Is Spinal Muscular Atrophy a disease of the motor neurons only: pathogenesis and therapeutic implications?

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Abstract

Spinal Muscular Atrophy (SMA) is a genetic neurological disease that causes infant mortality; no effective therapies are currently available. SMA is due to homozygous mutations and/or deletions in the Survival Motor Neuron 1 (*SMN1*) gene and subsequent reduction of the SMN protein, leading to the death of motor neurons. However, there is increasing evidence that in addition to motor neurons, other cell types are contributing to SMA pathology. In this review, we will discuss the involvement of non-motor neuronal cells, located both inside and outside the central nervous system, in disease onset and progression. These contribution of non-motor neuronal cells to disease pathogenesis has important therapeutic implications: in fact, even if SMN restoration in motor neurons is needed, it has been shown that optimal phenotypic amelioration in animal models of SMA requires a more widespread SMN correction. It will be crucial to take this evidence into account before clinical translation of the novel therapeutic approaches that are currently under development.

Keywords

Spinal Muscular Atrophy; pathogenesis; therapy; central nervous system

1. Introduction

Spinal muscular atrophy (SMA) is the most common genetic neurological disease leading to infant mortality. It is characterized predominantly by spinal motor neuron loss, muscle

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atrophy and motor impairment [1,2]. There is currently no effective treatment for this disease, although several promising therapeutic strategies are under development [3,4]. SMA is caused by mutations in the Survival Motor Neuron 1 (*SMN1*) gene, which result in functional SMN protein deficiency, leading to motor neuron degeneration.

SMN is ubiquitously expressed and its well-characterized function is linked to a critical pathway of RNA metabolism: the small nuclear ribonucleic proteins' (snRNP) biogenesis [5]. Briefly SMN, in collaboration with partner proteins, catalyzes the assembly of snRNPs, which are the building blocks for pre-mRNA splicing [5]. Highlighting the importance of this function, which is essential for every cell, the complete loss of SMN protein results in embryonic lethality in knockout animals [6]. In contrast, SMA is caused by various degrees of reduction in SMN levels. In line with this, Cre-Lox transgenic rodent models in which full-length SMN is completely ablated in specific tissues, have shown that the absence of SMN protein leads to dramatic tissue-specific defects that are more severe than the typical SMA phenotype, where residual SMN protein levels are still present [7,8].

SMA is a neurodegenerative condition and is widely referred to as a motor neuron disease. Although some researchers consider that the motor neuron is the primary site of pathology in SMA and SMN restoration in these cells is sufficient for therapy [7,9,10]; other work suggests that multiple organs contribute to the phenotype, in particular in the most severe forms of SMA [11,12]. From a clinical point of view, SMA is classified into three types based on age of onset and clinical course. Type I SMA is the most severe infant form, type II is an intermediate form affecting toddlers, and type III is a mild juvenile form. Type 1 and type 0 SMA patients, can present some systemic signs and symptoms such as autonomic dysfunction, cardiac impairment, and, rarely, skin necrosis [13,14]. Indeed, these systemic features are even more prominent in the most widely used severe transgenic mouse models of SMA [15-17]. When SMN levels are dramatically diminished, this more widespread pathology is probably linked to the general RNA metabolism pathways described above [17]. On the other hand, in the mildest forms of SMA, in which SMN levels are relatively higher, only motor neurons are affected, suggesting that this cell type is more sensitive to SMN reduction. Thus, it is legitimate to hypothesize that there is a variable threshold of susceptibility to SMN reduction in different cell types. The level of SMN in other tissues could be important to better understand the role of SMN protein, and the effects of its depletion could be useful to develop new therapeutic strategies against SMA.

Humans and bonobos are the only species in which two copies of paralogous inverted *SMN* genes in chromosome 5 are present [18]. The paralogous gene SMN2 differs from SMN1 by a single C-to-T transition in exon 7 that modifies a splicing modulator and causes deletion of exon 7 in 90% of SMN2 mRNA transcripts [19]. The SMN protein lacking exon 7 does not oligomerize efficiently and is quickly degraded, leading to the reduction of total SMN levels. The SMN2 gene produces approximately 10% of full-length SMN2 protein [19], which is a major modulator of SMA clinical phenotype

As a result, only a small percentage of full-length, functional and stable SMN protein is produced from *SMN2*. Due to this partial SMN production, the number of *SMN2* copies is inversely correlated with disease severity in patients [20]. In fact, it is known that there are asymptomatic subjects carrying homozygous *SMN1* gene mutations and multiple copies of *SMN2*; while at the other extreme, the total absence of SMN products is invariably fatal [5].

In this review, we will discuss the involvement of non-motor neuronal cells, located both inside and outside the central nervous system (CNS), in SMA pathogenesis. We will discuss the different mechanisms that could play a role in disease initiation and progression, as well as the important implications for the optimal design of novel effective therapeutic strategies. Motor neurons are the predominant target in the majority of therapeutic approaches. However, the consideration of other cell types affected by SMN deficiency, at different time points of the disease, could be crucial for therapeutic success in SMA patients. Taking into consideration all these aspects, it is crucial to focus on targeting both motor neurons and other cell types and to define the therapeutic window when interventions can still be meaningful, when developing new therapeutic strategies.

2. Therapeutic implications in non-motor neuron cell types

Rodents, in contrast to humans, carry only the smn gene. For the generation of mouse models of SMA, scientists have combined the genetic deletion of endogenous mouse smn with the insertion of several copies of human *SMN2 [21]*. The genetic insertion of two copies of *SMN2* and of a transgene missing the exon 7 sequence (SMNΔ7) in smn knockout murine embryonic cells has led to the generation of a mouse strain known as SMNΔ7 [22]. SMNΔ7 mice are widely employed in pre-clinical studies of SMA since they recapitulate many key aspects of the disease, including severe progressive muscle weakness and an average lifespan of about 2 weeks [22]. Although there is limited muscle denervation and overall motor neuron loss, specific muscle groups and motor neuron subsets in these mice show greater vulnerability compared to others [23-25].

