Molecular Mechanisms of Neurodegeneration in Spinal Muscular Atrophy

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ABSTRACT: Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease with a high incidence and is the most common genetic cause of infant mortality. SMA is primarily characterized by degeneration of the spinal motor neurons that leads to skeletal muscle atrophy followed by symmetric limb paralysis, respiratory failure, and death. In humans, mutation of the Survival Motor Neuron 1 (SMN1) gene shifts the load of expression of SMN protein to the SMN2 gene that produces low levels of full-length SMN protein because of alternative splicing, which are sufficient for embryonic development and survival but result in SMA. The molecular mechanisms of the (a) regulation of SMN gene expression and (b) degeneration of motor neurons caused by low levels of SMN are unclear. However, some progress has been made in recent years that have provided new insights into understanding of the cellular and molecular basis of SMA pathogenesis. In this review, we have briefly summarized recent advances toward understanding of the molecular mechanisms of regulation of SMN levels and signaling mechanisms that mediate neurodegeneration in SMA.

KEYWORDS: SMA, SMN, JNK, ROCK, ZPR1, MND

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder of early childhood caused by the deletion or mutation of Survival Motor Neuron 1 (SMN1) gene. SMA affects 1 in 6,000 to 1 in 10,000 individuals worldwide.¹ Humans have two copies of SMN gene located on chromosome 5g13 that are identified as SMN1 (telomeric) and SMN2 (centromeric).² The SMN2 gene is almost identical to the SMN1 gene but is unable to produce sufficient amount of full-length transcripts because of a C to T transition in the coding exon 7 that causes alternative splicing and skipping of exon 7, resulting in a truncated protein lacking exon 7 (SMN7) that is not fully functional and degrades rapidly.²⁻⁴ However, SMN2 produces low levels (5%–10%) of the full-length SMN protein that are sufficient for survival but result in SMA. The severity of SMA disease inversely correlates with the SMN2 copy number.⁵⁻⁷ Low levels of SMN protein result in the degeneration of spinal motor neurons and cause muscle weakness that is followed by symmetric limb paralysis, respiratory failure, and death.⁸⁻⁹

Currently, there is no treatment for SMA. The development of therapeutic treatments requires understanding of the molecular mechanisms involved in the regulation of gene expression and neurodegeneration. The molecular mechanisms of regulation of SMN2 gene expression and the mechanisms of motor neuron degeneration caused by low levels of SMN in SMA are unclear. However, recent studies have provided insights into the regulation of SMN2 gene expression that may help develop suitable therapeutic strategies. In addition, recent advances toward understanding the signaling pathways activated by low levels of SMN that might mediate neurodegeneration in SMA have provided insights into non-SMN targets as potential therapeutic targets to prevent neurodegeneration.

This review focuses on the role of cellular signaling pathways, extracellular regulated kinase (ERK)/ELK-1, JAK2/signal transducer and activator of transcription 5 (STAT5), and AKT/cAMP response element-binding protein (CREB), in the regulation of transcription of SMN2 gene. In addition, this review discusses the role of Rho kinase (ROCK) and the recently identified c-Jun NH²-terminal kinase (JNK) signaling pathways in mediating neurodegeneration associated with the pathogenesis of SMA.

Regulation of SMN2 Gene Expression

All forms of SMA are caused by insufficient levels of full-length SMN protein, ranging from the most severe type 0 (onset in utero), severe type I (onset 0–6 months), intermediate type II (onset ~6–18 months), mild type III (onset >18 months), and mildest type IV (onset >30 years).⁸⁻¹⁰ The onset and severity of SMA disease inversely correlate with the amount of full-length SMN protein produced by varying SMN2 copy numbers.
present in patients with severity ranging from type I to type IV.\textsuperscript{5–7} Restoration of SMN levels within the central nervous system (CNS), including spinal motor neurons, using transgenic expression of SMN results in the rescue of phenotype, alleviation of SMA pathologies, and increase in lifespan of mice with SMA-like disease.\textsuperscript{11–14} These findings suggest that restoration of SMN levels in the CNS is sufficient to reduce the severity of disease and improve the SMA phenotype. The SMN2 gene represents a positive modifier and an attractive therapeutic target for producing higher amounts of SMN protein by manipulating the transcription of SMN2 gene.\textsuperscript{7,15} Understanding the mechanisms of control of the transcriptional regulation of SMN2 gene is one of the important areas of investigation that may lead to identification of viable cellular therapeutic targets to generate sufficient amounts of SMN for the treatment of SMA.

