EXPLORING MODIFIERS IN SPINAL MUSCULAR ATROPHY: THE POWER OF AAV9

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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presented by Jolill Ross,

a candidate for the degree of master of sciences,

and hereby certify that, in their opinion, it is worthy of acceptance.

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LIST OF ABBREVIATIONS

AAV9	Adeno associated virus serotype 9
ALS	Amytrophic Lateral Sclerosis
ASO	Antisense oligonucleotide
CBA	Chicken-β-actin
CBC	Cap binding complex
CNS	Central nervous system
COP1	Coat protein 1
DMEM	Dulbecco's Modified Eagle's Medium
dSmn	*Drosophila melanogaster* Smn
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
HDACi	Histone deacetylase inhibitor
HEK	Human Embryonic Kidney
hnRNP	Heterogeneous nucleic ribonucleicproteins
ICV	Intracerebroventricular
ISS	Intron splicing silencer

IV	Intravenous
LB	Lysogeny broth
MO	Morpholino
m^7g	7-methylguanosine
NAC	Non-amyloidogenic core
NF	Neurofilament
NMJ	Neuromuscular junction
PEI	Polyethylenimine
PHAX	Phosphorylated adaptor for RNA export
pICln	Chloride conductance regulatory protein
PND	Post natal day
PRMT5	Protein arginine methyltransferase 5
RAN	Ras related nuclear protein
RNAi	RNA interference
RNPs	Ribonucleicproteins
SAHA	Suberoylanilide hydroxamic acid
scAAV9	Self complementary adeno associated virus serotype 9

siRNA	Small interfering RNA
Sm	Smith antigen
SMA	Spinal Muscular Atrophy
SMN	*Survival Motor Neuron*
SMNΔ7	Survival Motor Neuron delta 7
SMNrp1	Survival Motor Neuron related protein 1
SNARE	Snap receptor
snRNA	Small nuclear RNA
snRNPs	Small nuclear ribonucleicproteins
SIP1	SMN interacting protein 1
Syt1	Synaptotagmin 1
SV2	Synaptic vesicle 2
TGS1	Trimethlyguanosine synthetase 1
TSA	Trichostatin A
UNRIP	UNR-interacting protein
XPO1	Exportin 1
ZBP	Z-DNA binding protein

ABSTRACT

Spinal Muscular Atrophy (SMA) is the second most common autosomal recessive disorder. The rate of new occurring cases is 1 in 6,000, with a carrier frequency of 1 in 35. SMA is characterized by the loss of *Survival Motor Neuron 1 (SMN1)* gene, resulting in the degeneration of motor neurons located in the spinal cord, atrophy of muscles, paralysis, and eventually death. We utilize scAAV9, a viral vector, as a tool for introducing heterogeneous genes as a method for delivery. We and others have shown that delivery of full length SMN1 to the "delta 7" SMA mouse model fully rescues the disease. Therefore, we employ scAAV9 to introduce genes that are designed to modify the disease phenotype. Furthermore, we will investigate which domains of SMN are important for its function with scAAV9. SMNrp1 is a putative paralogue to SMN; the Tudor domain of SMNrp1 is highly similar to the Tudor domain of SMN. Both SMNrp1 and SMN are involved in spliceosome formation, therefore we hypothesized that SMNrp1 may be able to restore the function of SMN, making it a potential candidate as a modifier for SMA. Another example of a potential modifier of SMA is α-synuclein, which functions with synaptic vesicle recycling at the pre-synaptic membrane. Reduced α-synuclein protein and mRNA levels have been shown to correlate with reduced levels of SMN protein. Therefore, we hypothesized that α-synuclein may work with SMN to stabilize and protect motor neurons from SMA. In order to determine which functional domains within the SMN protein are particularly relevant for the development of SMA, we interrogated the ability of phylogentically diverse SMN proteins to ameliorate the

SMA phenotype in mice. Phylogentically more distant species, such as Drosophila or *S. pombe* were hypothesized to be unable to restore SMN function, because of the reduced conservation of SMN protein domains. For this study, the severe SMNΔ7 mouse model was utilized. These studies will provide insight into the function of SMN as well as provide evidence for potential therapeutics for SMA.

Chapter 1: INTRODUCTION

Spinal Muscular Atrophy Background

Spinal Muscular Atrophy (SMA) is a neurodegenerative disorder, second in frequency to cystic fibrosis, and the most common cause of infantile death, with an incidence of 1:35 and a carrier frequency of 1:6000 [1, 2]. SMA affects all populations; however Caucasians are more likely to be affected, with a carrier frequency of 1:35 compared to Hispanics, with a 1:117 chance of being a carrier. This disease is caused by the loss or functional inactivation of *Survival Motor Neuron 1* (*SMN1*) gene, resulting in the loss of lower motor neurons in the anterior horn of the spinal cord [3, 4]. Eventually muscle wasting (atrophy) of proximal voluntary muscle sets in, followed by the weakened distal muscles. Paralysis soon follows muscle atrophy, and systemic defects from multiple organ failure are the cause of death.

Survival Motor Neuron genetics

SMN1 gene has a nearly identical copy called *SMN2*, which is classified as a disease modifier. *SMN1* is located near the telomeric portion, while *SMN2* is near the centromeric area of chromosome 5q13. They are a part of a 500 kb inverted duplication and share over a 99% nucleotide identity [5]. The human SMN genes have 9 exons, where Exon 2 is broken into 2a and 2b [6]. The nucleotide differences that do arise are located on the 3' end of the genes. In *SMN2,* there is a C to T transition or non-

polymorphic nucleotide difference located at position +6 of Exon 7, within the coding region. This silent mutation, which does not affect the amino acid sequence, leads to alternatively spliced mRNA lacking Exon 7 [7]. From this mutation in *SMN2*, mRNA lacking Exon 7 (SMNΔ7) is produced about 85 to 90% of the time, where full length mRNA containing Exon 7 is only produced 10% of the time. Therefore, the loss of full length SMN protein is responsible for SMA.

Normally, full length mRNA containing Exon 7, transcribed from the *SMN1* gene is produced 100% of the time. As a result of SMNΔ7, the translated protein product is unstable and rapidly degraded. The *SMN2* gene is considered a modifier of the disease because the more copies of *SMN2* a patient has, the more full length SMN is produced. Variability in the copy number of *SMN2* is due to the inverted duplication region. It contains at least 4 genes and repetitive elements that are subject to rearrangements and deletions. Furthermore, it is thought that gene conversions are the main reason for variable copy numbers of *SMN2*.

SMA Clinical Types

Due to the complete loss of *SMN1*, the more copies of *SMN2* a patient has, the less severe the disease. Therefore, SMA is divided into five clinical subtypes. Type 0 is considered embryonic lethality. Type I SMA or Werdnig-Hoffman disease, results from 2 copies of *SMN2,* where symptoms arise anywhere from 0 to 6 months of age. These symptoms include the inability to sit up and the inefficiency of major bodily organs, especially the respiratory system. For these children, the main cause of death is

respiratory failure induced by pneumonia [8]. The life expectancy for Type I SMA is 2 to 4 years of age. In Type II SMA or Dubowitz disease, most patients have 3 copies of *SMN2*, with an onset of symptoms anywhere from 6 to 18 months of age. Children with Type II SMA gain the ability to sit up, and have a life expectancy into adolescence. Patients with Type III juvenile SMA or Kugelberg–Welander disease have 4 copies of *SMN2*, resulting in a later onset of symptoms anywhere from 18 months to 30 years of age. They are able to walk sometimes, but a wheel chair is still needed. Type III SMA patients have a normal life expectancy. Type IV SMA patients with 4 or more copies of *SMN2,* have an adult onset of symptoms, where muscles progressively get weaker. There life expectancy is also normal. If a patient has 5 or more copies of *SMN2*, they are classified as unaffected.

SMN Pre-mRNA Splicing: Major and Minor Pathways

Splicing is the process where introns are removed by the spliceosome from pre-mRNA leaving only the exons in mRNA. The major and minor pathways both utilize small nuclear ribonucleicproteins (snRNPs) to remove these introns. snRNPs including U1, U2, U4, U5 and U6 are part of the major splicing pathway, while U11, U12, U4atac and U6atac are part of the minor splicing pathway. The major splicing pathway accounts for 99% of splicing [9]. The minor splicing pathway though very similar to the major splicing pathway, splices rare introns with different splice site sequences [9]. Pre-mRNA splicing of SMN is regulated by cis- and trans- acting proteins that help to either enhance or repress the splicing of introns and exons. Specifically hTRa2-β1 and SF2/ASF are exon

splicing enhancers that direct accurate splicing of Exon 7 in the pre-mRNA. However, the C to T transition in the *SMN2* gene creates a binding site for a splicing silencer heterogeneous nuclear RNP-A1 (hnRNP-A1), that promotes the skipping of Exon 7 [3].

SMN Protein Functions: snRNP Biogenesis

The stable, full length, ubiquitously expressed SMN protein produced is localized in both the cytoplasm and the nucleus. SMN protein localized in the nucleus is located near both gems or subnuclear bodies, and cajal bodies. While localized in the nucleus it carries out one if its main functions, which is snRNP biogenesis [10-17]. snRNPs are composed of U small nuclear RNA (snRNA), which includes U1, U2, U4, U5, U11 and U12. They are also composed of additional proteins, specific to each snRNP.

Assembly of snRNPs is controlled by the SMN complex. This process begins in the cytoplasm, where 7 Smith antigen or Sm proteins bind and form two separate complexes with the chloride conductance regulatory protein (pICln) [18, 19]. pICln first binds SmB and SmD3 complex, and then binds the SmD1 and SmD2 complex. The SmD1 and SmD2 complex then bind SmE, SmF and SmG [20]. Once bound, the protein arginine methyltransferase 5 (PRMT5) complex and PRMT7 complex methylate SmB, SmD1 and SmD3 [18, 19]. Methylated Sm proteins are then released and bind the SMN complex, facilitated by PRMT5. The SMN complex consists of UNR-interacting protein (UNRIP), SMN and GEMIN 2-8. In this complex, SMN forms a self oligomer that is necessary for its function [21, 22]. From here, the SMN complex binds snRNA transcribed in the nucleus and exported to the cytoplasm by export proteins: phosphorylated adaptor for RNA

export (PHAX), Ras-related nuclear protein (RAN), exportin 1 (XPO1), and cap-binding complex (CBC) [23]. The SMN complex assembles Sm proteins onto the snRNA, followed by hypermethylation of the 7-methylguanosine cap (m^7G) on the snRNA by trimethlyguanosine synthetase 1 (TGS1) [23]. The SMN complex along with the snRNA binds importin-β and snuportin, which transports the complex into the nucleus. Once in the nucleus, the SMN complex and assembled snRNP localizes in cajal bodies, where the snRNP undergoes further development.