Several groups have shown that the restoration of *SMN1* in SMA mice, using a motor neuron specific promoter (homeobox gene 9 (HB9) or choline acetyltransferase (ChAT)), resulted only in a modest extension of survival [9,26,27]. Conversely, *SMN1* expression in SMA mice using a promoter highly expressed both in neurons and astrocytes (prion promoter), significantly extended their survival [7]. These crucial findings, together with others, suggest that astrocytes, sensory neurons, Schwann cells and skeletal muscle may all contribute to the expression of the disease and its associated motor neuron loss [28,29,27,30,25,31,32]. Additional evidence of the potential key role of non–motor neuronal cells in SMA pathogenesis was recently provided by an effort to up-regulate SMN protein, introducing the wild-type *SMN1* gene [33-36], or by modulating *SMN2* splicing with oligonucleotides or small molecules in mice (for review see [4], [37,38]. Several recent studies have demonstrated that these strategies can significantly increase survival of SMA mice [39-44,38]. In particular Foust and his group obtained the most profound phenotypic correction in terms of rescue of motor function, neuromuscular physiology and life span [40]. Here, vascular delivery of scAAV9 encoding SMN at postnatal day 1 in SMA pups was employed to increase levels of SMN protein. In contrast, Hua used a different strategy based on antisense oligonucleotides that effectively corrected SMN2 splicing and restored SMN expression in motor neurons. In agreement with the first study, the systemic administration of gene-correcting agents to neonates robustly rescued the severe SMA mice phenotype [16]. Also, in a recent paper Hua and collaborators demonstrated that increasing SMN exclusively in peripheral tissues completely rescued necrosis in mild SMA mice, and significantly extended survival of severe SMA mice, with noticeable improvements in motor neuron survival, neuromuscular junction integrity, and motor function. Accordingly, they conclude that the SMA phenotype in murine models is not the result of a cell-autonomous defect of motor neurons [45].

3. Role of non-motor neuronal cells located inside the CNS

3.1. Interneurons and sensory neurons

Numerous *in vitro* and *in vivo* studies have shed light on discrete alterations in sensory neurons and interneurons in SMA. For instance, Jablonka and collaborators (2006) (Table 1) have demonstrated that in Smn-deficient sensory neurons isolated from the severely affected SMA mouse model (Smn −/−; SMN2), growth cones are smaller, neurites are shorter, and levels of both β-actin mRNA and protein are reduced in comparison to neurons from control animals; without affecting the survival of these cells in culture [29]. *In vivo*, the sensory neurons of embryos from the same SMA mouse model did not develop normally. In particular, they exhibited smaller terminals in the skin and altered neurite growth and growth cone morphology compared to controls [29]. In the relatively less severe SMA mouse model, SMNΔ7, early alterations of monosynaptic connections between proprioceptive (sensory) neurons and motor neurons, such as reduction in the number of VGluT1+ synaptic terminals on motor neuron soma were shown to precede motor neuron loss [25]. Furthermore, a recent study performed by Zhang and colleagues, has found that motor neuron loss is preceded by a dysregulation of mRNAs critical for synaptic formation and sensory-motor circuitry in SMA mice spinal cord [46]. Importantly, abnormal conduction velocity and axonal degeneration of sensory neurons are also reported in severe SMA type I patients [47]. In a Drosophila model of SMA, the groups of McCabe (2012), and Pellizzoni (2012), have shown that when SMN is deleted in sensory neurons, their excitability is reduced causing secondary motor neuron dysfunction [28,48] (Table 1). In 2012, Pellizzoni’s group identified a novel gene called Stasimon, which is involved in U12 related splicing defects in the context of reduced SMN levels [48]. They demonstrated that aberrant splicing of Stasimon in cholinergic sensory neurons and interneurons causes SMN-related phenotypes, while its re-expression leads to the complete restoration of neuromuscular junction activity and muscle size.

In light of these studies suggesting that SMN levels and function in sensory neurons and interneurons contribute indirectly to motor neuron function, it has been hypothesized that some primary defects appearing in afferent sensory neurons may contribute to, or may exacerbate a secondary motor neuron dysfunction and loss [9,24,27]. In contrast, other studies point to motor neuron dysfunction triggering secondary sensory and interneuron deficits in SMA [9,27,49,50]. In a cellular model employing iPSC-derived neurons, Schwab and Ebert (2014) (Table 1) have used a direct co-culture approach to assess whether sensory neurons established from SMA-derived iPS cell lines can lead to WT motor neuron loss [51]. They found that SMA iPSC-derived sensory neurons have a negligible impact on motor neuron survival, or expression of glutamate transporters (VGLUT1, VGLUT2) in neurites and cell bodies of motor neuron; concluding that SMN-deficient sensory neurons are not the primary trigger of motor neuron loss [51]. As described above, it has been shown that motor neuron loss is preceded by a small reduction in VGLUT1+ bouton numbers on sensory neurons [24]. From a neuropathological point of view, at the end stage of the disease, the number of VGLUT1+ synapses decreases in motor neurons but not in sensory neurons, while VGLUT2 excitatory boutons reduce in L3–L5 lateral motor neurons, raising the possibility that synaptic defects might appear in interneuronal circuits [24]. This evidence lead Thirumalai and collaborators to conclude that the loss of proprioceptive synapses on motor neurons may be secondary to motor neuron pathology [50]. Similarly, amelioration of electrophysiological deficits and loss of sensory-motor synapses could be achieved through increasing SMN levels in motor neurons in SMNΔ7 mice, and even in the severe murine model, suggesting that the reduced levels of SMN in motor neurons contributes to the motor circuit dysfunction [9,52,27].

In SMA, the de-afferentation of motor neurons is an early event leading to motor impairment [25]. Moreover, Reanult and co-workers (1983) showed reduced H-reflexes in type I human SMA patients that is responsible for the muscle weakness caused not only by proprioceptive abnormalities but also complicated by neuromuscular denervation and motor neuron loss [53]. In addition to abnormal sensory nerve conduction velocity [54,55,47], nerve biopsies have revealed sensory axonal degeneration in SMA [47]. Overall, it remains unclear whether the loss of sensory-motor synapses is the cause, or the consequence of motor neuron loss; further studies are clearly needed to decipher the sequence of events in the pathophysiology of SMA.

