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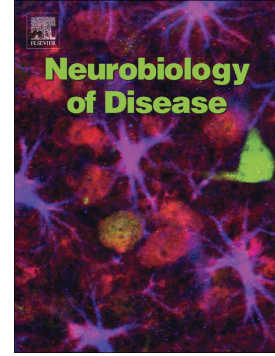


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Spinal Muscular Atrophy: Factors That Modulate Motor Neurone Vulnerability

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Abstract

Spinal muscular atrophy (SMA), a leading genetic cause of infant death, is a neurodegenerative disease characterised by the selective loss of particular groups of motor neurones in the anterior horn of the spinal cord with concomitant muscle weakness. To date, no effective treatment is available, however, there are ongoing clinical trials in place which promise much for the future. However, there remains an ongoing problem in trying to link a single gene loss to motor neurone degeneration. Fortunately, given successful disease models that have been established and intensive studies on SMN functions in the past ten years, we are fast approaching the stage of identifying the underlying mechanisms of SMA pathogenesis. Here we discuss potential disease modifying factors on motor neurone vulnerability, in the belief that these factors give insight into the pathological mechanisms of SMA and therefore possible therapeutic targets.

Keywords:

Selective vulnerability, SMA, SMN, disease modifier, motor neurone disease

Highlights:

- Factors that influence vulnerability of motor neurons in SMA.
- SMA disease modification.
- Impact of surrounding cells on neuronal death.
- Precise molecular defects in SMA; mRNA splicing and miRNA interactions.
- Cytoskeletal stability and axonal transport effects.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal-recessive neurodegenerative disorder, caused by homozygous mutations in survival of motor neurone 1 (*SMN1*). It is characterised by the loss of a large number of lower motor neurones and muscle denervation. In general, there are four different types of SMA categorised according to the age of onset and level of motor function achieved [1]. Type 1 (Werdnig Hoffman disease), the most severe type is also the most common genetic cause of infant mortality. Type 2 has a delayed onset around 0.5-1.5 years of age but still usually leads to death before adulthood. Patients with type 3 or type 4 diseases typically can live a normal life with little assistance. The molecular basis for disease severity is associated with both the quality and quantity of SMN protein. In man, a unique gene called *SMN2*, which is a duplication of *SMN1* and can be present in multiple copies. *SMN2*, has a near identical sequence but a crucial C to T substitution in exon 7 frequently results in exclusion of this exon and an unstable transcript, thus causing a low yield of full-length protein product (**Figure 1**) [2, 3]. Also, some mutations in *SMN1* do not cause complete loss of its function [4]. As a result, the disease severity is determined by both the preserved function of mutated *SMN1* and the number of copies of *SMN2* found in the patient genome.

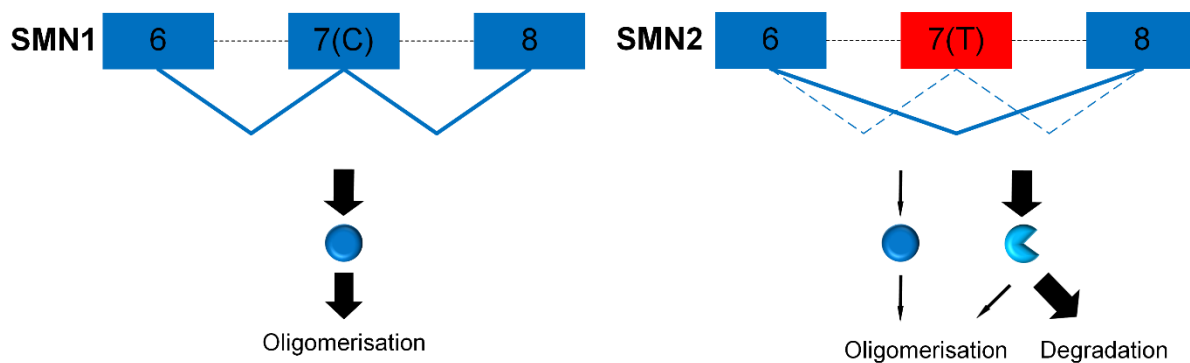


Figure 1. C to T conversion in SMN2 exon 7 results in a large part of protein products lacking exon 7. This exon contains a domain important for self-association. Without oligomerisation, free-SMN undergoes degradation rapidly, further reducing the total SMN levels.

The *SMN1* mutation primarily affects lower motor neurones, the resulting motor neurone loss causing paralysis and early death due to respiratory failure. However, the reason why loss of a ubiquitously expressed protein causes motor neurones to be particularly more vulnerable than other cell types is an intriguing subject.