Both SMN1 and SMN2 genes are regulated transcriptionally during cell growth and differentiation.\textsuperscript{16} Analysis of promoter regions of SMN1 and SMN2 genes shows identical sequences consisting of common cis-regulatory elements required for the initiation and regulation of transcription.\textsuperscript{17,18} However, both SMN genes show differential expression in neurons and nonneuronal cell types.\textsuperscript{17} The differential expression of SMN genes in different cell types might be because of the presence of two transcription initiation sites: the first transcription site is located 163 base pairs upstream of the translation start site and the second site is located 246 base pairs upstream of the translation start site.\textsuperscript{16} A regulatory region of approximately 5 kb upstream of the transcription start site might be involved in the transcriptional regulation of the SMN genes. The upstream regulatory regions (5’-UTR) of the SMN genes contain binding sites for known trans-acting factors, such as ELK-1 (E26 transformation specific [ETS] like or ETS domain containing), CREB, and STAT5 (signal transducers and activator of transcription) that could regulate transcription.\textsuperscript{17–20}

Recent studies have indicated the role of modulation of ELK-1 and CREB activities by mitogen-activated protein kinase (MAPK) signaling pathways in the regulation of SMN2 gene expression. The intracellular Calcium/calmodulin-dependent kinase II (CaMKII)/phosphatidylinositol-3 kinase (PI3K)/AKT/CREB cascade that is known to be a downstream mediator of N-methyl-D-aspartate (NMDA) receptor signaling was found to be activated in the spinal cord explant cultures from mouse models with SMA-like disease, the Taiwanese SMA type II mouse model\textsuperscript{21} and the severe SMA type I mouse model,\textsuperscript{22} upon treatment with NMDA.\textsuperscript{23} Treatments of the spinal cord cocultures with inhibitors for kinases, CaMKII (KN-93) and PI3K (LY294002), abolished NMDA-mediated increase in the levels of SMN. However, treatment with NMDA and U0126, inhibitor of MEK/ERK/ETS like (ELK) pathway that is known to be a target for CaMKII, did not change the levels of SMN expression induced by NMDA. In vivo studies show that the treatment of mice with NMDA improved phenotype, including lifespan of the SMA type II mice.\textsuperscript{23}

In vitro studies indicated that the presence of intracellular crosstalk between ERK and AKT pathways and shifting of balance of activation from ERK to AKT pathway by inhibition of MEK/ERK/ELK pathway result in increased SMN2 gene expression.\textsuperscript{24} In vivo inhibition of ERK pathway using the MEK inhibitor (U0126) resulted in the activation of CaMKII/AKT/CREB cascade and an increase in SMN levels in the spinal cords from severe SMA-like mice. Treatment of severe SMA mice with U0126 resulted in improvement of disease phenotype with reduced loss of motor neurons and increased lifespan.\textsuperscript{24} A recent study showed that the reduced expression of the insulin-like growth factor-1 receptor (Igf-1r) gene results in neuroprotection and improvement in the phenotype of SMA mice. Reduction in IGF-1R levels causes activation of the AKT/CREB pathway and inhibition of the ERK/ELK-1 pathway, which results in higher levels of SMN.\textsuperscript{25} Together, findings from these studies suggest that the activation of ERK/ELK-1 pathway negatively regulates SMN2 expression and the activation of AKT/CREB pathway stimulates SMN2 expression to increase the levels of full-length SMN. Simultaneous inhibition of ERK pathway and stimulation of AKT pathway results in the upregulation of SMN2 expression in SMA. A graphical summary of signaling pathways regulating the SMN2 gene expression in SMA is presented in Figure 1.