SMN Protein Functions: Axonal mRNA Transport

SMN function is not only seen with snRNP biogenesis, but with axonal mRNA trafficking [24]. SMN has been shown to associate with ribonucleicproteins (RNPs) outside of snRNPs and Sm proteins. These include Q and R heterogeneous nucleic ribonucleicproteins (hnRNPs) and Z DNA binding protein (ZBP) 1 and 2 located in the axons [25, 26]. This complex together with SMN is responsible for transporting β-actin mRNA to the growth cone. Other mRNAs transported to the growth cone include KSRP/ZBP2/MARTA1, FBP and hnRNPQ/R [3, 27, 28]. Transport of β-actin mRNA to the growth cone leads to its accumulation at the distal end of the neuromuscular junction (NMJ), resulting in well developed NMJs [29, 30].

Effects of SMN Loss: Axonal mRNA Transport

Well developed NMJs are important because they connect the nervous system to the muscular system through synapses. Action potentials or nerve impulses that

reach the end of motor neurons trigger the opening of voltage dependent calcium channels. An influx of calcium into the neuron binds sensor proteins located on synaptic vesicles. This prompts the synaptic vesicles to fuse with the pre-synaptic membrane and release neurotransmitters into the synaptic cleft. These neurotransmitters bind their receptors located on the plasma membrane of muscle fibers or the sarcolemma, which causes depolarization of the muscle fiber, resulting in a contraction of the muscle fiber [31].

Knockdown of SMN in a zebrafish model showed defects in NMJs [28, 30]. In theory, without SMN axonal transport of β-actin mRNA and various other mRNAs to the growth cone, poorly developed NMJs would not be able to release neurotransmitters into the synaptic cleft, thereby leading to decreased muscle contractions. Decreased muscle contractions will eventually lead to degeneration of muscles, which is a hallmark of SMA. However, contrasting evidence shows that in the severe mouse model with a knockdown of SMN, NMJ defects are not observed [32, 33]. Furthermore, in a zebrafish SMA model, NMJ defects were rescued with snRNP particles that did not contain SMN [34]. Therefore, an ongoing debate exists as to whether the function of SMN with axonal transport contributes to the SMA disease phenotype or if NMJ defects are just a downstream effect.

Effects of SMN Loss: snRNP Biogenesis

The loss of functional SMN clearly affects assembly of snRNPs, specifically those apart of the minor spliceosome pathway [35, 36]. U11 and U12 snRNP expression

showed a clear reduction in severe SMA mice compared to control animals [23]. Other snRNPs showed reduction as well, but not as statistically significant as U11 and U12 [23]. These snRNPs are a part of the minor spliceosome pathway, and function to remove introns from the pre-mRNA, similarly to U1 and U2 of the major spliceosome pathway. U1 and U2 snRNPs are functional analogs of U11 and U12 [36].

In the minor splicesome pathway, U11 and U12 bind each other to form a stable di-snRNP, which subsequently binds the 5' splice site and branch site forming the A complex. Unlike the minor spliceosome pathway, U1 binds the 5' splice site to form the E complex, followed by U2 binding the branch point forming the A complex. U11 and U12 do not form the E complex [36]. From here, U4/U5/U6 tri-snRNP bind U1 and U2 of the major pathway or U4atac/U5/U6atac tri-snRNP bind U11 and U12 of the minor pathway to form the B complex. Interestingly U5 is the only snRNP that functions in both pathways. Structural rearrangements occur, activating the B* complex, and U4/U6 or U4atac/U6atac base pairing is disrupted. U1/U4 or U11/U4atac snRNPs are displaced. A region of U6 or U6atac forms a stem loop, introns are removed from the pre-mRNA, and U snRNPs are disassociated and recycled [36]. It is thought that due to a lack of SMN, existing SMN may only assemble snRNPs in the minor spliceosome pathway, since the concentration of these snRNPs are reduced compared to those of the major splicesome pathway [3, 23].

Background on SMA models

Functional models engineered with *SMN2* are important for the study of SMA, since humans only carry this gene. The current well characterized species used to model SMA include: *Schizosaccharomyces pombe (S. pombe), Saccharomyces cerevisiae (S. cerevisiae), Caenorhabditis elegans (C. elegans), Drosophila melanogaster (D. melanogaster), Danio rerio (D. rerio),* and *Mus musculus*. Yeast, which have the least homology to human SMN were one of the first models to emerge for the study of SMA. *S. pombe* has been shown to contain Yab8p protein, functionally similar to human SMN, and Yip1p protein, which is homologous to SMN interacting protein 1 (SIP1 or Gemin 2) in humans [37]. Both Yab8p and Yip1p were found to colocalize in the nucleus, and suppression of *Yab8p* caused accumulation of poly(A) mRNA and inhibition of splicing [37]. *Yip1p* is 20% identical and 45% similar to SIP1, and an ortholog of *Yip1p*, *Brr1p* is found in *S. cerevisiae* [38, 39]. Furthermore, knockout of *Yab8p* in *S. pombe* is lethal; however it cannot be rescued with human SMN [38]. This data demonstrated that SMN function is less conserved at the eukaryotic level.

C. elegans are nematodes that have proven useful as a model for SMA. *C. elegans* only contain one ortholog of human SMN, *smn-1*, which is 36% identical to human *SMN1* [40, 41]. Reduction of *smn-1* was achieved by either exposing the worms to mutagens which cause genetic mutations or knocking down the gene with RNA interference (RNAi) by feeding the worms small interfering RNA (siRNA) libraries. Knocking out *smn-1* with RNAi causes larval lethality since a reduction in F_1 descendants was observed. This was explained by reduced levels of maternal and/or early zygotic

smn-1. Therefore, a null mutation was engineered to delete the coding region of the *smn-1* gene [42]. The resulting *smn-1*(*ok355*) mutation reduces the life span and leads to a progressive loss of motor function in worms [43]. Neuron specific defects were verified in these worms when a neuron or muscle specific transgene of *smn-1* was introduced, and the transgene specific to neurons partially rescued motor defects, while the muscle transgene did not [43]. This model of SMA has also been used for drug screening, which thereby required an intermediate model, so modifications of the phenotype would be apparent. A point mutation in *smn-1, smn-1(cb131)*, gave rise to a less severe phenotype where worms had weak motor defects with a slightly longer life span compared to the more severe worms [44].

D. melanogaster only have one copy of the SMN ortholog, Smn, and is a well substantiated SMA model. This gene has 41% similarity to the human *SMN1* sequence, and a complete loss of this gene also causes pupal lethality [45]. Point mutations in *D. melanogaster* similar to those seen in human *SMN1* gave rise to a phenotype that includes disorganization of motor neuron boutons, reduction in excitatory post-synaptic currents, glutamate receptor loss at the NMJs, and a reduction in motor function [46]. *D. melanogaster* with mutations that cause a partial loss of function of Smn exhibit defective axonal branching in motor neurons and a loss of thin filament formation in muscles [47]. This model has been used to investigate snRNP assembly and pre-mRNA splicing of Smn. Both major and minor snRNPs were not affected in this model, suggesting that snRNP deficiencies are dependent upon the species [47, 48]. However recent reports in the Smn-deficient *D. melanogaster* model have shown that U12

containing genes including *Stasimon*, display defective splicing leading to poor NMJ and muscle development [49]. *D. melanogaster* have also been used to screen for genes that interact with Smn, as well as uncovering other functions of Smn, proving to be a useful model [44, 48, 50] .

Danio rerio or zebrafish are also a useful SMA model, since neuromuscular defects can be easily studied. Mutations in this SMA model not only give rise to motor defects seen in Amyotrophic Lateral Sclerosis (ALS), but knockdown of the Smn protein causes defects in outgrowth and path finding of motor axons seen in SMA [28, 51, 52]. This model is well characterized due to the ability of easily studying axon morphology and neuromuscular junction formation. Knockdown of Smn protein was achieved through the use of antisense morpholinos (MO) against the only copy of the *smn* gene in zebrafish. As a result, increased axon branching and truncated axons were observed. Human SMN mRNA was able to partially rescue some defects, where axonal branching was slightly reduced after administration [28]. Additional zebrafish mutants have been engineered *smnY267stop*, *smnL265stop*, and *smnG264D* which are two stop mutations and a missense mutation in Exon 7. These mutants exhibited reduced Smn protein and synaptic vesicle 2 (SV2) protein suggesting that Smn may play a role in the integrity of the pre-synapse [30]. These studies are just a few to corroborate the usefulness of the zebrafish model with SMN studies.

Mus musculus or mouse SMA models are well accepted, with the ability to mirror clinical phenotypes seen in SMA patients that span from severe to mild cases. Mice, similar to other species stated earlier only have one copy of SMN, *Smn1*. A very severe

mouse model was generated by disrupting the first 40 nucleotides of Exon 2 with a neomycin cassette and a *lacZ* fused gene. These $Smn^{-/-}$ mice at the early embryonic stage displayed massive cell death, or embryonic lethality [53]. Therefore, the SMN gene is extremely important for the biologic functionality of mice. Following this mouse model, many more transgenic lines were generated with phenotypes similar to those seen in Type I, II and III SMA patients. Deletion of Exon 7 (Δ7) in mouse *Smn1* using the Cre-loxP system restricted to the skeletal muscle, gave rise to muscle necrosis, paralysis and eventually death. These muscle defects suggested a major role of skeletal muscle in human SMA [54]. A similar study was conducted to target neurons, but not skeletal muscle. The C terminus of Exon 7 in mouse *Smn1* was deleted using the Cre-loxP system, which led to motor neuron and axon loss [55].