Fortunately, given the studies on SMN function and a number of disease models established in the past ten years, we are beginning to understand what factors cause motor neurones to more prominently succumb to disease. These factors can be categorised into three major groups depending on external and internal effects on diseased motor neurones: First, it is known that as with other neurodegenerative diseases, other cell types contacting with the primary affected target cell also play a role in modulating disease severity: Motor neurones are surrounded by, and interact with, glia, such that faulty communication between these cells may exaggerate motor neurone pathology – so-called non-cell autonomous effects. Second, SMN is a multifunctional protein involved in a number of processes including RNA maturation and transportation in axons. The low quality or quantity of SMN protein may dysregulate genes which are crucial for motor neurone development and survival, but less crucial for other cell types. Thirdly, there may be motor neurone-specific disease modifiers of

SMN effects or gene production. Here, we review factors that have either been demonstrated to, or have the potential to, influence motor neurone vulnerability.

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2. Non cell-autonomous effects on motor neurone vulnerability

Since the identification of the *SMN* gene and its role in SMA [2], multiple efforts have been made to understand how SMN restoration or deprivation in the motor neurone affects the disease phenotype. It has been shown that specifically elevating SMN in the motor neurones of SMA mice profoundly improves many morphological and physiological defects associated with motor neurones such as neuromuscular junction, (NMJ), breakdown, abnormal synaptic transmission, motor function, and motor neurone viability. However, there is still room for further functional improvement [5-7]. In addition, specific SMN deprivation in mouse motor neurones or delaying the induction of *smn* expression in fish does not necessarily generate manifestations of disease [8], thereby implying some other factor(s) or cell type(s) play a part in motor neurone vulnerability.

To produce a movement, spinal motor neurones propagate the signal generated from the sensory neurone and inter-neurone, and then coordinate the signal to muscle fibres. Their normal function is highly regulated by neuroglia cells. In other words, the communication between all of these cell types is essential not only for effective motor movement but also for cell survival. In SMA, cells communicating with motor neurones are also under the stress of SMN malfunction, and as a result, they may contribute to motor neurone vulnerability. How these contacting cells respond to SMN malfunction and whether they negatively regulate motor neurone health will be considered in turn.

2.1. The role of muscle

The bi-directional nature of communication at the NMJ has long been shown to play an essential role in the function of both the axon terminal and innervated muscle [9-12]. Cultures of neonatal chicken spinal neurones treated with muscle extracts from SMA patients show inhibition of neurite outgrowth [13]. Because of the accessibility and apparent malfunction of SMA muscle, many experiments have been carried out to determine whether muscle could be an effective therapeutic target or if there might be a retrograde effect from the muscle to the motor neurone compartment.

Thus, selective knockdown of SMN levels in mouse skeletal muscle recapitulates the atrophic muscle fibres seen in SMA whilst motor neurone number and NMJ are spared [14]. Similarly, increased expression of SMN specifically in mature muscle (driven by the promoter of human skeletal actin, HSA, which is active only in mature myofibres) shows no benefit in nerve or muscle preservation, and little extension in lifespan in the SMN2 mouse model (*smn*^{-/-}; *SMN2*^{+/+}) [15]. A further investigation used MyoD (myogenic differentiation), whose expression begins at embryonic stage, to drive the expression of SMN in muscle in SMN Δ 7 mice (*smn*^{-/-}; *SMN2*^{+/+}; *SMN Δ 7*^{+/+}). While this resulted in slightly increased survival and fully rescued muscle size, it again did not restore the motor neurone number, NMJ pathology, or motor behaviours such as the righting reflex [7].

A further study demonstrated that whilst muscle could grow and function normally even when SMN is reduced to the disease level, and again no rescue was seen using an alternative promoter, (*Myf5*), to drive the muscle SMN expression in SMA Δ 7 mice [16]. This result combined with previous work suggests the muscle weakness seen in SMA is a secondary change to the motor neurone pathology and there is minimal retrograde impact of defective SMN protein levels in muscle to motor neurones.

2.2. Is a sensory neurone defect involved in inducing motor neurone pathology?

The significance of communication between sensory and motor neurones has been widely demonstrated. For example, NMJ formation is greatly facilitated by the presence of dorsal root ganglion neurones (DRG) in a co-culture system [17, 18]. Evidence for an impaired sensory system, including myelination loss and ganglion cell degeneration [19, 20], and absence of the refractory reaction following muscle spindle stimulation (H-reflex) are reported in some severe SMA cases [21]. Correspondingly, in mouse models of SMA, deafferentation from sensory inputs onto motor neurone results in lower input from presynaptic activity, which can account for the impaired motor activity of SMA [22-25]. In addition, SMN deprivation causes overlapping defects in both motor and sensory neurones, including reduced axonal hnRNP-R mRNA and growth cone size [26].

These studies raise the question whether any abnormal communication onto the motor neurone might aggravate motor neurone pathology. This hypothesis received some initial support from an SMA *Drosophila* model, which has an obligate requirement for SMN in cholinergic neurones, proprioceptive neurones and partial interneurones but not in motor neurones, for recovering motor behaviours [27], suggesting that normal sensory or other inputs play an important role in regulating motor neurone impairment.