The effect of different classes of small cell permeable compounds has been examined on increasing levels of SMN protein by enhancing transcription that improve disease phenotype in mice with SMA. These compounds include quinazoline compounds (eg, RG3039) that function as inhibitors of RNA decapping enzyme (DcpS)\textsuperscript{26–28} and have been shown to improve the disease phenotype, including the lifespan of mice with SMA in different SMA mouse models, severe SMAβ model\textsuperscript{29–31} and intermediate Smn\textsuperscript{β–γ} model.\textsuperscript{32} Benefits of RG3039 treatment were observed in the improvement of SMA phenotype, such as increase in the number of spinal motor neurons and increase in the number of SMN-containing gems.\textsuperscript{32,33} However, in vivo increase in SMN levels was not significant in mice with SMA.\textsuperscript{33} Another set of compounds known as histone deacetylase (HDAC) inhibitors are valproic acid (VPA), trichostatin A (TSA), LBH589, M344, suberoylanilide hydroxamic acid (SAHA), sodium butyrate, and phenylbutyrate that are shown to increase the levels of SMN.\textsuperscript{33–41} Other small compounds that have been shown to increase SMN levels include hydroxyurea,\textsuperscript{42,43} resveratrol,\textsuperscript{44} and a new class of compound, LDN-76070, whose precise mode of action remains to be examined, which improved the phenotype of SMA mice.\textsuperscript{45} However, the detailed mechanism of action of these compounds on the regulation of SMN2 expression remains to be studied.

The role of Janus kinase (JAK)/STAT signaling pathway is also shown in the regulation of SMN2 expression. The JAK tyrosine kinase interacts with cytokine and prolactin (PRL) receptors and relays signal downstream by phosphorylation of
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The STAT group of transcription factors; which regulates transcription and are essential for mammalian developmental process, including cell survival, proliferation and differentiation, migration, apoptosis, neuroprotection, and immune cell and mammary gland development. Cell permeable compounds, sodium valproate, TSA, and aclarubicin, have been shown to activate STAT5 in SMA-like mouse embryonic fibroblasts and motor-neuron-like (NSC34) cells transfected with human SMN2 and induce SMN2 expression. In addition, a peptide hormone PRL that is known to activate JAK2/STAT5 pathway is shown to increase SMN2 expression in neuronal (NT2) cells. In vivo activation of JAK2/STAT5 pathway by administration of PRL in mice with severe SMA (SMAΔ7 mice) causes an increase in SMN levels that improves disease phenotype and increases the lifespan of SMA mice. The role of JAK2/STAT5 pathway in the regulation of SMN2 expression is presented in graphical form (Fig. 1).

An alternative method has been developed to generate full-length SMN from the SMN2 gene by modifying the processes involved in RNA biogenesis, such as transcription and pre-mRNA processing and splicing using transcriptional activators, small nuclear U RNA, small compounds, and antisense oligonucleotides (ASO) to correct splicing.

The small compounds, pseudocantharidins, a phosphatase (PP2A) activator, which dephosphorylates Tra2-β1, a splicing factor, and VPA, a drug approved by the U.S. Food and Drug Administration, which upregulates the levels of Tra2-β1, result in increased incorporation of exon 7 and enhances the levels of full-length transcripts by partially correcting splicing. A new class of cell permeable compounds (SMN-C1, SMN-C2, and SMN-C3, developed by PTC Therapeutics) has also been shown to correct SMN2 splicing and improve the phenotype of SMA mice.

The ASO-based approach to correct splicing and increase the levels of SMN is one of the promising therapeutic approaches currently under different phases of clinical trials.

Intracellular Signaling Pathways that Mediate Motor Neuron Degeneration in SMA

In SMA, muscular atrophy is a result of degeneration of spinal motor neurons caused by low levels of SMN protein. SMN is a

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ubiquitously expressed protein, but why selectively lower spinal cord motor neurons degenerate remains unclear. The degeneration of motor neurons suggests that the low levels of SMN are unable to support the essential cellular functions required for the survival and maintenance of neurons. The defects in cellular functions, including mRNA biogenesis caused by reduced levels of SMN, might result in the activation of intracellular stress signaling pathways that mediate neurodegeneration in SMA. The intracellular mechanisms that are triggered by the low levels of SMN and mediate neurodegeneration remain unclear. However, a noticeable progress has been made recently to understand the intracellular signaling cascades activated by the low levels of SMN that might mediate neurodegeneration in SMA. The ROCK and the JNK signaling pathways have been shown to be activated by the low levels of SMN in in vitro and in vivo SMA models.

