome [Mezzina and Merten, 2011], exogenous SMN could be lost within a few replicative cycles. Consequently, this could require repetitive infections to maintain constantly high SMN levels in dividing tissues. It is documented that host immunization against AAV occurs naturally, and this leads to the production of neutralizing antibodies that reduce the transduction efficiency of viral particles [Treleaven et al., 2012]. As in the case of SMN-ASOs, this aspect could potentially be a more significant issue in type I patients compared to patients with chronic SMA.

CONCLUDING REMARKS

There has been significant progress made in our understanding of SMA; but, there are significant gaps that may impact on the efficacy of any therapeutic strategy. There is a need to continue to understand the multiple functions of SMN, characterize the composition of SMN-containing multimeric complexes, and determine their potential contribution to SMA pathology. It is clear that SMN fulfills multiple functions; there is good evidence for a role in snRNP biogenesis and evidence is accumulating to indicate that SMN is involved in the maturation and maintenance of the NMJ by an as yet undefined mechanism. Thus, it would not be surprising that the underlying pathogenic mechanisms leading to clinically heterogeneous SMA will be complex. The SMN protein may contribute to SMA pathology in distinct ways at distinct stages of the disease in a SMA type-specific manner.

The *SMN2* gene is exclusive to humans; the evolutionary meaning of the fixation of *SMN2* from non-human to human primates is unknown. The differential regulation of *SMN1* and *SMN2* is unknown as well: understanding this might help shed light on the developmental role of SMN.

- Because there is probably no single pathogenetic mechanism, the best therapeutic window and regimen may be distinct for each SMA type. For example, early and combinatorial therapy is likely to be needed to effectively treat type I SMA.
- It is conceivable that small molecules increasing SMN levels with systemic biodistribution might be the best for patients with type I SMA, although the very negative aspect of lifelong administration might introduce unexpected toxicities in these very fragile children.
- The need for biomarkers (molecular? Electrophysiological? Imaging?) that reliably track treatment response is undeniable; biomarkers that predict clinical improvement of patients could be used as surrogate measures or even as primary endpoints in clinical trials. A priori, there is a need to define significant preclinical outcomes that would justify transitioning medicines to human clinical trials. In addition, a battery of meaningful therapeutic outcomes must be established for all forms of SMA.
- In living with SMA, there is a need for evidence-based standardized assessment and management protocols for the care of patients with SMA.

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This review is dedicated to the memory of Dr. Christina Brahe, who has dedicated most of her professional life to the dream of seeing patients with SMA walking, and to all families and patients with SMA worldwide.

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