However, there is concern that *Drosophila* has a nervous system that is not representative of higher organisms. For example, the neurotransmitters acetyl choline and glutamate are proprioceptive and motor in function in *Drosophila* respectively, but have converse functions in vertebrates. Thus, whether the role of SMN in *Drosophila* proprioceptive neurones is equivalent to that in vertebrate animals needs further investigation.

Other SMN models have failed to support a role for sensory neurones in motor neurone degeneration. VGlut1 puncta on motor neurones are the contact point where motor neurones receive input from sensory afferents, and are reduced in number in SMA mouse

models [6, 7, 25]. Boosting SMN protein in motor neurones is sufficient to fully rescue VGlut1 puncta number in SMA mouse models, suggesting motor-sensory deassociation may be secondary to motor neurone pathology [6, 7, 28]. Furthermore, motor neurone viability is independent of the motor-sensory communication when motor neurones are co-cultured with SMA sensory neurones derived from induced pluripotent stem cells (iPSCs) [29].

The interaction of sensory and other neuronal cells with the motor neurone requires further investigation as a number of issues remain unresolved. For example, whether specific expression of SMN in other neuronal cells in a mammalian model affects motor neurone phenotype is yet to be investigated.

2.3. Glial cells

There is a growing body of evidence implicating non-neuronal cells (glia) in various neurodegenerative diseases. There are various types of glia (principally astrocytes, oligodendrocytes and microglia) which have varied roles including the regulation of homeostasis, myelination and immune response [30].

Various astrocytic defects, including shorter process length and increased GFAP protein expression have been reported in pre-symptomatic SMA Δ 7 mice [31]. Furthermore, both SMA iPSC-derived and primary astrocytes reveal abnormal Ca²⁺ homeostasis, an important molecule affecting cytoskeleton [32, 33]. A study using mixed and matched co-cultures of motor neurones and astrocyte from wild type and SMA strains shows the importance of astrocyte in synapse formation and electrophysiological properties of motor neurones [33]. This is further verified in an *in vivo* study which demonstrated a remarkable improvement in motor functions, NMJ occupancy ratio and life span when SMN is specifically restored in SMN astrocytes [33]. However, the functions of SMN in astrocytes did not include the mitigation of motor neurone death in both studies [33, 34].

In a study of Schwann cells (the myelinating glial cell of lower motor neurones in the periphery) in SMN2 and Taiwanese SMA mouse models, there was defective secretion of myelin and laminin proteins [35]. The former is required for effective motor axon transmission. Interestingly, this defect was not demonstrated in the corticospinal tract, indicating the peripheral nervous system is more sensitive to a myelination defect. Laminin is a known factor exerting a strong influence on neurite growth and motor neurone viability *in vitro* [36]. Moreover, laminin deficiency is a possible cause of motor axon shortening due to inhibition of local axonal translation [37].

Although there has been no systematic study made on SMA microglia, there is *in vivo* evidence showing that microglia cells are also increased in number and activity in the spinal

cord of SMA Δ 7 mice, suggesting the involvement of microglia in regulating motor neurone function by stripping synaptic input [25].

Although there is no direct link between motor neurone death and SMN malfunction in any kind of glial cells, a conclusion that can be drawn is that the interplays between motor neurone and glial cells may largely contribute to the clinical manifestations, suggesting SMA is a multi-system disorder. However, more evidence will be required to fully understand their roles. For example, It is known that ALS can be recapitulated by overexpressing mutant Sod1 in astrocytes [38]. As such, it would be interesting to see how selective SMN reduction in glial cells could affect motor neurones.

3. Autonomous motor neurone vulnerability

SMA has long been considered as an autonomous motor neurone disease as there is considerable motor neurone loss whereas other cell types are relatively spared. The exact function of SMN, specifically in motor neurones as opposed to other cell types is unresolved. Such functional characterisation of the pathways that are affected by SMN would contribute considerably to our understanding of SMN pathogenesis (**Table 1**).

Modifying factor	Modulating mechanism	Model	Ref.
Stasimon	Unknown	Drosophila Zebrafish	[39]
Chondrolectin	Neural development	Zebrafish	[40]
SMN2	Exon 7 inclusion	Mouse	[41]
hnRNP-R	mRNA transportation	Zebrafish	unpublished
HuD	Neural development mRNA transportation	Cultured cell	[42]
IMP1 (ZBP1)	mRNA transportation	Cultured cell	[43]
CPG15 (NEURITIN)	NMJ maturation	Zebrafish	[44]
PLS3	Actin dynamic	Mouse Human	[45, 46]
Htra2-β1	Exon 7 inclusion	Human	[47]
ROCK	Actin dynamic	Cultured cell Mouse	[48, 49]
PTEN	Excitotoxicity	Cultured cell Mouse	[50, 51]
NMDA	Exon 7 inclusion	Mouse	[52]
β-catenin	Protein metabolism	Zebrafish Mouse	[53]
UBA1	Protein metabolism	Zebrafish Mouse	[53, 54]
UPR genes	ER stress	Cultured cell Mouse	[55]
IGF-1	SMN expression	Mouse	[56]