3.2. Astrocytes

Astrocytes are the most abundant cell type in the CNS and are present both in white and grey matter. These cells are characterized by a star-shaped cytoplasm, comprised of intermediate filaments (glial fibers), extending numerous processes that surround adjacent blood vessels and neurons. Astrocytes have different roles, such as maintaining neuronal functions, supporting synaptic interactions, and serving as neural precursors in adult neurogenic regions (Figure 1). Moreover, through the reuptake of neurotransmitters, they can contribute to overall synaptic activity; and are a critical component of the blood-brain barrier (BBB), regulating the transport of different molecules across this barrier. [56]. Multiple cellular studies have demonstrated that astrocytes are able to release proteins involved in the control of neuronal maturation and differentiation. Through the release of neurotrophic factors, including neurotrophin-3 (NT-3) [57], brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), astrocytes can enhance neural survival and control both neuronal differentiation and growth [58]. Furthermore, other molecules, such as S100B which is a glial-specific protein expressed primarily by astrocytes, can be released and exert neuroprotective effects against glutamate excitoxicity [59].

In a neurodegenerative disorder such as SMA, there is growing evidence that astrocytic functions are disrupted, giving rise to the hypothesis that astrocytes could contribute to the disease process. In fact, during disease progression, astrocytes can become reactive exhibiting both morphological and functional changes. McGivern and collaborators recently demonstrated that astrocytes in the SMAΔ7 mouse spinal cord and even astrocytes derived from SMA-induced pluripotent stem cells show morphological and cellular alteration indicative of activation before the motor neuron loss [30] (Table 1). This indicates that there may be a direct consequence of SMN deficiency intrinsic to astrocytes, and that disruption of their function may be due to this loss rather than because of altered neuronal-glial signaling. No significant differences were reported between wild-type (WT) and SMA mouse astrocytes in early post natal days, but at post natal day 9 SMA astrocytes exhibited augmented cell bodies, increased expression of GFAP in the cytoplasm, and thin retracted processes. Moreover, reactive astrocytes produce pro-inflammatory cytokines, which can trigger an apoptotic cascade and motor neuron loss, in particular through ERK1 and ERK2, which are members of the MAPK signaling pathway [30]. McGivern and colleagues (2013) have hypothesized that increased phosphorylation of ERK1/2 observed in SMA iPSC-derived astrocytes, can have a direct role in apoptosis' induction through an increased expression of pro-inflammatory and pro-apoptotic cytokines such as TNFα, IL-1 and IL-6 [60-62]. In the context of growth factor deprivation, the same group also showed reduced secretion of GDNF in SMA iPSC-derived astrocyte cultures, which can be an indirect signal for the activation of the apoptotic pathway involving ERK. Astrocyte communication with other astrocytes and neurons can also take place through gap junctions, adenosine triphosphate (ATP) release, and calcium-dependent glio-transmission [63]. In a cellular model, McGivern and colleagues found that there is a dysregulation of basal calcium and decreased response to ATP stimulation, confirming abnormal astrocyte function [30] (Figure 1). Rindt and colleagues examined the involvement of SMN deficiency specifically in astrocytes in post-mortem human tissue and *in vivo* models of SMA. They reported prominent astrogliosis in end-stage SMA mice as well as post-mortem patient spinal cords. Importantly, restoration of SMN protein levels in astrocytes, using a viral vector-based approach, resulted in increased survival in both severe and intermediate models of SMA. In addition to an improvement of neuromuscular circuitry, the increased expression of proinflammatory cytokines was partially normalized in treated mice, suggesting that astrocytes directly contribute to the pathogenesis of SMA [64].

It is important to note that some groups have demonstrated that motor neuron loss is detectable only at the end stage of SMA [23,22]. As is commonly observed in other neurodegenerative diseases, the earliest structural defects appear distally, involving the neuromuscular synapse in the case of SMA. Prior to death of the motor neuron, there are pre-synaptic defects that include loss of terminal arborization as well as intermediate filament aggregation, which causes intermittent neurotransmission failures [65]. For this reason, it may be inferred that MN death is a late event in SMA pathogenesis, preceded by the activation of astrocytes leading to secondary motor neuron degeneration [56]. As discussed above, cellular studies have demonstrated that astrocytes participate in the regulation of synaptic function by providing an optimal presynaptic environment, triggering synaptic maturation, and maintaining synaptic activity and stability [66]. In neurodegenerative diseases, their function becomes deregulated, leading to the loss of central synapses, as showed by Tarabal and collaborators [67] (Table 1).

Overall, there is growing evidence suggesting that although the expression of SMN in motor neurons is essential, its restoration is not sufficient to significantly enhance survival in mouse models of SMA. When SMN1 is expressed highly in both astrocytes and MNs, survival is maximized [26,30]. These data implicate astrocytes in the pathogenesis of SMA and indicate that astrocytes could be an additional target for therapeutic intervention.

3.3. Microglia

Microglia represent the endogenous immune cells in spinal cord and in brain and mediate the innate immune response in the CNS to various types of pathogenic insults. Microglia are derived from hematopoietic stem cells, particularly precursors of the monocyte and mesodermal lineages. Micro and macroglial cells have an important role in the formation, function and elimination of synapses under normal and pathologic conditions (Figure 1) [68].. Neuroinflammation plays a key role in the pathology of several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (ALS) [70-75]. In the case of SMA, microglial activation is observed early in the patient's spinal cord [67]; however the pathological role of microglial cells in SMA has not yet been fully characterized. Recently, Tarabal and colleagues [67] (Table 1) detected activated microglia in lumbar spinal cord at different stages of a murine model of SMA [early post-natal, presymptomatic (P4-5), early symptomatic (P7-8), and end stage (P14-15)]. Immunoreactivity for the specific microglial marker, IbaI, was markedly increased, surrounding motor neurons in the ventral horn, in a time-dependent fashion in the lumbar spinal cord of SMNΔ7 in comparison to WT animals. This increase was significant at P7–8, and persisted until the late stages of the disease.. Moreover, in agreement with previous studies, they demonstrated an increased loss of glutamatergic terminals in SMNΔ7 spinal cord during postnatal development [76,24,25,10]. Importantly, in agreement with the findings of Mentis [25], but in contrast to Ling and colleagues, the study of Tarabal and co-workers showed that the damage of central synapses in SMA precedes MN death [67], as discussed above. In contrast, Ling and colleagues (2010) showed that while glutamateric terminals on motor neurons are reduced in SMA mice, this occurs in the absence of microgliosis in the spinal cord; leading these authors to conclude that neuroinflammation is not a major contributing factor to the disease mechanism in models of severe SMA [10].