One of the most widely used models is the SMNΔ7 (deletion of Exon 7) mouse model, which correlates to a severe form of SMA. SMNΔ7 transgenic mice were crossed with mice containing human *SMN2* and mouse *Smn* knockout allele to obtain double transgenic mice ($Smn1^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$). These mice were crossed with each other to get SMNΔ7 mice ($Smn1^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$), which have a life span on average of approximately 13 days [56]. Phenotype changes became apparent around post natal day (PND) 5, including muscle weakness, impaired gait, hind limb instability, and a tendency to fall. By PND9 these mice displayed severe defects in the NMJs and a loss of more than 50% of spinal motor neurons [56]. The phenotype seen in this model, made it very useful to many labs to study SMA. A more recent, well characterized intermediate model is the $Smn^{2B/-}$ mouse model. The 2B mutation results from a 3

nucleotide substitution in the exonic splicing enhancer hTra-2β1, in Exon 7 of mouse *Smn*. On average, these mice live 28 days, with an average onset of disease phenotype around 17 days. The phenotype observed included physical weakness, tremors, and ambulating difficulty in the hind limbs. Decreased weight gain, along with tail necrosis and kyphosis were also observed [57]. On the molecular level, mice exhibited a higher percentage of degenerating axons, with a reduction in the number of axons, along with loss of NMJs [57]. Therefore, this mouse model is well suited to study SMA, and potential disease modifiers.

Potential SMA Therapeutics

Spinal Muscular Atrophy is a disease currently without a cure. Therefore, methods of prediction and intervention are rigorously being studied. Molecular genetic testing and carrier screening is recommended to SMA patients and their families, due to the variability of onset. These tests aid in the diagnosis of SMA, as well as determining the chances of one inheriting the *SMN1* deletion [58]. SMA results from the loss of the *SMN1* gene. Due to mutation in *SMN2* and the alternative splicing, only small amounts of functional SMN protein are generated. Therefore intervention is directed at replenishing and/or compensating for the lack of fully functional SMN protein. Various methods are being heavily studied geared towards this type of intervention, including small molecule drugs, gene therapy, and antisense oligonucleotide (ASO) therapy.

Small molecule drugs are utilized to activate the expression of *SMN2*. Because in all cases of SMA, *SMN1* expression is completely lost which severely limits the amount

of full length SMN protein produced, increasing expression of full length SMN protein from SMN2 could compensate for this loss. To increase the amount of full length SMN protein translated from SMN2, either *SMN2* expression should be activated, and/or inclusion of Exon 7 transcript should be improved. Small molecule drugs are mainly targeted at increasing expression of *SMN2.* One example of a small molecule drug is histone deacetylase inhibitors (HDACi). Normally, HDACs transcriptionally repress chromatin by deacetylating chromatin histones. Therefore, it was reasoned that inhibiting HDACs may activate the *SMN2* gene. Specifically sodium butyrate was the first HDACi that could activate *SMN2* [59]. HDACi with the same ability following sodium butyrate included: valproic acid, phenylbutyrate, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) [60-65]. They were all tested using either patient derived cultures or animal models. Though TSA and SAHA were able to extend the survival of SMA mice by 30% to 40%, the real problem arises with the toxicity of TSA, especially for clinical trials [65, 66].

Quinazoline derivatives also showed promise increasing expression of *SMN2*. These small molecules were identified using a high-throughput screening approach and found to inhibit the degradation of the 5' cap during mRNA decay. When tested on SMA mice, results showed promise with increased survival and weight gain, as well as modest improvement with motor defects [67, 68]. Therefore, this compound advanced to Phase I clinical trials. For the inclusion of Exon 7, hydroxyurea was investigated since it increased the ratio of full length SMN to truncated SMN protein [69]. However, contradicting evidence published suggested that it had no effect on patient cells after

treatment [70]. Albarubicin was another drug that was able to increase Exon 7 inclusion in SMA derived patient fibroblasts, where it increased SMN protein and gem numbers. However it had a high toxicity level when used long term [71]. Various other drugs have also been investigated such as the aminoglycoside TC007, the proteasome inhibitor Bortezomib, and SMN C1-C3 compounds which have the ability to increase Exon7 inclusion and SMN protein expression [72-74]. But the main issue to overcome is still the toxicity level in human patients.

Gene therapy is another method that has proven very useful in animal studies and was recently advanced to patient clinical trials. Adeno associated viral vectors with specific tropisms have been used as a delivery method for SMN, through intracerebroventricular (ICV) or intravenous (IV) injections [75-78]. Self- complementary adeno associated virus serotype 9 (scAAV9) has been used widely because of its ability to transduce motor neurons [75]. scAAV9 is also useful because it easily crosses the blood brain barrier [78, 79]. Delivery of either scAAV9-SMN by IV injections or scAAV8-SMN by ICV injections extended the survival of severe SMA mice [76, 79]. Furthermore, in these studies IV injections proved to be more beneficial then ICV, since IV injected pups lived over 250 days, while IV injected pups lived an average of 157 days [76, 79]. Other labs have also shown that early injection of scAAV9-SMN on PND1 was able to increase weight gain and extend survival of severe SMA mice [80]. However, ICV injected mice gained more weight and had fewer early deaths than IV treated mice, suggesting that delivery through ICV injections was more beneficial then IV delivery [81]. Gene therapy with scAAV9-SMN has recently advanced to Phase 1 clinical trials. Type I

SMA patients will receive either a low dosage (6.7 X 10^{13} vg/kg) or a high dosage (3.3 X 10^{14} vg/kg) of scAAV9-SMN through IV infusion [82]. Because this clinical trial is ongoing, results have not been collected as of yet. However this study will help corroborate the power of scAAV9 IV delivery as a promising therapeutic for SMA.

Another promising strategy to increase Exon 7 inclusion is the use of antisense oligonucleotides (ASOs). They are modified nucleotides that bind specific mRNA sequences that interfere with RNA binding proteins. As an SMA therapeutic, ASOs are able to bind specific cis-acting splicing regulatory motifs, which can promote Exon 7 inclusion [68]. Targeted splicing regulatory motifs include intron splicing silencers (ISS) located at downstream of Exon 7, such as ISS-N1 [83]. With the use of various ASOs that targeted this region, a significant increase of SMN protein was observed in the brain of SMNΔ7 mice, along with extension in survival and increased weight gain [83]. Other targeted ISS sequences are located at the 5' end of introns 7 [84-86]. These ASOs were able to promote Exon 7 inclusion and increase SMN protein levels. Because ASOs showed such promise in animal models, some were extended to clinical trials. ISIS Pharmaceuticals developed a 2'-O-2-methyoxyethyl-modified ASO (ASO-10-27 or ISIS SMNRx), that not only corrects tail and ear necrosis in a mild mouse model of SMA, but extends the life span of severe SMA mice from 16 to 26 days with ICV injection [76, 87]. This ASO is currently a part of rigorous clinical trials in various stages. Two Phase 1 completed trials with ISIS SMNRx were determining how tolerable this ASO was to SMA patients. The results are still being determined and analyzed (Isis Pharmaceuticals). ASO targeted therapy is a lucrative strategy that could potentially compensate for the loss of

SMN protein in SMA patients in the future. Together, these strategies along with others may help in the fight against SMA.

Chapter 2: SURVIVAL MOTOR NEURON RELATED PROTEIN 1 AS A MODIFIER IN SPINAL MUSCULAR ATROPHY

Introduction

Functional U snRNPs, required for pre-mRNA splicing, are composed of small nuclear RNA (snRNA), Sm core proteins, and non snRNPs. These U snRNPs include U1, U2, U4/U6 and U5 [88]. U1 and U2 develop into a complex with the pre-assembled tri-snRNP U4/U5/U6, forming the spliceosome. The process of U snRNP assembly is mediated by the SMN complex, which includes SMN, GEMIN2-8 and UNR-interacting protein (UNRIP) and a heptameric ring of Sm proteins [9, 12, 89]. Together, the SMN complex and assembled U snRNPs are imported into the nucleus, where the spliceosome executes RNA splicing to remove introns from the pre-mRNA in a step wise process [90].

Survival Motor Neuron Related Protein 1 (SMNrp1) SPF30, or SMNdc1 is a putative paralogue (gene originated from intragenome duplication events) to SMN. The SMNrp1 gene is located on chromosome 10, and encodes a protein highly expressed in the skeletal muscle, along with minor expression in the heart, pancreas, brain and spinal cord. This tissue specific expression of SMNrp1 is in contrast to ubiquitous expression of SMN. SMNrp1 protein is localized in the nucleus. It is composed of approximately 239

amino acids, and shares a 34% identity to over 94 residues in the human SMN sequence [91].

SMNrp1 and SMN are homologous at the Tudor domain. This region in both proteins share 51% identity and 57% similarity, while flanking regions are less conserved [14]. The Tudor domain mediates binding to Sm proteins, by preferentially recognizing dimethylated arginine motifs. This is important for Sm assembly, and necessary for the formation of the spliceosome [91-94]. Therefore, it has been suggested that SMNrp1 and SMN both participate in spliceosome function [91]. SMNrp1 has been shown to be a 17S U2 snRNP-associated splicing factor, and essential for pre-mRNA splicing. It is required for mature spliceosome complex formation, and interacts with U4/U5/U6 snRNPs [14]. To explore SMNrp1 function, we produced scAAV-CBA-SMNrp1 virus, and ICV injected a 1×10^{11} v.p. dosage into $Smn^{2B/-}$ mice. Because SMNrp1 and SMN share homology and function, we hypothesized that overexpressed SMNrp1 might be able to compensate for the function of SMN. We found that SMNrp1 did not increase the survival or weight gain of the mice. This would suggest that the Tudor domain alone is not sufficient enough to functionally compensate for the loss of SMN.

Materials and Methods

Cloning

SMNrp1 cDNA was obtained from Thermo Fisher Scientific and cloned into the scAAV viral vector, driven by the chicken-β-actin (CBA) promoter. The restriction sites used were 5' *AgeI*, and 3' *HindIII* and *XhoI* blunt end sites for the scAAV vector and

SMNrp1 insert. The plasmid clone was transformed in the DH5α strain of competent E. coli bacteria cells, and plated on Ampicillin resistant Lysogeny Broth (LB)/Agarose plates, grown overnight in 37°C incubator. scAAV-CBA-SMNrp1 clones were screened using enzyme digestion, and verified using sequencing analysis, provided by the DNA Core facility.