CNTF	NMJ formation	Mouse	[57]
CT-1	NMJ formation	Mouse	[58]
Bcl-XL	Anti-apoptosis	Mouse	[59]
miR-183	mToR pathway	Cultured cell Mouse	[60]
miR-431	<i>Chold</i> regulation	Cultured cell	[61]

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Table1. Known factors that modulate SMA severity

In addition to the direct effects of SMN, other modifying factors have functions that are independent of SMN functions but may be relevant to motor neurone diseases.

Finally, it is apparent that motor neurones are not uniformly susceptible to SMA. Elucidation of the factors which render different populations of motor neurone vulnerable and resistant to SMA usefully contributes to our understanding of disease pathogenesis.

In this section, we will focus on SMN-dependent functions. SMN is known to be an RNP assembly protein involved in the formation of snRNP and mRNP indicating potential roles in both RNA splicing and transportation. One can expect SMN loss would impair RNA splicing and transportation [62-65].

3.1. RNA splicing defect

SMN has a key role in the assembly of small nuclear ribonucleoproteins (snRNPs), key components of the spliceosome machinery [66]. It is thus likely that disruption of RNA splicing is involved in SMA pathobiology [67]. Accordingly, studies have shown a direct correlation between the ability to assemble snRNP and SMA disease severity [68, 69]. Furthermore, restoring normal splicing function by delivering mature snRNP that do not contain SMN is sufficient to rescue SMA phenotypes in *smn*-depleted fish embryos [70].

This raises the question: Why does SMN loss of function cause selective motor neurone vulnerability if it affects such a fundamental process that occurs in all cell types? In answer to this, early studies found tissue- and spliceosome-specific splicing defects [71] that stress the differential influence of two complementary spliceosome complexes: The major spliceosome is involved in excising the majority of introns, whilst the minor spliceosome is responsible for splicing only a few hundred genes in the genome [72]. There is growing speculation that the minor spliceosome may have a more pronounced role in neuronal homeostasis [73]. Minor spliceosome components are significantly reduced in the spinal cord of late-stage SMA mice whilst the major pathway remains normal [68, 71]. This raises the possibility that cell

populations expressing a greater proportion of genes that preferentially use this minor splicing pathway are more selectively affected, thereby contributing to selective motor neurone vulnerability. In support of the importance of the minor spliceosome to motor neurones, we, and others have found significant reductions of minor spliceosome components in amyotrophic lateral sclerosis [74, 75].

Further support of this observation is the demonstration of mis-splicing of a number of minor spliceosome introns from a number of genes. For example, aberrant splicing of a minor spliceosome intron has been described in Stasimon (*Stas*) both in a *Drosophila* model of SMA and a dorsal root ganglion neurones of SMA Δ 7 mice [39]. Stasimon is a transmembrane protein that appears to be essential for normal motor function. Co-injection of a *Stas* mRNA and *smn* morpholino is capable of rescuing the axonal growth defect seen in SMN-deficient Zebrafish, suggestive of its modifying role in the disease [39].

Whilst there is significant evidence that the minor spliceosomal pathway is affected in SMA, it is still expected that the major pathway will also be disrupted owing to the crucial role of SMN in snRNP biogenesis [67]. An exon array analysis of late stage SMN Δ 7 mice spinal cord showed widespread splicing errors affecting both the major and minor spliceosome pathways but only in late stage disease [76]. For example, the gene *Chodl*, which encodes Chondrolectin, was identified as mis-spliced in this study. *Chodl* is normally spliced via the major pathway, is expressed at a high level in motor neurones and appears to be necessary for axonogenesis in zebrafish [77, 78]. A further study showed that its overexpression was able to partially rescue the neurite number and length in an *smn*-depleted NSC-34 cell line, and axonal growth in SMA fish [40]. However, the alternative splicing event seen in the mouse homologous gene does not occur in humans, so the precise relevance of this to human disease is yet to be determined [79].

Cell-type specific RNA-seq analysis has identified several genes whose splicing is selectively disrupted in motor neurones, but not other neuronal populations, in spinal cord at a very early disease stage in SMA Δ 7 mice (before post-natal day 1). Of these incorrectly spliced genes, *agrn* (agrin) is of particular interest [80]. *AgRN* is a stimulator of acetyl choline receptor (AChR) clustering, and as such is responsible for NMJ maturation [81]. Gene splicing of *Gria4*, which encodes the AMPA-type glutamate receptor 4, generates two splice isoforms, known as ‘flip’ and ‘flop’. Receptors of the latter form desensitise more slowly. The ratio of flip to flop receptors is increased in motor neurone of SMA Δ 7 mice [80], potentially explaining altered electrophysiological properties in SMA motor neurones [8, 22].