The phagocytic function of microglia cells for the clearance of degenerating neurons has not been fully considered in the past, but it is an increasingly recognized phenomenon in many neurodegenerative diseases [77]. This activity of microglia is also involved in synaptic elimination. In a mouse model of SMA, active microglial cells were detected engulfing structural complexes including damaged presynaptic terminals and postsynaptic dendrites [67]. These findings don't exclude the relevance of this cell type to the elimination of cellular fragments during synapse degeneration [67]. Examination of SMA post mortem spinal cord reveals a severe loss of myelinated fibers with numerous glial bundles in the anterior horns [78]. These glial bundles are most abundant in the lumbar regions and are observed only in the anterior spinal roots and not in the posterior roots. Glial bundles are characteristic, but not specific, to SMA and it is hypothesized that they are secondary to neuronal degeneration, rather than causing the degeneration of anterior horn cells [78]. Another interesting finding raised in this study is that the loss of GABAergic and glutamatergic synapses on MNs could result from the induction of neuronal nitric oxide synthase (nNOS), which is upregulated in SMNΔ7 MNs [67]. It is known that nitric oxide (NO) can induce synaptic degeneration following a physical injury to motor nerves; and is also found in ALS [79]. Moreover NO produced via up-regulation of nNOS could lead to activation of the RhoA/Rho kinase (ROCK) pathway [80]. This pathway is abnormally activated in the SMA mice, and ROCK inhibitor treatment improves maturation of the NMJ, and increases survival in treated mice [81].

4. Other cell types outside the central nervous system involved in SMA

4.1. Peripheral nervous system involvement: Schwann cells

Schwann cells are the main glial cells of the peripheral nervous system (PNS). There are two types of Schwann cells, myelinating and non-myelinating, that are both essential to axon stability and neuronal survival, notably through the production of proteins involved in the formation and maintenance of the extracellular matrix (ECM) [82,83]. In addition to this, myelinating Schwann cells also form the protective myelin sheath wrapping around axons of motor and sensory neurons, which allows a substantial increase in nerve conduction velocity.

Myelinating Schwann cell dysfunction is known to play a crucial role in hereditary peripheral neuropathies that are usually associated with axonal atrophy and degeneration of lower motor and sensory neurons [84]. More recently, low levels of SMN were reported to cause changes in Schwann cells that lead to abnormal axon myelination and interrupted the deposition of extracellular matrix proteins in peripheral nerves (Figure 2) [85]. In this study, Hunter et al. (Table 1) demonstrated that Schwann cells, isolated from SMA mice, had reduced levels of SMN as expected, but did not respond normally to differentiation signals in culture. Concurrently, they observed morphological imperfections in myelination in SMA mice that were associated with alterations in the expression of myelin-associated proteins, such as peripheral myelin protein 22 (PMP22), myelin protein zero (MPZ), and myelin basic protein (MBP) [86]. They further demonstrated that primary Schwann cells derived from SMA transgenic mice cannot successfully myelinate healthy neurons in co-culture, and that they exert a detrimental influence on neuritic stability. One possible explanation for these changes could be that low levels of SMN impair the regulation of expression of myelin proteins, which normally occurs during postnatal development [85]. By genetically-correcting SMN levels in Schwann cells, Hunter et al. showed that these defects were SMN-dependent and reversible. Alternatively, these myelination defects could also be explained by the reduced capacity of SMA-derived Schwann cells to produce key proteins composing the ECM of peripheral nerves. This is supported by other studies showing that the expression levels of several ECM proteins, including laminin alpha 2 (LAMA2), are severely altered in the context of SMN deficiency [87,88]. Consistent with this, it was shown that impairment of extracellular laminin-mediated control of beta-actin translation is contributing to SMA motor neuron axonal defects [89].

To understand how SMN deficiency can disrupt Schwann cell physiological functions, Sarvestany and collaborators (2014) (Table 1) performed label-free proteomics analyses of Schwann cells obtained from SMA mouse peripheral nerve [90]. Through this approach, they found that several pathways were altered in SMA Schwann cells including growth, proliferation, cell death, survival and molecular transport. Of particular interest, functional cluster analysis revealed that ubiquitination pathways are profoundly disrupted in SMN-deficient Schwann cells. For example, reduced levels of ubiquitin-like modifier activating enzyme 1 (uba1) were observed [90]. In fact, suppression of Uba1 through pharmacological treatment in WT Schwann cells is sufficient to recapitulate the defective myelination phenotype presented by SMA Schwann cells [90]. Microarrary analyses of Schwann cell myelination-related genes revealed disrupted gene expression in tissue from SMA mice in comparison to WT [87,88].

Altogether, these data highlight the important role of the SMN protein in regulating the ubiquitination pathway and maintaining the homeostasis of Schwann cells.

4.2. Neuromuscular junction and muscle cell involvement

Longitudinal studies in a severe mouse model of SMA have revealed that early symptomatic stages of the disease are characterized by morphological and functional anomalies at the synapse; affecting both NMJs [76,65,23,91,24,92] and synaptic inputs to motor neurons in the spinal cord [24,25] (Table 1). To understand the underlying mechanisms of this early synaptic pathology in SMA, Martinez and colleagues have recently generated three lines of SMA mice that express increased levels of SMN in motor neurons and/or muscle [27]. In this study, they showed that the increase of SMN expression in motor neurons improved synaptic function both at the NMJ and motor neuron somas, whereas selective over-expression of SMN in muscle restores muscle mass without affecting synaptic integrity [27]. They concluded that SMN could have a role in the growth of muscle that is independent of its effect on motor neuron synaptic function; suggesting that SMN has multiple roles in the motor neuron unit (fig.2).