Virus Production

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U Penicillin/ 100 µg Streptomycin per mL (Invitrogen). Cells were incubated in 37°C with 5% CO_2 until ≈ 90% confluency before transfection. Triple transfection was performed by using Polyethylenimine (PEI; Polysciences), to deliver scAAV-CBA-SMNrp1, pHelper, and Rep2/Cap9 plasmids into cells. 48 hrs post transfection cells were harvested. Virus produced from harvested cells was purified by three consecutive isopycnic CsCl gradient ultra-centrifugations. Real-time PCR was used to determine the viral titer following each ultra-centrifugation. CsCl was removed from purified virus during dialysis with HEPES buffer, repeated 3 times. Final titers of purified virus were determined using real-time PCR.

Real-Time PCR

Viral fractions obtained following ultra-centrifugation were diluted 1:2000. Quantification of viral genomes occurred using the Applied Biosystem® 7500 Real-Time PCR System. SYBR® Green and primers were utilized, to amplify a region following the

CBA promoter in the scAAV viral vector, using the forward (5'-CCGGTGGTGGTGCAAATCAAAGAA 3') and reverse (5' AGCAGAAGTAACACTTCCGTACAGGC 3') primers. A standard curve was used to calculate the absolute number of viral genome particles in each fraction. The standard curve for real-time PCR was generated using serial dilutions of the scAAV-CBA-GFP plasmid. Following real-time PCR, the viral fraction with the highest titer was advanced to dialysis, and used for animal studies.

Animals

Animals used for these studies were all housed and treated according to the Animal Care and Use Committee guidelines at the University of Missouri. These regulations were established in the "Guide for the Care and Use of Laboratory Animals". The *Smn*$^{2B/-}$ intermediate mouse model was utilized for these experiments. These mice generated from cross breeding *mSmn*$^{+/-}$ to *mSmn*$^{2B/2B}$ [57]. The 2B allele was designed to generate a 3 nucleotide substitution in the exonic splicing enhancer hTra-2β1, in Exon 7 of mouse *Smn*. This mouse model average life span ranges from 28-31 days, where the average onset of disease phenotype began around 10-17 days. Mice exhibited an intermediate phenotype, including physical weakness, tremors, and ambulating difficulty in the hind limbs. Decreased weight gain, along with tail necrosis and kyphosis were also observed. Mice with the *Smn*$^{2B/+}$ genotype were used as unaffected controls for these experiments.

Genotyping

To determine the genotype of *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice, tail biopsies were taken on PND1 and used for PCR analysis with primers specific for the mouse *Smn* gene. The primer sets used for genotyping were: *mSmn*-WT forward (5' TCTGTGTTCGTGCGTGCGTGGTGACTTT 3'), *mSmn*-WT reverse (5' CCCACCACCTAAGAAAGCCTCAAT 3'), *lacZ mSmn* knockout allele forward (5' CCAACTTAATCGCCTTGCAGCACA 3') and the *lacZ mSmn* knockout allele reverse (5' AAGCG-AGTGGCAACATGGAAATCG 3'). PCR samples were analyzed by gel electrophoresis. *Smn*$^{2B/-}$ mice were identified by a 327 bp *Smn* and 626 bp *lacZ* band, while *Smn*$^{2B/+}$ is identified by only a 327 bp *Smn* band.

Survival, Weight Gain and Phenotype

All mice were monitored daily to record their survival and weight gain. Time to right was determined by placing the mice on their back, and timing how long it took them to right themselves. Time was capped at 30 seconds. Motor function was scored and evaluated based on the time recorded for the righting reflex. Mice unable to right themselves, within 30 seconds were classified as failed. Wild-type mice with good motor function were usually able to right themselves in 1 to 2 seconds.

Results

SMNrp1 did not extend the life span of *Smn*$^{2B/-}$ mice

In order to determine the function of SMNrp1, the cDNA was cloned in the scAAV viral vector. This clone along with pHelper and Rep2/Cap9 plasmids were transfected into HEK 293T cells, and harvested 2 days post transfection. The cells were lysed, and the virus was extracted and purified. A 1×10^{11} v.p. dosage of scAAV-CBA-SMNrp1 was delivered via intracerebroventricular (ICV) injection to *Smn*$^{2B/-}$ mice on PND2. The average life span for scAAV-CBA-SMNrp1 treated mice was 36.5 days. The median life span for treated mice was 31 days. Untreated *Smn*$^{2B/-}$ mice had an average life span of 44 days, with a median life span of 31 days. Collectively, we found that scAAV-CBA-SMNrp1 did not show any significant difference in survival between untreated and treated *Smn*$^{2B/-}$ mice (Figure 1).

SMNrp1 did not rescue the weight gain of *Smn*$^{2B/-}$ mice

The *Smn*$^{2B/-}$ mice treated with 1×10^{11} v.p. dosage of scAAV-SMNrp1 were weighed daily following treatment. There was no significant difference in weight gain daily between scAAV-CBA-SMNrp1 treated mice and untreated mice (Figure 2). The percent weight gain from birth to peak was also calculated, to determine how much weight was gained during development. The scAAV-CBA-SMNrp1 treated mice did not show any significant difference in the weight gain from birth to peak, compared to untreated *Smn*$^{2B/-}$ mice (Figure 3). Therefore, SMNrp1 had no impact on the overall weight gain of *Smn*$^{2B/-}$ mice.

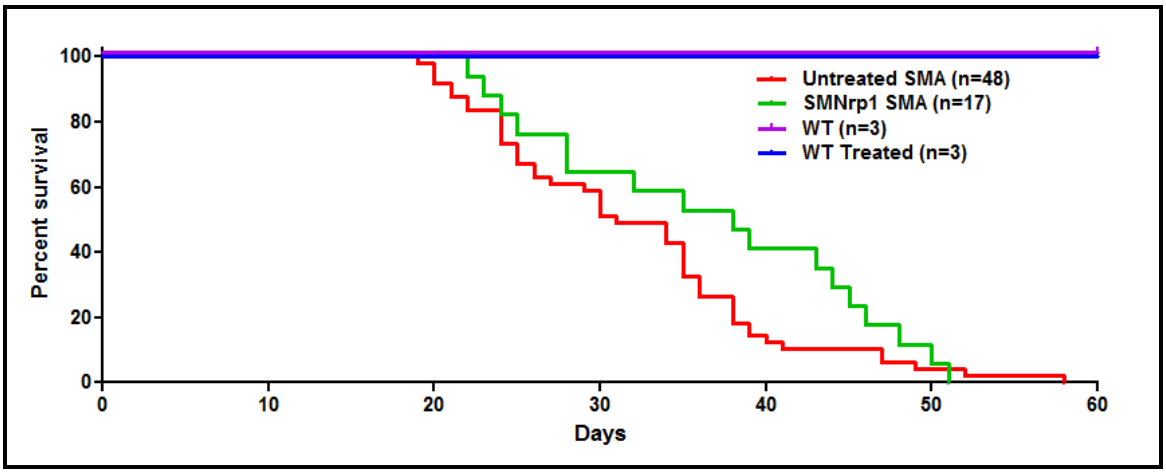

Figure 1: SMNrp1 did not extend the survival of *Smn*$^{2B/-}$ mice.

Kaplan Meier survival curve showed the life span in days of Untreated SMA, SMNrp1 SMA, WT, and WT treated mice with or without 1 x 10^{11} v.p. of scAAV-CBA-SMNrp1.

p = 0.177 Untreated vs SMNrp1

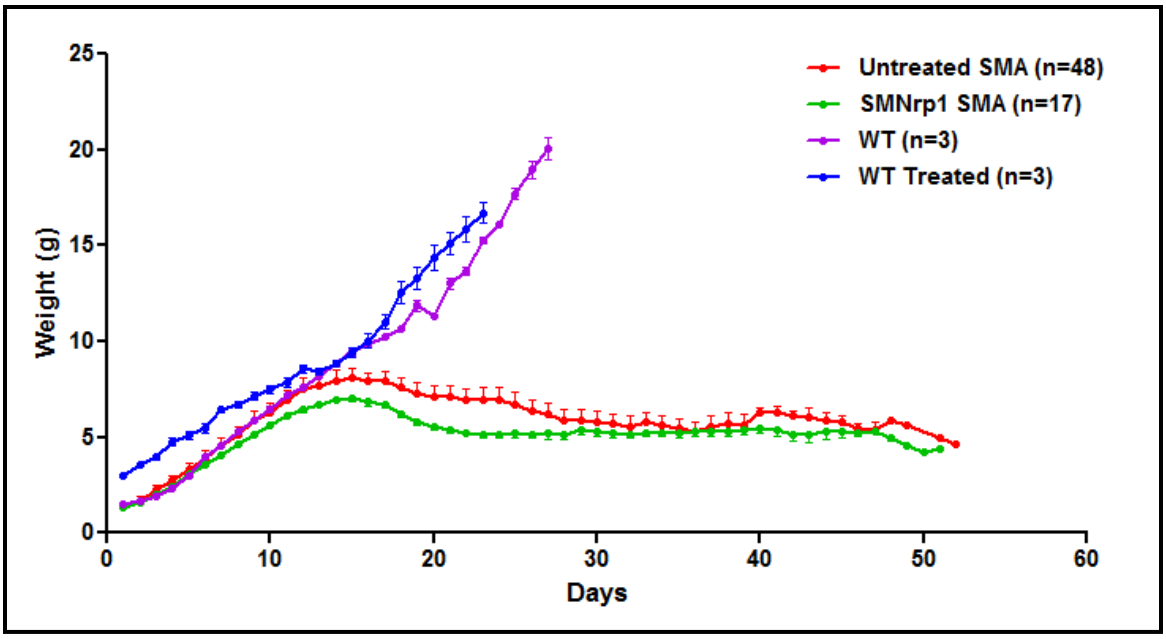

Figure 2: SMNrp1 daily weight gain curve.

Untreated SMA, SMNrp1 SMA, WT and WT treated mice with or without 1 x 10^{11} v.p. of scAAV-CBA-SMNrp1 were monitored daily. Recorded weights for unaffected and unaffected treated mice were stopped after 28 days.