The RhoA/ROCK signaling pathway in SMA. The role of RhoA (a small GTPase) and the immediate downstream target ROCK, RhoA/ROCK signaling, is established in the regulation of cytoskeleton dynamics essential for neuronal growth, differentiation, pathfinding, retraction, and degeneration. Alterations in the activity of ROCK and its downstream targets, including profilin IIa, cofilin, lim kinases (LIMK), myosin regulatory light chain, and myosin light chain phosphatase (MYPT), are associated with human diseases. SMN has been shown to interact with ROCK and profilin. It is suggested that the low levels of SMN result in a free pool of profilin IIa and cause an increase in ROCK/profilin complexes that leads to hyperphosphorylation of profilin IIa in SMA. In vitro studies with knockdown of SMN in neuron-like cells (PC12 and NSC34) indicated the activation of RhoA/ROCK and the phosphorylation of downstream targets, such as profilin IIa, cofilin, LIMK, and MYPT, and suggested that the ROCK pathway might be associated with the pathogenesis of SMA. It is clear that the low levels of SMN result in the activation of ROCK; however, there is some inconsistency in the literature on the modulation of downstream targets of ROCK that might be because of the use of different cellular and animal SMA models.

The activation of both ROCK and ERK pathways in SMA indicates a possibility of crosstalk because ERK and ROCK can inhibit each other. However, in in vitro SMA cell model, activated ERK was unable to affect the levels of activated ROCK. It is possible that in SMN-depleted neuronal cells, ERK activation contributes toward promoting neuronal outgrowth and negatively regulates SMN2 expression with phosphorylation of ELK-1, whereas hyperactivation of ROCK may inhibit neurite outgrowth. A possibility of crosstalk between neurotrophic growth factor signaling and ROCK pathway to regulate neurite outgrowth is also indicated in SMA. Another possibility of crosstalk exists between ROCK and phosphatase and tensin homolog (PTEN deleted on chromosome 10) pathways because ROCK interacts and phosphorylates PTEN. PTEN hydrolyzes phosphatidylinositol (3,4,5)-triphosphate, a second messenger that activates PI3K, and inhibits the activation of AKT mediated by PI3K. The downregulation of phospho-AKT is shown in the spinal cords of SMAΔ7 mice and human SMA patients. Therefore, ROCK activation in SMA might be involved in the activation of PTEN that leads to inactivation of PI3K/AKT cascade. However, in vivo modulation of PTEN activity under SMA conditions remains to be examined. Nevertheless, studies with the knockdown of PTEN in cultured SMN-deficient motor neurons and in mice with SMA have shown beneficial effects on the growth of motor neurons, reduction in the severity of disease, and increase in the lifespan of SMAΔ7 mice. A graphical model representing the activation of RhoA/ROCK pathway in SMA is shown in Figure 2.

Interestingly, pharmacological inhibition of ROCK using inhibitors (Y-27632 or Fasudil) resulted in a marked increase in the lifespan of an intermediate SMA mouse model (Sma2/B) without any change in the SMN transcription and protein levels. However, ROCK inhibition did not result in an increase in the numbers of spinal motor neurons and did not prevent SMN-dependent neuromuscular junction (NMJ) denervation. The improvement in the SMA phenotype might be because of the improvement in the functionality of motor neurons and NMJs and the increase in the skeletal muscle fiber size due to reduction in the levels of phospho-LIMK and phospho-cofilin in SMA mice treated with ROCK inhibitors. The reduction in the levels of ROCK downstream targets, phospho-LIMK and phospho-cofilin, may help stabilize the actin cytoskeleton and improve the functionality of SMN-deficient neuronal and nonneuronal cells.