Similarly, by transcriptome microarray analysis, a more recent study using *smn* knockdown zebrafish first identified dysregulation of *neurexin2a* (*nrxn2a*) in both its overall expression quantity and isoform ratio, and then validated these findings in motor neurone of SMN2 mice [82]. This gene acts at the pre-synaptic terminal regulating exocytosis and pre- and postsynaptic adhesion [83]. Experimental knockout of *nrxn2a* in mice has revealed various NMJ defects similar to those seen in motor neurone diseases [84, 85]. In keeping with its influence on motor axon terminal, knockdown of *nrxn2a* in wild type fish and raising the *nrxn2a* mRNA levels in a *smn*-deficient fish showed detrimental and beneficial effects on motor axon growth respectively [82]. However, *nrxn2a* was dysregulated both quantitatively and qualitatively, as such it remains to be shown which results in the motor neurone defect or a combination of both [82].

In addition to SMN-deficiency mediated mRNA splicing disruption, it has also been suggested that the level of *SMN2* exon 7 inclusion efficiency by the splicing machinery plays a fundamental role in motor neurone vulnerability: It is possible that *SMN2* exon 7 inclusion is particularly low in some spinal motor neurones [41, 86] compared to other cell types.

Whilst aberrant splicing may occur in SMA, it may be contributory but not be centrally causative to the pathology. For example, SMA-like phenotypes cannot be induced by

disturbing snRNP assembly or function [87-89]. Further, Baumer et al. found that splicing errors are only present on a large scale late in the disease [76]. This would suggest that other SMN functions are relevant to selective vulnerability at earlier stages of disease.

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3.2. Axonal RNA transport defect

Within the cell, SMN protein is not restricted to the nucleus where the snRNP assembly mainly takes place, as a small proportion of SMN is found in the axon in granular form [90-94]. These axonal SMN granules are free of Sm ribonucleoproteins which are core components of the snRNP assembly of the spliceosome [91]. Furthermore, truncated SMN that lacks the functional domain necessary for snRNP assembly is able to partially rescue the disruption of axon pathfinding in SMA fish [92]. These data, combined with the evidence of prominent axon degeneration and/or an outgrowth defect seen in various SMA models, is suggestive of a direct role of SMN in SMA pathology occurring at the distal part of motor neurone [95-99].

What does SMN do in the motor axon? It is possible that the RNA-binding feature of SMN and its associated molecules (many of them are RNA-binding proteins) allow them to transport RNA in cells beyond the snRNP biogenesis machinery [100, 101]. SMN co-localises with hnRNP-R in mouse motor neurones [102] and is associated with both hnRNP-R and β -actin mRNA in the motor axon and growth cone, and hence may affect local protein synthesis of β -actin [103, 104]. hnRNP-R is a member of hnRNP family and is involved in the various RNA regulatory functions including transport, splicing and metabolism. Knockdown of hnRNP-R has been shown to induce pronounced SMA-like phenotypes in zebrafish whereas other neuronal cells are less affected, similar to the phenotypes observed in *smn* morphant [104, 105]. Actin also plays an important role in axon structure and function [106-108]. However, whether and how actin-deficiency in a distal part contributes to axonopathy in SMA remains unclear [109].

In a further study of the axon, SMN was found to co-localise with Hu-antigen D (HuD) protein and *cpg15* (candidate plasticity-related gene 15, also known as neuritin) mRNA. [44]. HuD is a further RNA-binding protein with a pivotal role in neuronal cells that has been implicated in a wide range of activities including development, maintenance, and plasticity

[110]. This protein can act on the expression of a wide variety of mRNA species by binding to their 3'UTR [111]. This mechanism is regulated by methylation via CARM1 [112]. It appears that the SMN protein can increase CARM1 translation causing an increase in HuD methylation. As such, SMN loss reduces the affinity of HuD for its target RNA which is likely to contribute to the SMA axonopathy. This defect can be partially rescued by *HuD* overexpression [42, 43, 113].

cpg15 mRNA, which colocalises with SMN protein in the growth cone, appears to be involved in synaptic maturation in both sensory and motor neurones [114, 115]. *cpg15* overexpression in *smn*-deficient zebrafish partially rescued motor axon pathology [44]. However, although *cpg15* mRNA is shown to be translated in the growth cone, its levels are not only decreased in neurites but also in the cell body upon SMN reduction. Therefore, whether *cpg15* has a specific or more general impact on the motor axon remains unclear.