Some studies have reported that high levels of SMN expression in muscle can affect the SMA phenotype in Drosophila melanogaster [93,5]. To investigate whether this is also the case in mammals, Gavrilina and colleagues (2008) (Table 1) have used transgenic SMA mouse models in which SMN expression is restored in skeletal muscle fibers under the human skeletal actin (HSA) promoter [94-96] and in neurons under the prion promoter (PrP) [97], which is also known to lead to high expression in astrocytes [97,7]. In this study, both lines were null for the murine smn gene, and homozygous for the human *SMN2* gene. They found that full-length SMN expressed under the PrP promoter had a strong beneficial impact on survival and phenotype in the severe SMA mouse [7]. In contrast, restoration of SMN expression in muscle alone was unable to correct the phenotype of the disease. This confirms that re-establishment of SMN expression in neurons, and possibly astrocytes, is required for maximal disease-modifying effect in SMA [7]. Many studies have reported a direct or indirect interaction of SMN with molecular modulators of actin dynamics such as plastin 3, profilin, small Rho GTPases Cdc42 and RhoA, as well as ROCK, a direct downstream effector of RhoA [98-101]. If activity of the RhoA/ROCK pathway is increased in SMN-depleted neuronal cells and tissue, inhibition of actin-mediated neuronal outgrowth and differentiation is observed. Conversely, when SMA mice are treated with ROCK inhibitors, they show an improvement of skeletal muscle mass and NMJ morphological maturation and an accompanying increase in life span [103,81] (Table 1), with no change in the number of surviving motor neurons or in the expression of SMN protein [102].

SMN has also been implicated in myoblast fusion, proliferation and in the correct formation of myotubes [104-108] (Figure 2 and Table 1). Among its many functions, the RhoA/ROCK pathway is also involved at multiple points as, interestingly, it also regulates muscle contraction [109] and skeletal myogenesis [110]. It is possible that modulation of myogenic regulatory factors as well as actin-dependent myogenesis via regulation of ROCK signaling is sufficient to improve survival in mouse models of SMA. This raises the possibility that, to ameliorate disease progression, a modulation of myogenic regulatory factors and an actin-dependent regulation of myogenesis might be considered as a therapeutic target [102].

Altogether, these data demonstrate that the expression of high levels of SMN contribute to myofiber growth and development, but the full restoration of muscle fibers in severe form of SMA may require the targeting of other muscle cells, such as the satellite cells, which are crucial in the maintenance and regeneration of the muscle mass. This possibility is supported by the significant effects of ROCK inhibitors in SMA mice, which bypass the replacement of SMN full-length protein by targeting downstream pathogenic events.

Other targets, independent of efforts to enhance SMN protein levels in muscle tissue, have been explored as possible disease-modifying approaches. Lorson and collaborators [111] investigated the role of follistatin, a cystine-rich glycoprotein that binds to and inhibits several TGF-β family members [23,24], including myostatin [5,9,25], in SMA progression. They demonstrated that recombinant adeno-associated virus (AAV) expression of follistatin enhances muscle mass in WT and SMA mice. This finding suggests that the administration of follistatin and the subsequent inhibition of the myostatin pathway are beneficial in SMA models as they preserve the expression of muscle-derived neurotrophic factors in SMA mice.

Another substance beneficial for motor neuron survival is insulin-like growth factor I (IGF-1). Tsai and colleagues evaluated the efficacy of intravenous administration of a recombinant AAV1 vector encoding human insulin-like growth factor- 1 (IGF-1) in a severe mouse model of SMA. Affected mice treated with AAV1-IGF-1 on postnatal day 1 exhibited reduced motor neuron degeneration, cardiac and muscle atrophy as well as a greater extent of innervation at the neuromuscular junctions compared to untreated transgenic mice. Moreover they demonstrated that treated animals had prolonged lifespan, increased body weight and improved motor coordination. Importantly, IGF-1 overexpression led to an increase in SMN protein levels in multiple tissues including spinal cord and muscle [112]; although the relevant site of action of IGF-1 for boosting SMN levels was not determined.

Recently, our group [113] examined quadriceps muscle biopsies from 24 patients with genetically documented SMA, and paraspinal muscle biopsies from 3 patients with SMA-II. We found that cytochrome-c oxidase (COX) deficiency was more evident in muscle from patients with SMA-I and SMA-II compared to healthy controls, and that muscle mtDNA content and citrate synthase activity were also reduced in all 3 SMA types. Our results strongly support the conclusion that an altered regulation of myogenesis and downregulated mitochondrial biogenesis contribute to pathologic changes in the muscle of patients with SMA [113].

SMA patients also suffer from gastroesophageal reflux, constipation and delayed gastric emptying. Gombash and colleagues [114] employed two mouse models of SMA to determine whether functional gastrointestinal complications are a direct consequence of SMN deficiency or a secondary event. With this study they demonstrated that SMN deficiency in enteric nervous system (ENS) neurons caused constipation, delayed gastric emptying, slow intestinal transit and reduced colonic motility (similar to what is reported in SMA patients), in the absence of gross anatomical or histopathological abnormalities. In particular SMN deficiency led to disrupted ENS signaling to the smooth muscle of the colon but did not cause enteric neuron loss. Together these data implicate that the susceptibility of the ENS cells to SMN deficiency may underlie gastrointestinal symptoms often reported in SMA.

4.3. Immune system dysfunction in SMA

The extent of involvement of the immune system in SMA has not been studied in depth to this point. An original study from Dachs and colleagues (2011) (Table 1) investigated the stimulation of apoptosis and macrophage recruitment in SMA muscles. Surprisingly, macrophage infiltration in SMA muscle was approximately 25-50% lower in comparison to healthy controls. This observation was tissue-specific, as no differences were observed in the liver and spleen between WT and SMA mice [115]. The authors concluded that the muscle-specific reduction in macrophage infiltration results from a primary defect in the immune system of severe SMA mice, due to a specific atrophy of immune organs, such as spleen and thymus. Similar alterations, such as spleen atrophy and systemic immune aberrations were previously reported in murine models of ALS [116]. Considering the pathologic alteration identified in the immune system organs of SMA animals, this field of study will require further attention in SMA patients.

4.4. Cardiac involvement

The presence of heart alterations have been reported in the most severe forms of SMA; caused either by congenital anomalies manifesting during cardiogenesis [117-121], or secondary to autonomic nervous system defects [122-127]. Many authors suggest that in juvenile cases of SMA, in particular for SMA types I and II, the presence of cardiac involvement is likely secondary to chronic respiratory insufficiency, which is a common feature of the disease. Investigators hypothesized that ventricular arrhythmia, bundle-branch and atrioventricular blocks are provoked by pulmonary and respiratory defects, highlighting the importance of respiratory assistance in preventing the onset of cardiological alterations [128,118,126,119,127,121] (Table 1). While previous clinical reports of SMA I patients did not explicitly determine if the cardiac defects were secondary to respiratory distress; recently, however, congenital heart anomalies are being described more frequently upon autopsy and include: dilated right ventricle, atrial and ventricular septal defects. The most common congenital heart defect is an anomalous development of the heart, referred to as hypoplastic left heart syndrome [123,129,120]. Analysis performed by Rudnik-Schoneborn and collaborators support a crucial role for SMN protein in cardiac development. In fact, they found that among patients with a single copy of SMN2, the incidence of congenital septal defects is approaching 75%; whereas among people without SMA, its frequency is closer to 1 in 50 million [120].