The JNK signaling pathway in SMA. The role of JNK group of kinases has been established in neuronal cell growth, differentiation and apoptosis, CNS morphogenesis, memory, and synaptic plasticity. The JNK group of MAPK is encoded by three genes, Jnk1, Jnk2, and Jnk3, that generate a total of 10 transcripts for multiple isoforms. The Jnk1 and Jnk2 genes show ubiquitous expression, but the Jnk3 gene is mainly expressed in neurons, with some expression in the heart and testis. The role of JNK has been implicated in neurodegeneration caused by alteration of microtubule stability induced by JNK-mediated phosphorylation of microtubule-associated proteins, including MAP1B, MAP2, Tau, and stathmin (microtubule–destabilizing family of proteins); JNK pathway has been indicated as a potential therapeutic target for the treatment of neurodegenerative diseases, such as Parkinson’s and Alzheimer’s diseases.

The low levels of SMN in neurons cause neurodegeneration in SMA. The stress-activated protein kinases are known to be activated by a variety of extracellular stress signals, such as growth factors, cytokines, and ultraviolet light. SMN deficiency may result in intracellular stress that might activate intracellular signaling cascade and lead to neurodegeneration in SMA. We have recently shown the activation of the JNK
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eJnk3 deficiency resulted in the protection of cultured neurons with low levels of SMN, suggesting that JNK3 may be a potential target for SMA therapeutic interventions. This study identified two signaling modules, ASK1/MKK4/JNK3 and MEKK1/MKK7/JNK3, that may mediate JNK activation and neurodegeneration in SMA. A graphical model representing the activation of JNK by two signaling modules in SMA is shown in Figure 2.

Scaffolding of MEKK1/MKK7/JNK complex by neuron-specific JNK-interacting protein 3 may activate MKK7. The marked difference in the levels of activation of MKK4 compared to MKK7 suggests the tight regulation and specificity of the activation of signaling modules. Preferential activation of neuron-specific isoform, JNK3 (MAPK), was detected in SMN-deficient neurons. JNK3 deficiency resulted in the protection of cultured neurons with low levels of SMN, suggesting that JNK3 may be a potential target for SMA therapeutic interventions. This study identified two signaling modules, ASK1/MKK4/JNK3 and MEKK1/MKK7/JNK3, that may mediate JNK activation and neurodegeneration in SMA. A graphical model representing the activation of JNK by two signaling modules in SMA is shown in Figure 2.

Furthermore, in vivo studies by genetic inhibition of JNK3 in SMAΔ7 mice resulted in the systemic rescue of SMA phenotype, including reduction in the loss of spinal cord motor neurons.
cord motor neurons and muscle degeneration, improvement in muscle fiber thickness, muscle growth, gross motor function and overall growth, and increase in lifespan. Interestingly, genetic inhibition of the Jnk3 did not alter the levels of SMN in mice with SMA. The findings from this study suggest that the amelioration of SMA phenotype in SMA mice by Jnk3 deficiency is SMN independent, and Jnk3 represents a non-SMN target. Genetic elimination of the Jnk3 gene in SMA mice validated Jnk3 as a potential (non-SMN) therapeutic target.84

Other potential signaling molecules and pathways in SMA. A few other proteins have been identified that may be a part of the intracellular signaling mechanisms contributing toward SMA pathogenesis, including modifier proteins that alter disease phenotype. Humans with homozygous SMN1 deletion and identical SMN2 copy numbers show discordant phenotypes compared to their siblings, suggesting the possibility of SMA modifier genes in addition to SMN2.101-103 Recent studies have identified genes located outside of the 5q SMA locus, such as plasin 3 (PLS3, Chr Xq23) and zinc finger protein 1 (ZPR1, Chr 11q23.3), that have been shown to modify the severity of SMA disease.103,104 PLS3 levels were upregulated in unaffected female SMA patients compared to affected SMA patients (siblings). PLS3 is a calcium-dependent actin-bundling protein and shown to regulate axonogenesis by increasing the levels of F-actin.103,105 Overexpression of PLS3 in cultured SMN-deficient neurons corrected axonal growth defects. PLS3 overexpression moderately improved the SMA phenotype by delaying axon pruning that resulted in improved NMJ functionality in Taiwanese SMA mouse model.106 In another study, PLS3 overexpression did not modify the severity of SMAΔ7 mouse model.107