$p < .05$ PND7- PND14, PND20-PND26 Unaffected vs SMNrp1

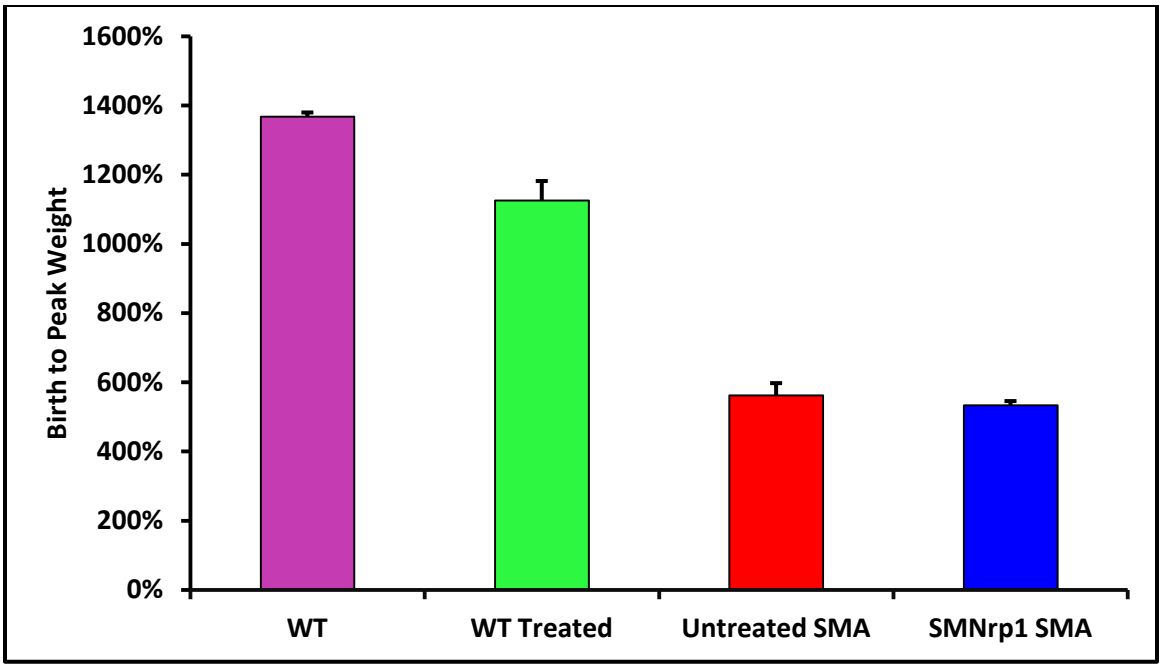

Figure 3: SMNrp1 did not rescue the percent weight gain of $Smn^{2B/-}$ mice.

Percent weight gain of each group was determined by the equation: ((peak weight - birth weight)/birth weight)*100.

Standard Deviations: WT 0.22, WT Treated 0.98, Untreated SMA 0.79, SMNrp1 SMA 0.52.

Standard Errors: WT 0.1254, WT Treated 0.5647, Untreated SMA 0.3543, SMNrp1 SMA 0.1251.

SMNrp1 did not change the motor function of *Smn*$^{2B/-}$ mice

To assess the motor function of scAAV- CBA-SMNrp1 treated mice, we performed the righting reflex test to assess the motor function of these mice. During this test, mice were placed on their back, and timed to see how long it took them to right themselves. They were allotted 30 seconds to right themselves. PND18 was chosen for analysis since variability was not observed until PND16, also when the disease phenotype sets in. We found the scAAV-SMNrp1 treated mice had a slower time to right than the untreated *Smn*$^{2B/-}$ mice (Figure 4).

Discussion

SMNrp1 is homologous to SMN at the Tudor domain, suggesting that it may function similarly to SMN by binding to Sm proteins and facilitating spliceosome formation. This function is possibly jeopardized in SMA patients, since the Tudor domain of SMN is mutated in some patients [95]. Here we wanted to determine whether the Tudor domain was sufficient enough to restore SMN function, by overexpressing SMNrp1 via viral delivery system in the intermediate SMN$^{2B/-}$ mouse model. Our results showed that SMNrp1 is not sufficient enough to improve the life span and motor function, but partially rescues the weight gain of these mice. This data suggested that SMNrp1 alone may not be enough to rescue the SMN$^{2B/-}$ mouse model. Furthermore, the Tudor domain, necessary for the formation of the spliceosome, may not be enough to restore the function of SMN.

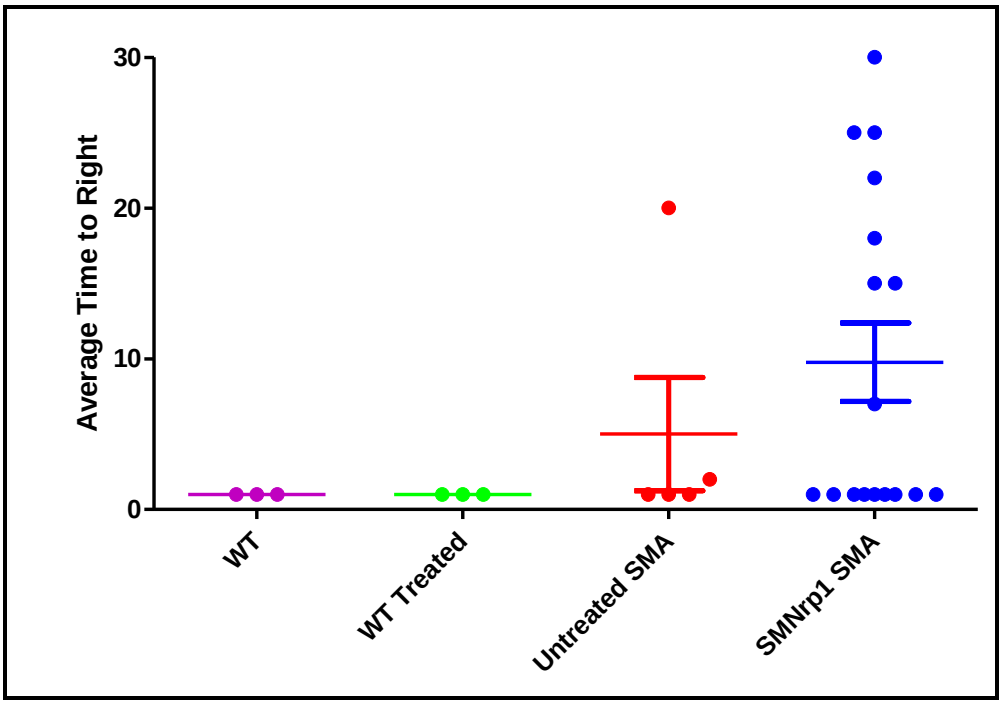

Figure 4: SMNrp1 did not rescue motor function of $SMN^{2B/-}$ mice.

Time to right of WT, WT treated, Untreated SMA, and SMNrp1 SMA mice were averaged on PND18.

$p > .05$ SMNrp1 vs Untreated

WT n=3; WT Treated n=3; Untreated SMA n= 5; SMNrp1 SMA n=17

Mutations in the Tudor domain, including E134K, have been reported in SMA patients, where it interrupts Sm protein binding to SMN [96]. The highly conserved Tudor domain plays a distinct role with functional SMN. SMN has been shown to bind glycine-arginine rich C terminal tails on Sm proteins at its Tudor domain [97]. Assembly of the Sm protein heptameric ring is essential for SMN mediated snRNP biogenesis.

Alongside the Tudor domain in Exon 3, the nucleic acid binding domain is located in the amino terminal of Exon 2. Oligomerization domains, located in both Exon 2 and Exon 6, are responsible for SMN self association [3]. This function is of high importance because without oligomerization, SMN cannot assimilate into the snRNP complex, thereby leaving SMN protein unstable. Other important domains and regions of the SMN protein include the Y/G box and the poly-proline motif. Deletion of the Y/G box abolishes SMN function, and the poly-proline motif binds profilin protein, which binds and sequesters inactive globular ATP [3, 98]. These functional domains are highly characterized; however it is still unclear which domains further contribute to SMA. Therefore, SMNrp1 was a good candidate to help understand this dilemma.

Even though SMNrp1 did not extend the survival and increase the weight of the $SMN^{2B/-}$ mice phenotype, protein expression of SMNrp1 needs to be confirmed. It would be interesting to determine whether SMNrp1 could improve the survival and weight of other SMA mouse models. Furthermore, coupling SMNrp1 with other potential modifiers might prove to be beneficial, rather than SMNrp1 alone. Understanding the role of SMNrp1, would help elucidate the function of SMNrp1 in SMA. This would shed light on the disease, the function of SMN, and potential therapeutics for SMA.

Chapter 3: ALPHA-SYNUCLEIN AS A MODIFIER IN SPINAL MUSCULAR ATROPHY

Introduction

Alpha-synuclein is a 140 amino acid protein encoded by the *SNCA* gene [99]. The protein consists of three domains: an amino terminal lipid binding α-helix domain, a non-amyloidogenic core (NAC) domain, and an unstructured carboxy-terminus [100]. These domains have been shown to be important for its natively unfolded structure [100]. It is expressed throughout the central nervous system (CNS), predominantly in the substantia nigra, neocortex, cerebellum, hippocampus and the thalamus [101]. Alpha-synuclein is a key factor in Parkinson's disease, where duplications and triplications of the *SNCA* gene are linked to early onset of this disease [102, 103]. Mutations in this gene can contribute to a misfolded α-synuclein protein, where the NAC domain forms β-sheets. The formation of β-sheets leads to protofibril and fibril formation of α-synuclein protein [100]. These fibrils have been shown to localize at a high concentration in abnormal aggregates, called Lewy bodies. Aggregates and aggregation is a hallmark of Parkinson's disease, which have proven to be cytotoxic [102].

Normally, α-synuclein is associated with synaptic vesicles, and recycling the pool of vesicles in the pre-synapse. Its proposed function is to increase the rate of Snap Receptor (SNARE) complex formation at the pre-synapse by binding the synaptobrevin-2, which is a SNARE protein [102]. From here, the pre-synaptic membrane fuses with the synaptic vesicle membrane, mediated by the synaptic-SNARE complex. This complex includes synaptobrevin-2, syntaxin-1 and SNAP-25. Synaptic vesicle fusion to the pre-synaptic membrane leads to neurotransmitter release from the pre-synapse, and recycling of synaptic vesicles [104].