Similarly, another protein, insulin-like growth factor mRNA-binding protein 1 (IMP1) (also known as zipcode-binding protein 1 or ZBP1), has also been found to colocalise with SMN in axons [116]. This protein has some mRNA targets in common with HuD including *β -actin* and *Gap43* (microtubule-associated protein tau and the growth-associated protein 43), of which *Gap43* is of particular interest because of its versatile role in regulating axonal functions. More recently, an *in vitro* study showed decreased levels of *Gap43* in SMA axons, which were restored by overexpressing either HuD or IMP1, which also rescued the short axon phenotype [43].

Another SMN molecular partner involved in axonal transportation is coatamer subunit alpha (α -cop). α -cop belongs to the coat protein complex I (COPI) that is responsible for Golgi-mediated transportation and also has the ability to bind a number of mRNAs [117]. SMN/ α -cop granules are found in neurites and this association is likely to have begun in the Golgi apparatus [118, 119]. Knockdown of α -cop causes SMN accumulation in the Golgi

whereas over-expressing α -cop increases neurite length in *smn*-depleted NSC34 cells [118, 120]. These pieces of evidence further demonstrate there is a direct link between motor neurone vulnerability and axonal SMN.

In line with the potential role of SMN in RNA transport, two studies used microarray analysis combined with microfluidic techniques to specifically isolate axonal RNA species. The studies identified about 400 RNA species potentially binding with axonal SMN. A further study found more than 1000 genes dysregulated in neurites of NSC-34 cells and *smn*-depleted primary mouse motor neurones [121-123]. Among these genes are a number involved in axonal outgrowth, synaptogenesis, neurogenesis and neurotransmitter release [121, 122].

4. Other modifiers

In addition to SMN itself and its interacting partners, a number of genes have been identified that are able to ameliorate the SMA phenotype, as shown in **table 1**. These genes are putatively independent of SMN functions or appear to modify motor neurone vulnerability via less well defined pathways. In general, these modifiers are known to be essential for reducing neurodegenerative disease, with actions ranging from stabilising cytoskeleton, reducing excitotoxicity to regulating gene expression.

4.1. Cytoskeleton stability

Analysis of clinically discordant family members that carry homozygous deletions of *SMN1* and identical *SMN2* copy number enable investigation of these modifiers. Following this approach, the expression of the F-actin bundling protein gene, *Plastin 3 (PLS3)*, is significantly higher in unaffected females from six discordant families suffering with mild type of SMA (type II or III) [45]. However, further investigations demonstrated that overexpression of *Pls3* in relatively severe SMA mouse models (Taiwanese and SMA Δ 7) only shows marginal or no improvement [46, 124]. Conversely, a more recent study demonstrates its strong beneficial effects on Taiwanese SMA mice when SMN protein quantity is slightly boosted [125]. These suggest PLS3 may serve as a protector when SMN protein is above a certain threshold level, or further factors are involved [126]. Along with the same PLS3 study, CORONIN 1C or CORO1C, another F-actin binding protein was also identified as a disease modulator through *in vitro* protein-protein interaction assays [125]. CORO1C displays a comparable beneficial result to that of overexpressing PLS3 in SMN-deficient zebrafish [125]. The mechanism underlying the rescue effect of both PLS3 and CORO1C might involve an increase in the stability of the cytoskeleton and(or) restoring the endocytosis defect seen in SMN-deficient cells [125].

In addition to the role of SMN as an RNA carrier in the axon, another putative SMN function is the direct regulation of other protein activity in the axon. For example, SMN is able to bind non-phosphorylated profilin 2a protein, which is one of the substrates of rho-associated protein kinase (ROCK), an important regulator of actin dynamics [48, 127]. As a result of low SMN, it may upset the substrate balance for ROCK and in turn impairing actin dynamics. Inhibition of ROCK ameliorates the SMA-like phenotype both in cell culture and animal models [49], but knockout of one or both *profilin 2a* alleles does not cause any amelioration in a SMA mouse model [128]. As such, the exact mechanism of this effect remains to be explained.

In addition to the impairment of actin dynamics, microtubule destruction has also been proposed to be a pathophysiological feature of both SMA and amyotrophic lateral sclerosis (ALS) [129, 130]. A regulator of microtubule dynamics, Stathmin, causes depolymerisation of microtubules, inhibiting axon outgrowth and organelle movement in the neuronal processes [131]. In a proteomic analysis of an SMA mouse model, stathmin was specifically upregulated in spinal cord but remained unchanged in the brain [130]. More importantly, the down regulation of *stathmin* in SMA-like motor neurones significantly recovered defects in the axonal transport of organelles such as mitochondria [130], supporting the idea that cytoskeletal changes exacerbate disease progression.