The reasons for moderate to no improvement in different mouse models are unclear. It is possible that in addition to PLS3 overexpression in unaffected individuals, there would be other proteins/factors whose levels could be altered in a gender-specific manner that contribute to PLS3-dependent discordant phenotype in SMA type II/III patients. It is unclear whether PLS3 overexpression will also provide beneficial effects in severe SMA but warrants further studies. However, identification of the molecular mechanism that upregulates PLS3 levels in unaffected individuals with homozygous SMN1 deletion will provide insights into the alteration of levels of other potential targets that may be operating synergistically with PLS3 in SMA.

ZPR1 is an evolutionary-conserved essential protein108 that is a component of the receptor tyrosine kinase signaling pathways and interacts with the epidermal growth factor receptor and platelet-derived growth factor receptor in quiescent cells.109,110 Treatment of quiescent cells with mitogens or serum results in the formation of ZPR1 complexes with translation elongation factor EF-1α and SMN proteins and translocation to the nucleus.111,112 ZPR1 interacts with SMN and is required for accumulation of SMN in subnuclear bodies, including gems and Cajal bodies. Interaction of ZPR1 with SMN is disrupted in SMA patients, and both ZPR1 and SMN fail to accumulate into nuclear bodies. The defect in nuclear accumulation of SMN is the cellular defect in SMA that may affect the biochemical function of SMN associated with its localization to nuclear bodies. Notably, the severity of SMA disease correlates negatively with the number of SMN bodies.6

ZPR1 is downregulated in SMA patients.104,113 The reduced expression of ZPR1 causes progressive loss of spinal motor neurons in mice.114 The low levels of ZPR1 increase the severity of disease and decrease the lifespan of mice with SMA.104 Overexpression of ZPR1 in fibroblast derived from SMA type I patients restores the accumulation of SMN in subnuclear bodies and increases the levels of SMN. ZPR1 overexpression in spinal motor neurons from SMA mice rescues axonal growth defects.

The role of ZPR1–SMN complexes in the growth and maintenance of neurons is unclear. However, ZPR1 may contribute to the functions of SMN, including mRNA splicing because ZPR1 is a part of the SMN containing cytoplasmic spliceosomal small nuclear ribonucleoprotein (snRNP) complexes and interacts with snurportin 1.115 ZPR1 deficiency causes defects in cellular distribution of snRNPs and in pre-mRNA splicing similar to SMN deficiency.112,116,117 In addition, ZPR1 complexes may also contribute to overall RNA biogenesis, including splicing and transcription.109,118

A recent study showed that ubiquitin-like modifier activating enzyme 1 (UBA1) interacts with SMN and disruption of ubiquitination pathway contributes to the severity of SMA disease.119 The ubiquitination pathway is shown to regulate the stability of SMN protein120 and is involved in mediating synaptic and axonal degeneration.121 Mutations in the human UBE1 (UBA1) gene cause X-linked infantile SMA.122 The reduced levels of UBA1 and the increased levels of β-catenin in SMA mouse models [severe SMA (Smn−/−; SMN2+/−) and Taiwanese SMA (Smn−/−; SMN2+/−)] indicate an increase in β-catenin signaling that may influence the transcriptional regulation of critical genes.119 However, the expression of specific genes altered by the increased levels of β-catenin that may contribute to SMA pathogenesis remains to be examined. Interestingly, pharmacological inhibition of β-catenin with quercetin, a cell permeable flavonoid,123 improves neuromuscular pathology in different animal models, Drosophila SMA model,124 Zebrafish SMA model,125 and Taiwanese SMA mouse model21 by Gillingwater’s group.119 However, the inhibition of β-catenin did not improve systemic pathology in SMA mice. Nevertheless, the alteration of ubiquitin homeostasis and β-catenin signaling in SMA suggests that targeting of this pathway may have therapeutic potential to reduce the severity of SMA disease.119