SMN has been shown to be associated with vesicle trafficking, specifically with the vesicle coat protein I (COPI). This protein coats vesicles, and together with the COPI complex transports proteins from the cis- Golgi apparatus complex back to the rough endoplasmic reticulum (ER). From here the budding process of synaptic vesicles is initiated on the cis-Golgi membrane. SMN function is necessary for β-actin mRNA axonal transport to the growth cone of motor neurons [24]. β-actin is important for sufficient NMJ development [3]. SMN, α-COP (coatomer component of COPI), and β-actin were all shown to co-immunoprecipiate with each other [105]. Furthermore, in primary mouse neurons both SMN and α-COP were shown to travel together within the axons, and localize in the growth cone. When α-COP was depleted, SMN was mislocalized to the cytoplasm and nucleus as granules [105]. This suggests that α-COP association is necessary for the transport of SMN within axons.

In previous reports, a gene expression array associated with neurodegeneration was performed on mouse NSC-34 cells, and it displayed a reduction of α-synuclein

expression levels, in a cell line with low levels of SMN. Further investigation showed that in SMA patient fibroblasts and spinal tissue, low SMN levels correlated to low mRNA and protein levels of α-synuclein [106]. Therefore, we sought to understand the relationship between SMN and α-synuclein further, by overexpressing α-synuclein in the intermediate $Smn^{2B/-}$ mouse model. We produced scAAV-CBA-α-synuclein virus, and IV injected a 1×10^{11} v.p. dosage into $Smn^{2B/-}$ mice on PND2. We hypothesized that α-synuclein and SMN work together to stabilize and protect motor neurons in SMA. Our data showed that overexpressed scAAV-CBA-α-synuclein with a 1×10^{11} v.p. dosage increased weight gain of the mice. A 3×10^{11} v.p. dosage was able to extend survival and increase the daily and percent birth to peak weight gain of these mice.

Materials and Methods

Cloning

Alpha-synuclein cDNA obtained from Thermo Fisher Scientific was cloned into the scAAV viral vector. The restriction sites used were 5' *NcoI* and 3' *HindIII*. The plasmid clone was transformed into competent DH5α bacteria cells, and plated on Ampicillin resistant Lysogeny Broth (LB)/Agarose plates, grown overnight in 37°C incubator. scAAV-CBA-α-synuclein clones were screened using enzyme digestion and sequence analysis, provided by the DNA Core facility.

Virus Production

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U Penicillin/ 100 µg Streptomycin per mL (Invitrogen). Cells were incubated in 37°C with 5% CO_2 until ≈ 90% confluency before transfection. Triple transfection of scAAV-CBA-α-synuclein, pHelper, and Rep2/Cap9 plasmids into cells was performed by using Polyethylenimine (PEI; Polysciences). Forty-eight hrs post transfection cells were harvested. Virus produced from harvested cells was purified by three consecutive isopycnic CsCl gradient ultra-centrifugations. Real-time PCR was used to determine the viral titer following each ultra-centrifugation. CsCl was removed from purified virus during dialysis with HEPES buffer, repeated 3 times. Final titers of purified virus were determined using real-time PCR.

Real-Time PCR

Viral fractions obtained following ultra-centrifugation were diluted 1:2000. Quantification of viral genomes occurred using the Applied Biosystem® 7500 Real-Time PCR System. SYBR® Green and primers were utilized with collected fractions, to amplify the forward (5'- CCGGTGGTGGTGCAAATCAAAGAA 3') and reverse (5' AGCAGAAGTAACACTTCCGTACAGGC 3') region following the chicken β-actin (CBA) promoter in the scAAV viral vector. A standard curve is used to calculate the number of viral genome particles in each fraction. The standard curve for real-time PCR was generated using serial dilutions of the scAAV-CBA-GFP plasmid, which has a known copy

number. Following real-time PCR, the viral fraction with the highest titer was advanced to dialysis, and used for animal studies.

Animals

Animals used for these studies were all housed and treated according to the Animal Care and Use Committee guidelines at the University of Missouri. These regulations were established in the "Guide for the Care and Use of Laboratory Animals". The *Smn*$^{2B/-}$ intermediate mouse model was utilized for these experiments. These mice result from crossing *mSmn*$^{+/-}$ to *mSmn*$^{2B/2B}$ [57]. The 2B mutation results from a substitution of 3 nucleotides in the exon splicing enhancer hTra-2β1 of Exon 7. This mutation increases the life expectancy on average from 28-31 days, where phenotypic changes occur around 10-17 days. This mouse models average life span was from 28-31 days, where the average onset of disease phenotype began around 10-17 days. Mice exhibited an intermediate phenotype, including physical weakness, tremors, and ambulating difficulty in the hind limbs. Decreased weight gain, along with tail necrosis and kyphosis were also observed. Mice with the *Smn*$^{2B/+}$ genotype were used as unaffected controls for these experiments.

Genotyping

To determine the genotype of *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice, tail biopsies were taken on PND1 and used for PCR analysis with primers specific for the mouse *Smn* gene. The primer sets used for genotyping were: *mSmn*-WT forward (5' TCTGTGTTCGTGCGTGCGTGGTGACTTT 3'), *mSmn*-WT reverse (5'

CCCACCACCTAAGAAAGCCTCAAT 3'), *lacZ mSmn* knockout allele forward (5' CCAACTTAATCGCCTTGCAGCACA 3') and the *lacZ mSmn* knockout allele reverse (5' AAGCG-AGTGGCAACATGGAAATCG 3'). PCR samples were run on agarose gels during gel electrophoresis. $Smn^{2B/-}$ mice were identified by a 327 bp *Smn* and 626 bp *lacZ* band, while $Smn^{2B/+}$ was identified by only a 327 bp *Smn* band.

Survival, Weight Gain and Phenotype

All mice were monitored daily to record their survival and weight gain. Time to right was determined by placing the mice on their back, and timing how long it took them to right themselves. Time was capped at 30 seconds. Mice unable to right themselves, with a time of 30 seconds or more were classified with poor motor function. Mice able to right themselves in 1 to 2 seconds had good motor function.

Results

α-synuclein 1 x 10^{11} v.p. dosage did not extend the survival of $Smn^{2B/-}$ mice

To explore the function of α-synuclein, the cDNA was cloned in the scAAV viral vector. This clone paired with pHelper and Rep2/Cap9 plasmids were transfected into HEK 293T cells, and harvested 48 hrs following the transfection. The cells were lysed and the virus was extracted and purified. A 1 x 10^{11} v.p. dosage of scAAV-CBA-α-synuclein was delivered by IV injection to $Smn^{2B/-}$ mice on PND2. This dosage is considered the standard dosage since our lab has shown that a 1 x 10^{11} v.p. dosage of full length SMN rescues the phenotype of SMA mice [80]. The average life span for scAAV-CBA-α-

synuclein treated mice was 29 days. The median life span for scAAV-CBA-α-synuclein treated mice was 28 days. Untreated *Smn*$^{2B/-}$ mice had an average life span of 32 days, with a median life span of 31 days. This data showed that scAAV-CBA-α-synuclein did not show any significant difference in survival between untreated and treated *Smn*$^{2B/-}$ mice (Figure 5).

α-synuclein 1 x 10^{11} v.p. dosage increased the weight gain of SMN$^{2B/-}$ mice

Following treatment with standard 1 x 10^{11} v.p. dosage of scAAV-CBA-α-synuclein, *Smn*$^{2B/-}$ mice were weighed daily. There was no significant difference in weight gained between scAAV-CBA-α-synuclein treated mice and untreated mice (Figure 6). The birth to peak weight gain for standard dosage was also determined to assess overall weight gain. Surprisingly, the scAAV-CBA-α-synuclein treated mice showed a significant increase in birth to peak weight gain, compared to that of untreated mice (Figure 7). Determining the birth to peak weight gain reveals subtle differences that may expose overlooked variations in weight. Due to the significant increase in weight gain, we investigated if an increase in viral dosage of scAAV-CBA-α-synuclein could elicit a more profound phenotypic improvement in the *Smn*$^{2B/-}$ mouse model.

α-synuclein high dosage extended the survival of Smn$^{2B/-}$ mice

A high dosage of 3 x 10^{11} v.p. scAAV-CBA-α-synuclein was delivered by IV injection to *Smn*$^{2B/-}$ mice on post natal day 2 (PND2). There life span was monitored daily. The average life span for scAAV-CBA-α-synuclein treated mice was 74 days. The median life span for scAAV-CBA-α-synuclein treated mice was 50 days. Compared to

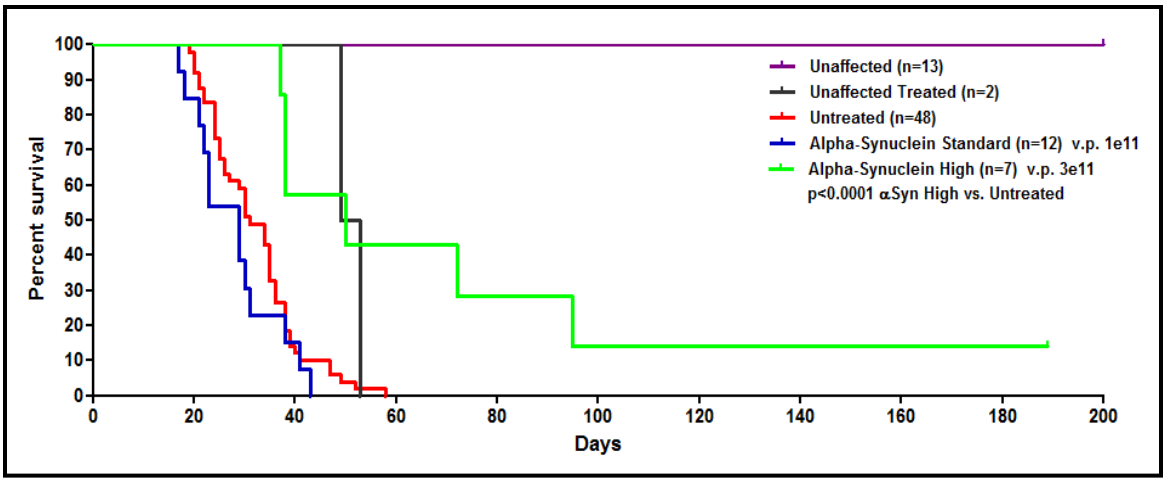

Figure 5: α-synuclein extended the survival of $Smn^{2B/-}$ mice.

Kaplan Meier survival curve showed the life span in days of unaffected, unaffected treated, untreated, scAAV-CBA-α-synuclein standard 1 x 10^{11} v.p. treated, and scAAV-CBA-α-synuclein high 3 x 10^{11} v.p. treated mice.