Preclinical studies in animal models of SMA have confirmed the presence of cardiac dysfunction [130,131,15,132]. Shababi and collaborators (2010) (Table 1) compared the very severe model of SMA (Smn−/−, SMN2+/+) and the well-established severe model for SMA, SMAΔ7 model (Smn−/−, SMN2+/+; SMNΔ7+/+), to examine the role of SMN protein in cardiogenesis and the contribution of heart anomalies to the SMA pathological phenotype. In the very severe model, as early as the embryonic stage, there was evidence of cardiac remodeling, well in advance of motor neuron loss. In the SMAΔ7 mouse model, similar structural heart defects have been described in the early post-natal period. The authors suggested that oxidative stress could lead to cardiac fibrosis, which starts at the time of symptom onset and increases significantly in the following days (Figure 2) [132].

In addition to structural defects, there is increasing evidence that arrhythmia and cardiomyopathy are widespread in SMA patients. In light of this, Heier and collaborators (2010) (Table 1) investigated whether such phenotypes are also present in SMA mouse models. Transgenic mice developed a severe bradyarrhythmia characterized by heart block and reduced ventricular depolarization as early as 2 days of age, before the onset of neuromuscular symptoms (Figure 2). However, at later stages of the disease, dilated cardiomyopathy and as well as sympathetic innervation defects are involved in heart failure. Moreover, mice treated with trichostatin A, a histone deacetylase inhibitor, showed improved heart rate and cardiac size. An improvement in neuromuscular function was also noted; however, affected mice died as a result of persistent bradyarrhythmia. This work further strengthens the notion that the involvement of cardiac arrhythmia is an early and progressive feature of SMA [15].

Previously, Foust and collaborators (2010) demonstrated that early post-natal delivery of SMN1 in SMNΔ7 mice using self-complementary AAV type 9 (scAAV9) improves both life span and neuromuscular function [133]. Based on these findings, Bevan and colleagues (2010) (Table 1) studied the effects of scAAV9 treatment on heart defects in the SMNΔ7 mouse model. They reported that treatment with scAAV9-SMN, also at the early post-natal stage, prevented bradycardia and the early development of dilated cardiomyopathy. They hypothesized that the success of this treatment was not only due to the targeting of spinal motor neurons, for which this subtype of AAV has a high tropism, but also to the transduction of autonomic nervous system neurons. However, as treated animals still exhibited contractility impairment, additional mechanisms, potentially initiated prior to the administration of the virus, are also involved in SMA-related heart defects. In fact, because the delivery of SMN1 was performed post-natally, it is difficult to conclude whether the remaining heart defects are due to embryonic autonomic nervous system dysfunction, or other cardiogenesis deficits [130]. Addressing this question, in 2012, Biondi and collaborators (Table 1) showed that cardiac alterations in type 2 SMA transgenic mice are largely caused by intrinsic heart defects and not by autonomic impairment [131]. They examined the effects of physical exercise on cardiorespiratory function in this SMA mouse model and observed many benefits, including a reduction in the delay of cardiac muscle maturation, a lessening of fibrosis, enhancement of cardiac electrical conduction velocity, and a decrease in arrhythmias and bradycardia; resulting in a partial restoration of cardiac function. In addition, their data show that the sympathetic system contributes to the amelioration of arrhythmia and bradycardia in this model [131,17].

In addition to direct effects on the structure and function of the heart itself, investigators have observed abnormalities in the vascular system of infants affected by SMA. Autonomic dysfunction and, particularly sympathetic dysfunction, has been hypothesized to be a primary source of the vascular perfusion abnormalities in SMA [134]. Vascular defects include coolness and poor perfusion of distal extremities; and occasional digital necrosis has been reported in SMA type I children [122]. Because vascular and innervation patterning are closely related, it is intuitively evident that there is an integral and codependent relationship between innervation and vascularization in motor neuron disease [135]. This occurs in particular in congenital or early infantile onset SMA when the growth and development of the vascular network is critical.

Taken together, the findings from multiple preclinical *in vivo* studies and the emerging clinical findings in SMA patients, suggest a key role of SMN protein in cardiogenesis, providing a new area of investigation that could be essential for optimal clinical success in the treatment of SMA.

4.5 Bone, pancreas and liver involvement

The impact of SMN deficiency in bone, pancreas and liver has also been documented. It is known that for patients affected by SMA as well as in the various SMA animal models, there is a high incidence of fractures and hypercalcemia, indicative of a role of SMN protein in bone function. In particular, children affected by SMA often suffer from severe osteopenia and fractures after a minimal trauma. In the most severe cases, congenital bone fractures and thinner ribs have been reported [136]. Moreover, a decrease of bone mineral density with increasing age in SMA patients has also been described [137]. Recently, a study revealed a key role of SMN protein in skeletal development critically dependent upon an interaction between SMN protein and osteoclast stimulatory factor. It was hypothesized that osteoclast stimulatory factor and SMN play a joint role in the cellular signal transduction cascade regulating the release of factors that stimulate skeletal development [138].