Recent advances made to understand the molecular mechanisms that regulate the expression of SMN2 gene and the cellular mechanisms triggered by the low levels of SMN
### Table 1. Signaling pathways and the molecular targets of spinal muscular atrophy.

| MOLECULAR TARGETS/PATHWAYS | CELLULAR MODEL* | COMPOUNDS/GENES | OUTCOMES | ANIMAL MODEL* | COMPOUNDS/GENES | OUTCOMES | REFERENCES |
|---------------------------|-----------------|-----------------|----------|---------------|-----------------|----------|------------|
| HDAC (Histone deacetylase) | SMA patient fibroblast Organotypic hippocampal slice cultures from rat | Valproic acid (VPA) (Inhibitor) | Increase in FL-SMN2 mRNA, splicing factors Htra2β1 and SR SF2/ASF and SRp20 protein levels. | SMA-A7 mouse model \(^{22}\) | Trichostatin A (TSA), Sodium butyrate SAHA (Inhibitors) | Increase in SMN levels. Improvement in SMA phenotype and increase in lifespan. | 35\(^a\), 34\(^b\), 37\(^b\) |
| ROCK (Rho kinase) | PC12 cells NSC34 (motor neuron-like cells) HEK293 cells | Y-27632 (Inhibitor) | Enhanced neurite outgrowth in SMN-deprived NSC34 cells. | Smn \(^{39}\) - SMA mouse model \(^{89}\) | Y-27632, Fasudil | Increase in skeletal muscle fiber and postsynaptic endplate size. Improvement in SMA phenotype and increase in lifespan. No change in SMN levels (SMN-independent). | 75\(^a\), 76\(^a\), 79\(^b\), 87\(^b\), 88\(^b\) |
| NMDA receptor | Co-cultures of spinal cord explants and muscle cells | NMDA | Increase in SMN levels. | Severe SMA-like mouse model \(^{22}\) Taiwanese SMA mouse model \(^{21}\) | NMDA | Increase in SMN levels. Improvement in SMA phenotype and increase in lifespan. | 23\(^a,b\) |
| MEK/ERK/ELK-1 | Co-cultures of mouse spinal cord explants and muscle cells Myogenic precursor cells from SMA type I patients | U0126 (MEK inhibitor) | Increase in SMN levels. | Severe SMA-like mouse model \(^{22}\) Taiwanese SMA mouse model \(^{21}\) | U0126, AZD6244 (MEK inhibitors) | Increase in SMN levels. Improvement in SMA phenotype and increase in lifespan. | 24\(^a,b\) |
| JAK2/STAT5 | SMA-like MEFs SMA2-NSC34 cells SMA-patient lymphocytes MN-1 cells NT2 cells | Sodium vanadate, TSA and aclarubicin Prolactin, Aurintri-carboxylic acid (ATA; STAT5 activator) | Increase in SMN levels and nuclear gems, and enhanced axonal outgrowth. Increase in SMN levels. | SMA-A7 mouse model \(^{22}\) | Prolactin | Increase in SMN levels. Improvement in SMA phenotype and increase in lifespan. | 51\(^a\), 20\(^a\), 19\(^b\) |
| DcpS (RNA decapping enzyme) | NSC34 (motor neuron-like cells) | D156844 (Inhibitor) | Increase in SMN levels. | Taiwanese SMA mouse model \(^{21}\) and Smn \(^{39}\) - SMA mouse model \(^{89}\) SMA-A7 mouse model \(^{27}\) SMA-A7 mouse model \(^{27}\) | RG3039 (Inhibitor), D156844, D156844+ follistatin | Improvement in SMA phenotype and increase in lifespan. Improvement in SMA phenotype and increase in lifespan with minimal change in SMN levels. Improvement in SMA phenotype and increase in lifespan. | 28\(^b\), 32\(^b\), 31\(^b\), 29\(^b\), 30\(^b\) |
| UBA1/β-catenin | NSC34 (motor neuron-like cells) | UBEI-41 (UBA1 inhibitor) | Increase in β-catenin levels | Taiwanese SMA model \(^{21}\) Zebrafish SMA model \(^{25}\) Drosophila SMA model \(^{24}\) | Quercetin (β-catenin inhibitor) | Improvement in neuromuscular, but not systemic pathology. No change in SMN levels (SMN-independent). | 119\(^a,b\) |