4.2. Micro-RNA dysregulation

A growing body of evidence has pointed to a role of micro-RNA (miRNA) in axonal outgrowth probably by regulating local translation [132-134]. Disruption of miRNA biogenesis causes SMA-like phenotypes [135]. Upon SMN loss, miR-183, which is known to target inhibition of *mTor* translation [136], is up-regulated in rat, human and mouse cells [137]. Knockdown of miR-183 results in a significant improvement in axonal length in *smn*-deficient rat motor neurones. This improvement is present but small when the effect is

studied *in vitro* with the inhibition of miR-183 expression in CNS neurones of SMA Δ 7 mice [60].

The upregulation of miR-431 has been observed in smn-knockdown primary mouse motor neurones and occurs in a motor neurone-specific manner [61]. One of miR-431 targets is the aforementioned *Chodl*. Manipulation of miR-431 expression is able to increase and decrease neurite length under smn-depleted and normal conditions respectively [61].

4.3. PTEN pathway

Downregulation of phosphatase and tensin homolog (PTEN) is well known to have cytoprotective properties via its action on mTOR signalling, a pathway known to be involved in many neurodegenerative diseases. PTEN inhibition has been demonstrated to benefit ALS as well as SMA both *in vivo* and *in vitro* [50, 51, 138-140], by decreasing *GluR1* and *GluR2* expression and apparently thereby reducing AMPA-mediated excitotoxicity [139].

4.4. Endoplasmic reticulum (ER) stress

Although the activation of the unfolded protein response (UPR) under conditions of ER stress has been linked to many neurodegenerative disorders owing to its relationship with pathological protein aggregation [141]. iPSC-derived motor neurones from SMA patients have recently been shown to have higher ER stress activity compared to other induced spinal cord neurones and glia cells [55]. In SMA Δ 7 mice, either knocking down UPR-related genes *in vitro* or treatment with an ER-stress inhibitor generates improvements in many aspects of the disease process including innervation, survival, and lifespan. However, the increase in ER stress activity seems to be a post-symptomatic event and therefore probably be a general response to the pathogenic mechanism [55].

4.5. Increase of functional SMN

Because patients with SMA harbour an imperfect *SMN1* gene, raising functional SMN protein levels has long been a tempting therapeutic strategy [142]. In general, this can be achieved by delivering synthetic DNA-like molecules to correct the splicing pattern [143-156], or small chemical compounds to alter the gene structure by inhibiting histone deacetylase [157-167]. To this end, several drugs, such as Nusinersen (Ionis/Biogen) , AVXS-101 (AveXis) and RG7916 (Rosche), have been developed and have been achieving some success in clinical trials .

To compensate the weak association of *SMN2* exon7 with splicing factors, upregulation of relevant splicing factors seems to be effective. An early attempt to identify a disease modifying gene in nine discordant families with variable SMA phenotypes found that there was an inverse correlation between the protein levels of an exon splicing enhancer, Htra2- β 1, and disease severity [47, 168]. This observation raises the possibility that all the elements subsequently identified to be involved in exon 7 inclusion are capable of modulating SMA [169-175].

A further strategy to find potential modulators of SMN protein and/or expression levels is to identify a common pathway among several drugs that are frequently used to treat SMA. Thus, *Stat5* (signal transducers and activators of transcription 5) was identified as a *trans*-element that regulated SMN expression: The amelioration of axon growth in SMA-like motor neurones was observed when transfecting with a continuously activated mutant of *Stat5* [176].

In addition, *SMN2* expression is raised upon the activation of the NMDA receptor achieved by exercise [177]. The increased level of *SMN2* expression is most probably due to elevating the NMDA receptor-mediated PI3K/AKT/CREB cascade downstream activity [52, 177-179]. Taken together, it is possible that these factors are differentially regulated in motor neurones and other cell types as a weaker inclusion of *SMN2* exon7 in motor neurone was reported [41, 86]. Therefore, these factors might play an important role and may underlie selective motor

neurone vulnerability. Insulin/IGF-1 (Insulin-like growth factor 1) signalling has long been implicated in modulating neurodegenerative diseases [180]. In this regard, studies show increasing IGF-1 levels in SMA mice can have many phenotypic benefits including increased muscle size, motor function, lifespan, NMJ innervation and motor neurone number [56, 181]. Interestingly, although the mechanistic basis is unclear, both full-length SMN transcript and its protein product are considerably increased by this intervention [56]. However, the improvement in function becomes insignificant when delivering IGF-1 into a mild SMA mouse model, suggesting IGF-1 may only have a basic improvement that is only detectable when SMN protein is severely reduced. [182].