$p < 0.0001$ Untreated vs scAAV-CBA-α-synuclein high

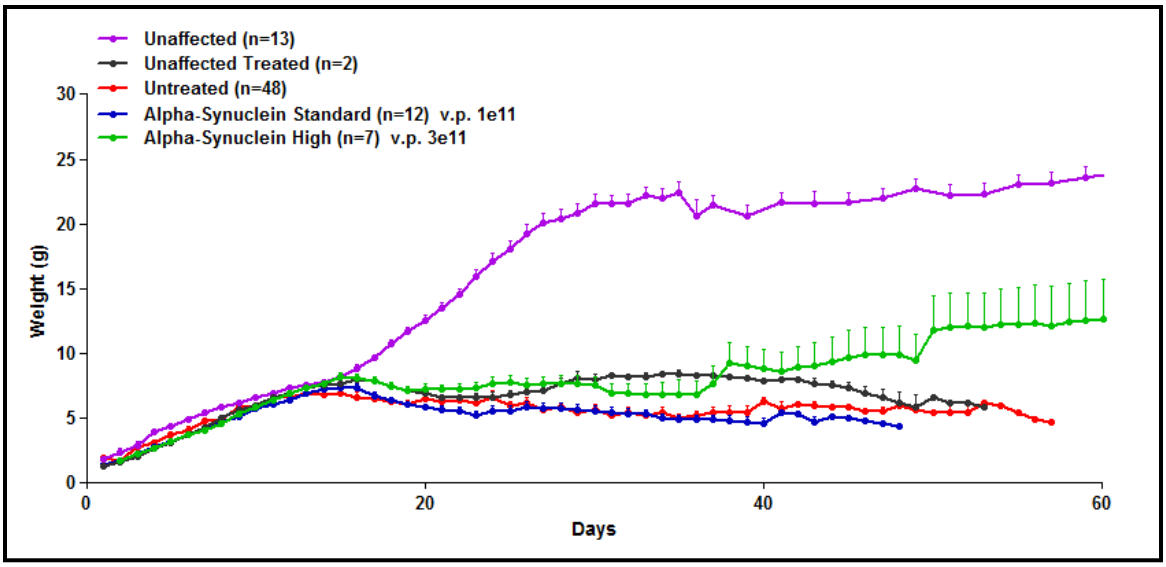

Figure 6: α-synuclein increased the daily weight gain of $SMN^{2B/-}$ mice.

$Smn^{2B/-}$ unaffected, unaffected treated, untreated, scAAV-CBA-α-synuclein standard 1 x 10^{11} v.p. treated, and scAAV-CBA-α-synuclein high 3 x 10^{11} v.p. mice were monitored and weighed daily.

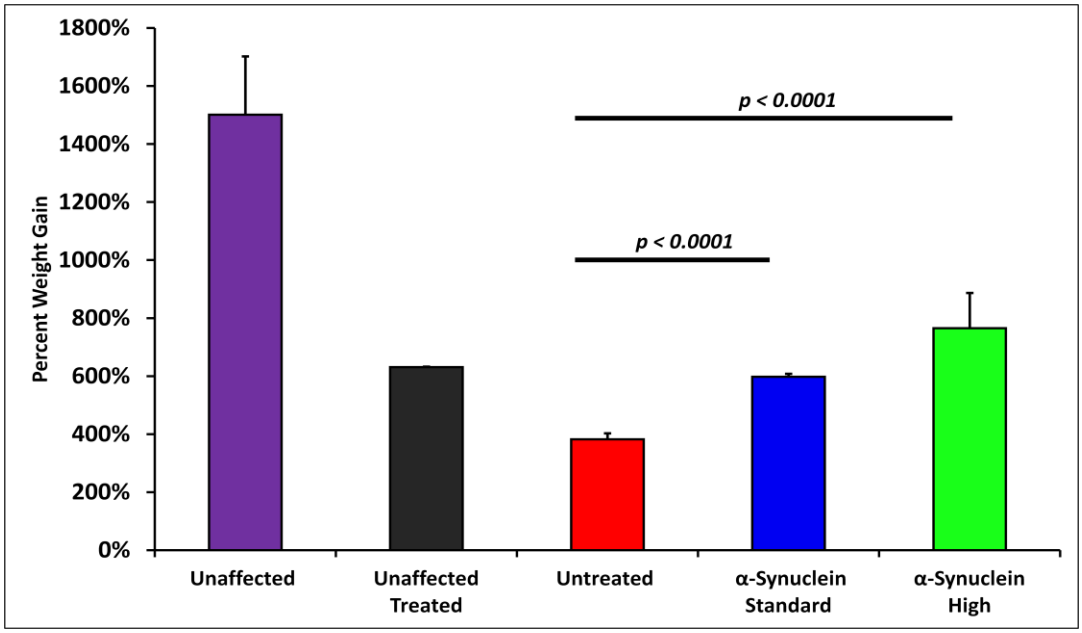

Figure 7: α-synuclein increased the peak weight gain of $SMN^{2B/-}$ mice.

Percent weight gain of each group was determined by the equation: ((peak weight - birth weight)/birth weight)*100.

Standard Deviations: Unaffected 2.84 , Unaffected Treated 0.067, Untreated 0.931, α-synuclein standard 0.362, α-synuclein high 3.22; Standard Errors: Unaffected 2.01 , Unaffected Treated 0.01, Untreated 0.20, α-synuclein standard 0.10, α-synuclein high 1.22.

untreated *Smn*$^{2B/-}$ mice with an average life span of 32 days and a median life span of 31 days, there was a significant increase (Figure 5).

α-synuclein high dosage increased the weight gain of SMN$^{2B/-}$ mice

Mice with the same 3 x 10^{11} v.p. dosage were monitored and weighed daily. scAAV-CBA-α-synuclein treated mice showed a significant increase in daily weight gain compared to that of untreated mice over time (Figure 6). Furthermore, mice treated with a high dosage of scAAV-CBA-α-synuclein had a higher percent weight gain than both untreated and standard dose treated mice (Figure 7). Therefore, it can be concluded that scAAV-CBA-α-synuclein high dosage was more beneficial than the standard dosage, due in part to restoration of reduced α-synuclein.

Discussion

SMN function has been linked to axonal trafficking, specifically with transporting β-actin mRNA and α-COP to the growth cone of axons. This transport is necessary for well developed NMJs. Here, we show that α-synuclein modifies the phenotype of SMA mice, since overexpression of α-synuclein extends the survival, and increases the weight gain of Smn$^{2B/-}$ mice. Since α-synuclein levels are reduced in both patient fibroblasts and spinal tissue, along with mouse NSC-34 cells, it was thought that this may be a consequence of low SMN levels [106]. It was interesting to observe a significant increase in the weight gain of mice injected with a standard 1 x 10^{11} v.p. dosage of scAAV-CBA-α-synuclein, but have no effect on their survival. The increased weight gain occurred between PND10 and PND16, comparable to wild type mice without any treatment. Phenotypic differences become evident during this time, suggesting that treatment was

able to partially overcome these differences. However the dosage was not enough to extend life span, since their survival was comparable to untreated mice. This prompted the increase in dosage from 1 x 10^{11} v.p. to 3 x 10^{11} v.p. This moderate increase in dosage was able to extend life span, and further increase weight gain of $Smn^{2B/-}$ mice.

A theory as to why this occurs includes SMN not only transporting β-actin mRNA and α-COP to NMJs, but also α-synuclein. Because α-synuclein is necessary for synaptic vesicle fusion and recycling, this protein would need to be present at the growth cone and NMJs. It is possible that low levels of α-synuclein can be attributed to decreased SMN protein levels with the ability to transport α-synuclein to the growth cone. This could lead to a large pool of synaptic vesicles at the NMJs unable to fuse to the pre-synaptic membrane to release neurotransmitters [107].

Alpha-synuclein is actively transported in both directions of axons, via both fast and slow axonal transport [100]. It has already been shown to associate with kinesin and dynein, which are axonal transport proteins [108]. Furthermore, it has also been proposed that α-synuclein, synapsin 1 and GAPDH form a complex with other potential proteins, and may function as a scaffold microtubule-dependent protein, that mediates axonal transport of a macromolecular complex [108, 109]. In SMNΔ7 mice, where decreased levels of full length SMN are seen, synaptotagmin 1 (Syt1) and synaptic vesicle 2 (SV2) proteins were reduced. This was observed two days before synaptic vesicle density decreased [110]. Syt1 is responsible for binding calcium, which is needed for the synaptic vesicle to fuse with the pre-synaptic membrane. Dynein protein was also seen at reduced levels, along with neurofilament (NF) accumulation [110]. NF

accumulation and decreased synaptic vesicles are abnormalities that have also been observed in SMA [107, 111, 112]. Together, this data implicates that SMN, α-synuclein and various other proteins could act together in a complex, specifically to transport other proteins and mRNAs across the axons.

Future directions include examining protein levels of both SMN and α-synuclein, and examining the NMJs in α-synuclein treated animals. If SMN protein levels are increased, it is one reason why the mice improved with weight gain and survival. However if SMN protein levels remain the same, it would suggest that α-synuclein is functionally compensating for the loss of SMN. If the NMJs are well developed, this would suggest that more α-synuclein is being transported to the growth cone, facilitating the fusion of synaptic vesicles to the pre-synaptic membrane to release neurotransmitters. It would also be interesting to determine if α-synuclein and SMN physically associate with each other, which would corroborate the earlier proposed theory involving SMN transport of α-synuclein to the growth cone. Determining if any other axonal transport associated proteins potentially form a complex with SMN and α-synuclein would also be of interest. From here, we could further elucidate other functions of SMN, how they contribute to SMA disease formation, and how they could be targeted for therapeutic intervention.