Occasional cases of pathologic pancreatic defects associated with SMA have been reported; in particular, diabetes and alterations in glucose metabolism in SMA Type II and III patients [139], and acute pancreatitis in SMA Type I patients [140]. Also, the intermediate SMA mouse model is characterized by metabolic defects such as fasting hyperglycemia, glucose intolerance, hypersensitivity to insulin, and hyperglucagonemia [139]. In an *in vivo* model in which Smn (+/−) heterozygous mice were created, it was proposed that metabolic and pancreatic defects are independent of late stage SMA neuromuscular pathology [141]. Since hepatic and pancreatic dysfunction appeared after one month of age, and only in response to dietary challenge, it was reasoned that defects in these systems occur independently of SMA-associated neurodegeneration. Although suggestive of SMN-dependency in these tissues, further confirmation using conditional SMN knock-out mouse lines, or reversal of the phenotype by viral mediated restoration of SMN protein levels selectively within these tissues would be beneficial. Both mice and human specimens show an abnormal localization of glucagon-producing α-cells within the pancreatic islets and increased insulin-producing β cells, hyperinsulinemia and increased hepatic glucagon sensitivity. Because SMA patients often suffer from pancreatic defects, there is an added risk of employing valproic acid in clinical studies since it is well known to be associated with an increased risk of pancreatitis [142].

The most common metabolic defect reported consists of alterations in fatty acid metabolism, which occurs in severe patients and some younger SMA Type II patients [143,144]. Abnormal fatty acid metabolism is characterized by mild to moderate dicarboxylic aciduria and increased levels of esterified carnitine. However, the level of ketone bodies is normal, suggesting a specific defect in fatty acid metabolism of muscles rather than in the liver. In order to investigate whether a fatty acid metabolism defect is a consequence of muscle denervation and atrophy, fatty acid levels in the plasma of severe SMA infants were measured and compared with control patients. The investigators found an increased ratio of dodecanoic acid to tetradecanoic acid in all SMA patients. These data suggest that the fatty acid metabolism dysfunction in SMA is not the consequence of SMA-related immobility, systemic illness, muscle denervation, or muscle atrophy. Rather these abnormalities may be directly related to the loss of *SMN1* or neighboring genes [144]. The importance of SMN protein in the development and function of the liver was demonstrated by Vitte and colleagues in 2004 using the Δ7 SMA *in vivo* model. In this study, a mutation in exon 7 of murine Smn induced liver failure and late embryonic lethality [145]. Subcutaneous injection of therapeutic oligonucleotide (ASO), which restores SMN protein levels, increased IGF-1 levels in the liver and rescued the pathological phenotype [16], highlighting the involvement of liver in SMA. Defects in fatty acid metabolism can lead to a reduction in production, causing extreme muscle wasting in severe SMA patients. For this reason, the evaluation of lipid content in the liver will be informative with respect to the effects of SMN deficiency on metabolic abnormalities.

5. Conclusions

Currently, there are no effective treatments available for SMA. There are, however, multiple ongoing clinical trials showing promising efficacy in ameliorating certain disease phenotypes. Historically, for SMA as in other neurodegenerative diseases, neuronal degeneration was widely considered to be strictly cell autonomous and the sole contributor to the clinical expression of the disease. However, the past few years have seen the accumulation of an overwhelming body of evidence that other cell types in the CNS, such as astrocytes, microglia and sensory neurons; and even outside the CNS, such as Schwann cells, muscle cells or heart, can be key components in the disease initiation and/or progression, as well as in the emergence of clinical symptoms. Here, we have reviewed and discussed the increasingly recognized involvement of these different systems in SMA which can have great consequences for the development of optimal therapeutic strategies for this aggressive disease.

Until recently, astrocytes and microglia were thought to be solely involved in the secondary response to a primary neuronal injury, via a process known as “reactive gliosis”. While the pathologic roles of glial cells in SMA have not been characterized as extensively as in other motor neuron diseases such as ALS, numerous alterations in SMA astrocytes and microglia could potentially initiate neurodegeneration as well as propagate injury. For example, restoration of SMN expression *both* in motor neurons and in astrocytes is necessary to greatly extend animal survival in a mouse model of SMA. In keeping with this, astrocytes and microglia should be thought as prime additional targets for the development of therapeutic interventions. Similarly, the roles of sensory neurons and Schwann cells have also recently gained prominence in the pathogenesis of SMA. In fact, several clinical observations made in type I SMA patients have indicated that these cells are also affected. In addition, other tissues outside the CNS contribute to the overall phenotype of SMA pathology. For example, several preclinical studies and clinical findings in SMA patients suggest that SMN protein plays an important role in cardiogenesis; these findings provide a new field of investigation that could be helpful to improve clinical success in the treatment of SMA.

Thus, while it is likely that motor neurons have an increased vulnerability to SMN deficiency, the involvement of different tissue in SMA pathology, inside and outside the CNS, warrants further investigation. Currently, the accumulating evidence concerning the involvement of cell types other than motor neurons is creating a paradigm shift in SMA and many other neurodegenerative diseases; what should we target, when, how and for how long? As discussed in this review, a first important point regarding the future development of genetic therapy is to consider that restoration of SMN expression in both central compartments (neurons/astrocytes) and peripheral compartments (muscle/Schwann cells) will be essential for an ideal disease-modifying treatment for SMA. Thus, while it is now largely recognized that each cell type may require different levels of SMN to exert its proper biological role, the regulation of SMN levels within different tissues and systems during development in animal models and humans remains to be investigated. The elucidation of levels of SMN that are required, regionally and temporally, is central to the development of successful therapies (genetic or otherwise), not only for the most severe SMA patients but also for the milder type 2, 3 and 4 SMA patients who appear to be much less responsive to SMN-restorative strategies than Type 1 patients. In keeping with this, unraveling the molecular pathways in SMA-relevant cell types that are defective after the SMN requirement window has passed may open new therapeutic strategies downstream of SMN, which could be more beneficial to milder SMA patients with later onset. These are among the critical questions that future translational research must address to finally make a cure for SMA a reality.

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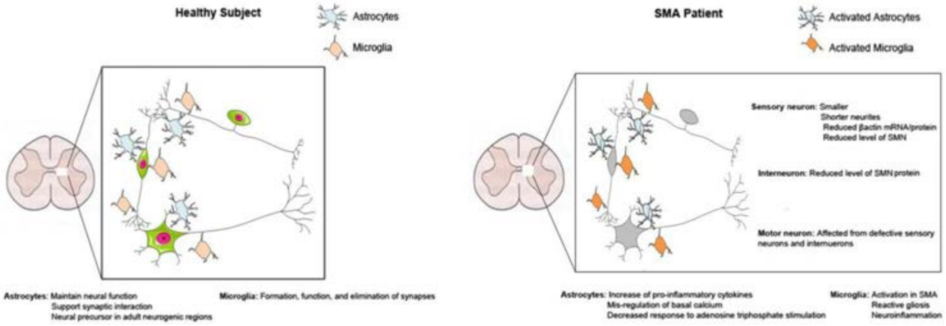

Figure 1.