4.6. Neurotrophin, growth factor and anti-apoptosis

Like IGF-1, other factors known to have positive effect on cell health and survivability have been shown to modify disease severity to different degrees. Ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1), two neurotrophins commonly used to prolong motor neurone survival *in vitro*, are shown to be able to mitigate denervation in SMA mice [57, 58]. Over-expressing anti-apoptotic factor, Bcl-X_L, can significantly ameliorate some SMA phenotypes, such as lifespan, motor functions and motor neurone number, in a mild SMA mouse model, but fail to recapitulate this effect in a severe one [59]. Notably, these studies of survival-promoting and anti-apoptotic factors were performed on less severe or mild SMA models. Whether they are still able to exert a protective effect in the more severe disease condition and whether the effect is motor neurone specific are questionable. Alternatively, they could act as an enhancer combined with other treatments.

4.7. Causative genes for other motor neurone diseases

The broadest sense of the term “motor neurone diseases” can refer to any disease that is characterised by progressive motor neurone loss and muscle weakness. Although they may

not be necessarily caused by the same mechanisms [183-185], these diseases may partially share common pathways with SMA and thus provide a possible opportunity to understand it. Mutations of ubiquitin-like modifier enzyme 1 (*UBA1*) cause X-linked infantile spinal muscular atrophy (XL-SMA), another form of motor neurone disease [186]. The protein product of this gene has also been found to be decreased in SMA mice. Pharmacological inhibition of its activity and raising its levels can reproduce and mitigate SMA phenotypes in fish respectively [53, 54]. In addition, comprehensive amelioration is observed upon increasing UBA1 levels in SMN-deficient mouse [54]. With respect to selective vulnerability, reduction of UBA1 affects ubiquitin homeostasis and causes accumulation of β -catenin in the spinal cord but not in other organs such as liver and heart [53]. Inhibition of *β -catenin* was shown to rescue NMJ pathology in SMA mouse whereas liver and heart showed no improvement in gross pathology, suggesting that *β -catenin* accumulation may be a key to selective motor neurone vulnerability [53].

Several other genes have been identified as causes of hereditary motor neuropathies (HMNs) [184]. Although the roles of these genes are not yet fully investigated, they may be highly relevant to our current understanding of the underlying mechanism by which not only SMA but also other neurodegenerative diseases develop. For example, bicaudal D homolog 2 (*BICD2*) [187-189], dynactin 1 (*DCTN1*) [190], vesicle-trafficking protein (*VAPB*) [191], and cytoplasmic dynein 1 heavy chain 1 (*DYNC1H1*) [192, 193] are identified as causative genes of some HMNs. Some are involved in cargo packaging and retrograde axonal transport [194], while mutations in some of heat shock protein family which may cause the dysregulation of protein metabolism also induce HMNs [195-197]. Hence, it would be interesting to investigate their roles with SMN, and whether these genes are differentially regulated, thereby leading to selective motor neurone vulnerability.

4.8. The nature of motor neurone vulnerability

Finally, clinical examination suggests that motor neurones are not affected equally during the disease course. Motor neurones are not entirely identical to each other, they develop, mature, locate, and innervate differentially. More specifically, the size [198, 199], NMJ maturation pathway [23, 200], motor column [22, 199], and motor pool [201-203] of motor neurones have been proposed to influence specific motor neurone vulnerability in SMA. As such, some modifying factors may be embedded and combined with the many other factors discussed above eventually leading to motor neurone vulnerability.

More recently, two studies using SMA mouse models have shown that even motor neurones with very similar characteristics can display very distinct vulnerability [204, 205]. These studies suggest there are intrinsic molecular differences between vulnerable and less vulnerable motor neurones, some of which may make some motor neuron subgroups primed to the SMN loss. Whilst systematic investigation of relatively preserved versus vulnerable motor neurone groups has been performed in ALS [206, 207], an equivalent study has only recently been performed in a SMA mouse model [208]. This has revealed numerous encouraging candidate mechanisms, many of which have been previously identified as putative disease regulators in other research, such as programmed cell death, oxidative phosphorylation and ubiquitination. Novel pathways of potential interest that were identified include DNA repair, ribosome and rRNA binding [208]. However, it is still difficult to distinguish whether the changes are primary or secondary to the SMN loss.

5. Conclusion

This review summarises the current knowledge of why some cells are more or less susceptible to SMA and describes some of the known factors that play a part in ameliorating disease severity. We believe that such investigations will not only shed light on SMA but also other motor neurone diseases such as ALS. For example, the protein products of disease causative genes for familial ALS such as *TARDBP* and *FUS* have been demonstrated to interact with SMN [209, 210]. In addition, SMN and SOD1 proteins interact within neurones and can potentially counteract functional deficits in each other [211, 212]. These suggest a shared pathway between these two most common motor neurone diseases.

What makes spinal motor neurones particularly prone to the disease? The answer to this question relies on a more detailed functional analysis of SMN not only in the motor neurone but also the other interacting cell types, and to identify the specific features that are present in motor neurones. Novel insights into motor neurone vulnerability together with greater understanding of SMN function will move us closer to designing effective treatments for SMA patients and this process might become a study paradigm of selective vulnerability for other neurodegenerative diseases.

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