\(^a\) - Human studies, \(^b\) - Animal studies.
| MOLECULAR TARGETS/PATHWAYS | CELLULAR MODEL* | COMPOUNDS/GENES | OUTCOMES | ANIMAL MODELb | COMPOUNDS/GENES | OUTCOMES | REFERENCES |
|---------------------------|-----------------|----------------|----------|---------------|----------------|----------|------------|
| PLS3                      | HEK293          | Overexpression of PLS3 | Increase in F-actin levels, stabilization of growth cones, improved axonogenesis and neurite growth. | Zebrafish SMA model125, Taiwanese SMA mouse model21, SMAΔ7 model127 | Overexpression of PLS3 | Rescued axonal outgrowth defects in motor neurons from SMA mouse and in zebrafish. Improved NMJs, stabilization of axons and increased muscle fiber size. No improvement in SMA phenotype. | 103ab, 106, 107b |
| ZPR1                      | SMA patient fibroblast | Overexpression of ZPR1 | Increase in SMN levels and number of gems. Neurite growth stimulation and rescue of axonal growth defects. | Generation of new mild SMA-like model (Smn−/−; Zpr1+−/−)106 | Reduced Zpr1+/− gene dosage. | Increased loss of motor neurons. Hyper-myelination of phrenic nerve. Decrease in lifespan of SMA mice. | 104ab |
| IGF-1R                    | MN-1 cells      | Mouse Igf-1r siRNA, Human IGF-1R siRNA | Increase in SMN levels. | Generation of Taiwanese SMA mouse model with Igf-1r−/− | Reduced Igf-1r−/− gene dosage. | Increase in SMN levels, Improvement in SMA phenotype and increase in lifespan. | 25ab |
| JNK3                      | Neuron-based SMA model (Primary cerebellar granule neurons and SMN knockdown with siRNA) | JNK3-deficiency | Reduced degeneration of SMN-deficient neurons. | Generation of new SMAΔ7 model with Jnk3+/−-null background (SMA−/−)127 | Genetic inhibition of Jnk3 by knockout of the Jnk3 gene. | Reduced spinal motor neuron degeneration, improved motor function and muscle growth. Systemic improvement in SMA phenotype with increase in lifespan. No change in SMN levels (SMN-independent). | 84ab |

Notes: *In vitro studies using cellular models. bIn vivo studies using animal models.
that mediate neurodegeneration in SMA have provided insights into SMN-dependent and SMN-independent mechanisms and the potential non-SMN therapeutic targets that laid a foundation to develop new strategies for therapeutic intervention in SMA. A summary of the signaling pathways regulating SMN2 expression and the molecular mechanisms mediating the neurodegeneration in SMA is presented in Table 1. In addition, the molecular targets that have been tested to examine the therapeutic potential in preclinical studies using SMA animal models are identified. Recent studies have also provided insights into the complexity of SMA disease as a multisystem disorder, in which the primary pathogenesis is the degeneration of the spinal cord motor neurons and muscle atrophy, accompanied by complications in the development and functioning of multiple nonneuronal organs, including the heart, liver, pancreas, vasculature, respiratory system (lungs, diaphragm, and phrenic nerve), and gastrointestinal system reviewed in recent publications. Collectively, these advances in the field of SMA point to the development of combinatorial treatments to simultaneously increase the levels of SMN and prevent neurodegeneration using non-SMN targets and SMN-independent mechanisms to restore the normal function of neuronal and nonneuronal tissues and organs.

Author Contributions
Conceived and designed the topic and structure of the review: LG. Prepared first draft of the manuscript: SA. Contributed to the writing of the manuscript: KB and AK. Made critical revisions and prepared final version: LG. All authors reviewed and approved of the final manuscript.

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