Role of astrocytes and microglia in healthy subjects and SMA patients

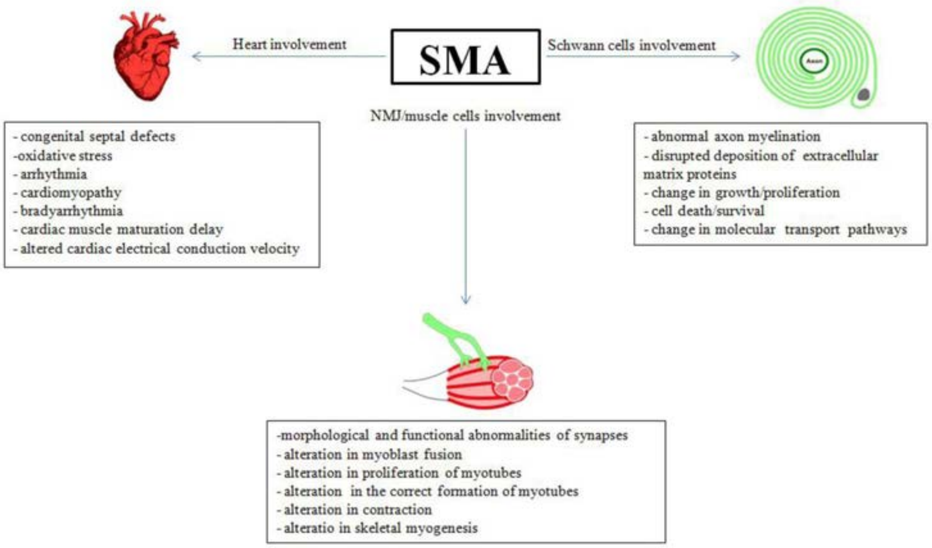

Figure 2.

The effects of SMA on non-motor neuronal tissue

Table 1

Summary of the participation different non-motor neuron cell types, intrinsin and extrinsic to the CNS, in SMA pathogenesis. PD: post natal day, MNs: motor neurons

Involvement of non-motor neuronal cells located inside the CNS			
Cell Type	**Citation**	**Disease model**	**Observation**
Astrocytes	McGivern 2013	SMAΔ7 mouse (Smn−/−, SMN2+/+; SMNΔ7+/+)	early PD: no differences between wt and SMA mice
			PD9 in SMA mice: augmented cell bodies; ↑ GFAP; thin and retracted processes
			production of pro-inflammatory cytokines through ERK1/2 activation
		SMA iPSC-derived astrocytes	mis-regulation of basal calcium
			decreased response to adenosine triphosphate stimulation
	Tarabal 2014	SMAΔ7 mouse	GFAP-positive profiles from PD4
			↑ components of Notch signaling pathway, in particular Jagged 1
Microglia	Tarabal 2014	SMAΔ7 mouse	↑ anti-Iba1 positive microglial cells from PD7
			CD68 positive microglial cells
			microglial clearance of cellular debris during synapse degeneration/elimination
Interneurons and sensory neurons	Jablonka 2006	SMA sensory neuron (E14 embryos derived)	smaller growth cones
			shorter neurites
			reduced βactin levels of mRNA and protein
	Schwab 2014	Co-colture with SMA iPS-derived sensory neurons and wt iPS-derived MNs	SMA sensory neurons doesn't contribute to MNs loss in this human stem cell system
	Lotti 2012	SMA Drosophila	aberrant splicing of Stasimon gene
		isolated sensory neurons from Smn −/−;SMN2 mouse embryos	smaller terminal in the skin and
			defects both in neurite growth and growth cone morphology
			abnormal sensory conduction velocity
			axonal degeneration
		SMAΔ7 mouse	alteration of monosynaptic connections between sensory neurons and motor neurons
Other cell types outside the CNS			
Heart involvement	Elkoen 1996 Distefano 1994 Roos 2009 Kimura 1980 Takahashi 2006 Takana 1976	SMA I patients	atrial and ventricular septal defects
			dilated right ventricle
			anomalous development of the heart
	Shababi 2010	severe model of SMA (Smn−/−, SMN2+/+) and	cardiac fibrosis due to oxidative stress

Involvement of non-motor neuronal cells located inside the CNS			
Cell Type	**Citation**	**Disease model**	**Observation**
		the SMAΔ7 mouse	
	Heier 2010	SMAΔ7 mouse	bradyarrhythmia from PD2 (heart block and reduced ventricular depolarization efficiency)
			sympathetic innervation defects and dilated cardiomyopathy at late stages of disease
	Bevan 2010	SMAΔ7 mouse	bradycardia
			dilated cardiomyopathy
	Biondi 2012	type 2 SMA-like mice	cardiac muscle maturation delay
			fibrosis
			alteration of cardiac electrical conduction velocity
			bradycardia and arrhythmia
Schwann cells involvement	Hunter 2014	Schwann cells derived from SMA mice	↓ levels of SMN protein
			failed to respond normally to differentiation signals
		SMA mice	alterations in the expression of key myelin proteins (MPZ, PMP22, MBP)
			↓ level of LAMA2
	Sarvestany 2014	Schwann cells isolated from SMA mice	SMN-dependent disruption changes in growth/proliferation, cell death/survival, and molecular transport pathways,
			changes in ubiquitination pathways proteins such as Uba1
Neuromuscular junction and muscle cells involvement	Kong 2009 Dachs 2013 Ling 2010 Kariya 2008 Lee 2011 Murray 2008 Mentis 2011	SMAΔ7 mouse	morphological and functional abnormalities of synapses
	Bowerman 2010	SMAΔ7 mouse	SMN levels in muscle fibers has no effects on SMA phenotype or on the survival
Neuromuscular junction and muscle cells involvement	Gavrilina 2008	SMAΔ7 mouse	defect in cytoskeleton organization
	Arlnold 2004 Boyer 2014 Cifuettes-Diaz 2001 Nicole 2003 Shafey 2005	SMAΔ7 mouse	defect in in myoblast fusion, proliferation and in the correct formation of myotubes
	Dachs 2013	SMAΔ7 mouse	↓ macrophage density