Chapter 4: PHYLOGENY OF SPINAL MUSCULAR ATROPHY: *DROSOPHILA MELANOGASTER*

Introduction

Currently, there are still unknown aspects of SMN function. It is clear that SMN plays a role in the assembly of snRNPs and axonal trafficking. However, it is still unclear about how different domains within the SMN protein contribute to SMA disease formation. Exons 2b and 6 contain oligomerization domains that are responsible for SMN self association. Without SMN oligomerization, snRNP assembly is abolished, thereby leaving the SMN protein unstable [3]. Exon 2b also encodes a domain necessary for nucleic acid binding, specifically binding GEMIN2 [113]. GEMIN2-8, SMN and UNR-interacting protein (UNRIP) form the SMN complex, which is responsible for placing Sm proteins on snRNA [13, 20]. The Sm binding region is located in Exon 3, along with the Tudor domain. The SMN Tudor domain is important because it folds into a conserved structure that binds the C terminal Arg and Gly-rich tails of SmD1 and SmD3 proteins. Together they form a multimeric complex with other Sm and SMN interacting proteins, necessary for splicesome formation [96]. The importance of the Tudor domain is highlighted by two novel mutations in Type III SMA patients, where a tyrosine is mutated to either a histidine or a cysteine [114]. The E134K point mutation has also been identified in the Tudor domain of a Type I SMA patient, along with several other

mutations [96, 115]. The poly-proline rich motif is located in Exons 6 and 7, which is responsible for binding the profilin protein. Profilin is important because it binds and sequesters ATP-actin in an inactive globular form [3]. Furthermore, there is a cytoplasmic localized motif 'QNQKE' in Exon 7, which is important for SMN stability [116-122].

To determine what domains are specific to SMA disease formation, we began investigating the role of the Tudor domain, since mutations in this domain have been observed in Type I and III SMA patients [96, 114, 115]. The Tudor domain from different species was first investigated to determine where functional differences began evolutionarily, and how disease manifestation developed with increasing sequence similarity. The species chosen include Smn from: *D. melanogaster* (*Drosophila*), *D. rerio* (zebrafish), *C. elegans*, *X. laevis* (*Xenopus*), and *S. pombe*. Because zebrafish and *Xenopus* Smn are more evolutionary similar to the Human Smn, we hypothesized that *C. elegans*, *Drosophila* and *S. pombe* Smn would show decreasing ability to compensate for the loss of SMN. We tested this hypothesis, by cloning the cDNA from each species into scAAV viral vectors, and performing ICV injections at a 1 x 1011 v.p. dosage to SMNΔ7 mice on PND2. We found that Smn from C. *elegans, Drosophila,* and *S. pombe* were not able to rescue the phenotype of SMNΔ7 mice. However zebrafish was able to rescue the phenotype and *Xenopus* partially rescued the phenotype of SMNΔ7 mice. This would suggest that the evolution of Smn towards human SMN began in *Xenopus*, which was able to partially restore the function of SMN.

Materials and Methods

Cloning

Drosophila Smn (dSmn) cDNA along with Smn from zebrafish, *C. elegans*, *Xenopus*, and *S. pombe* were cloned into the scAAV viral vector. The restriction sites used for dSmn were 5' *AgeI* and 3' *SacI*. The scAAV- CBA-dSmn plasmid clone was transformed in DH5α E. coli. bacteria cells, and plated on Ampicillin resistant Lysogeny Broth (LB)/Agarose plates, grown overnight in 37°C incubator. scAAV- dSmn clones were screened using enzyme digestion and sequencing analysis, provided by the DNA Core facility.

Virus Production

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U Penicillin/ 100 µg Streptomycin per mL (Invitrogen). Cells were incubated in 37°C with 5% CO_2 until ≈ 90% confluency before transfection. Triple transfection was performed by using Polyethylenimine (PEI; Polysciences), to deliver scAAV-dSmn, pHelper, and Rep2/Cap9 plasmids into cells. 48 hrs post transfection cells were harvested. Virus produced from harvested cells was purified by three consecutive isopycnic CsCl gradient ultra-centrifugations. Real-time PCR was used to determine the viral titer following each ultra-centrifugation. CsCl was removed from purified virus during dialysis with HEPES buffer, repeated 3 times. Final titers of purified virus were determined using real-time PCR.

Real-Time PCR

Viral fractions obtained following ultra-centrifugation were diluted 1:2000. Quantification of viral genomes occurred using the Applied Biosystem® 7500 Real-Time PCR System. SYBR® Green and primers were utilized with collected fractions, to amplify the forward (5'- CCGGTGGTGGTGCAAATCAAAGAA 3') and reverse (5' AGCAGAAGTAACACTTCCGTACAGGC 3') region following the chicken β-actin (CBA) promoter in the scAAV viral vector. A standard curve is used to calculate the number of viral genome particles in each fraction. The standard curve for real-time PCR was generated using serial dilutions of the scAAV-GFP plasmid, which has a known copy number. Following real-time PCR, the viral fraction with the highest titer was advanced to dialysis, and used for animal studies.

Animals

Animals used for these studies were all housed and treated according to the Animal Care and Use Committee guidelines at the University of Missouri. These regulations were established in the "Guide for the Care and Use of Laboratory Animals". The SMNΔ7 mouse model was used for these experiments. These mice were generated by introducing 2 copies of human *SMN2* and SMNΔ7 to $mSmn^{-/-}$ mice [56]. The average life span of this mouse model was from 14-15 days, and the average onset of disease phenotype begins around 5-10 days. Mice exhibited a severe phenotype, including physical weakness, tremors, and ambulating difficulty in all limbs. Heterozygous mice ($mSmn^{+/-,\ SMN2/SMN2,\ SMN\Delta7/SMN\Delta7}$) were used as unaffected controls.

Genotyping

To determine the genotype of SMNΔ7 mice, tail biopsies were taken on PND1 and used for PCR analysis with primers specific for the *mSmn* gene. The primer sets were: *mSmn*-WT forward (5' TCTGTGTTCGTGCGTGCGTGGTGACTTT 3'), *mSmn*-WT reverse (5' CCCACCACCTAAGAAAGCCTCAAT 3'), *lacZ mSmn* knockout allele forward (5' CCAACTTAATCGCCTTGCAGCACA 3') and the *lacZ mSmn* knockout allele reverse (5' AAGCG-AGTGGCAACATGGAAATCG 3'). PCR samples were run on agarose gels during gel electrophoresis. SMNΔ7 mice were identified by a 372 bp m$Smn^{+/-}$ band. Heterozygous mice were identified by both 372 bp m$Smn^{+/-}$ band and a 626 bp *lacZ* band. Wild type mice were identified by a 626 bp *lacZ* band.

Survival, Weight Gain and Phenotype

All mice were monitored daily to record their survival and weight gain. Time to right was determined by placing the mice on their back, and timing how long it took them to right themselves. Time was capped at 30 seconds. Mice unable to right themselves, with a time of 30 seconds or more were classified with poor motor function. Mice able to right themselves in 1 to 2 seconds had good motor function.

Results

dSmn did not extend survival of SMNΔ7 mice

To determine what domains of SMN are important for its function, *Drosophila* Smn along with Smn from zebrafish, *C. elegans*, *X. laevis*, and *S. pombe* were cloned into

the scAAV viral vector. scAAV-CBA-dSmn was then transfected into HEK 293T cells, producing virus. This virus was purified and ICV injected in to the SMNΔ7 mice on PND2. Their survival and weight was monitored daily. These mice were monitored daily and compared. *Drosophila*, *C. elegans* and *S. pombe* Smn did not show any significant extension in survival compared to untreated SMA mice. However, zebrafish and *Xenopus* showed a significant extension in survival compared to untreated SMA mice (Figure 8). Therefore, we concluded that evolutionary differences may become apparent within *Xenopus* Smn, since zebrafish rescued survival, while *Xenopus* only partially rescued the survival of SMNΔ7 mice.

dSmn did not increase SMNΔ7 mice weight gain

Mice injected with scAAV-dSmn were monitored and weighed daily. Compared to untreated SMNΔ7 mice, daily weight gain was not statistically significant in scAAV-dSmn treated mice. However, a spike in daily weight gain was seen around PND10, similar to that of human SMN. Furthermore, the weight gain of mice injected with *C. elegans* and *S. pombe* Smn did not show any statistical significance compared to untreated SMA mice. *Xenopus* and zebrafish Smn increased the weight gain of SMNΔ7 mice (Figure 9). The percent weight gain from birth to peak was also calculated, to determine how much weight was gained during development. scAAV-CBA-dSmn percent weight gain from birth to peak did not increase in SMNΔ7 mice, compared to untreated SMA mice. This was also true for *C. elegans* and *S. pombe* Smn. For zebrafish and

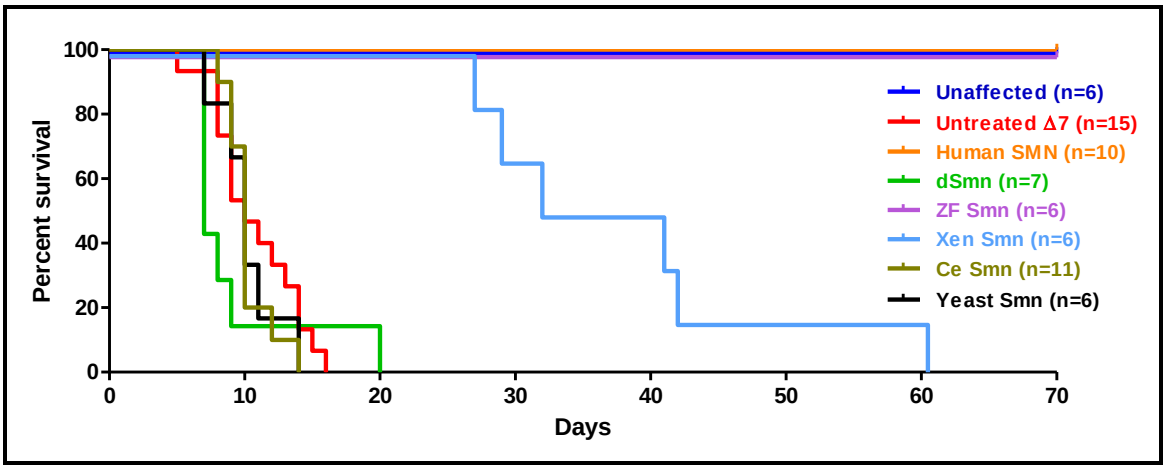

Figure 8: dSmn did not extend survival of SMNΔ7 mice.

Kaplan Meier survival curve showed the life span in days of unaffected, untreated Δ7, human SMN, *Drosophila* (dSmn), zebrafish (ZF), *Xenopus* (Xen), *C. elegans* (Ce), and *S. pombe* (Yeast) Smn treated SMNΔ7 mice with a 1 x 10^{11} v.p. dosage delivered via ICV injection on PND2.

p = 0.47 Untreated vs dSmn

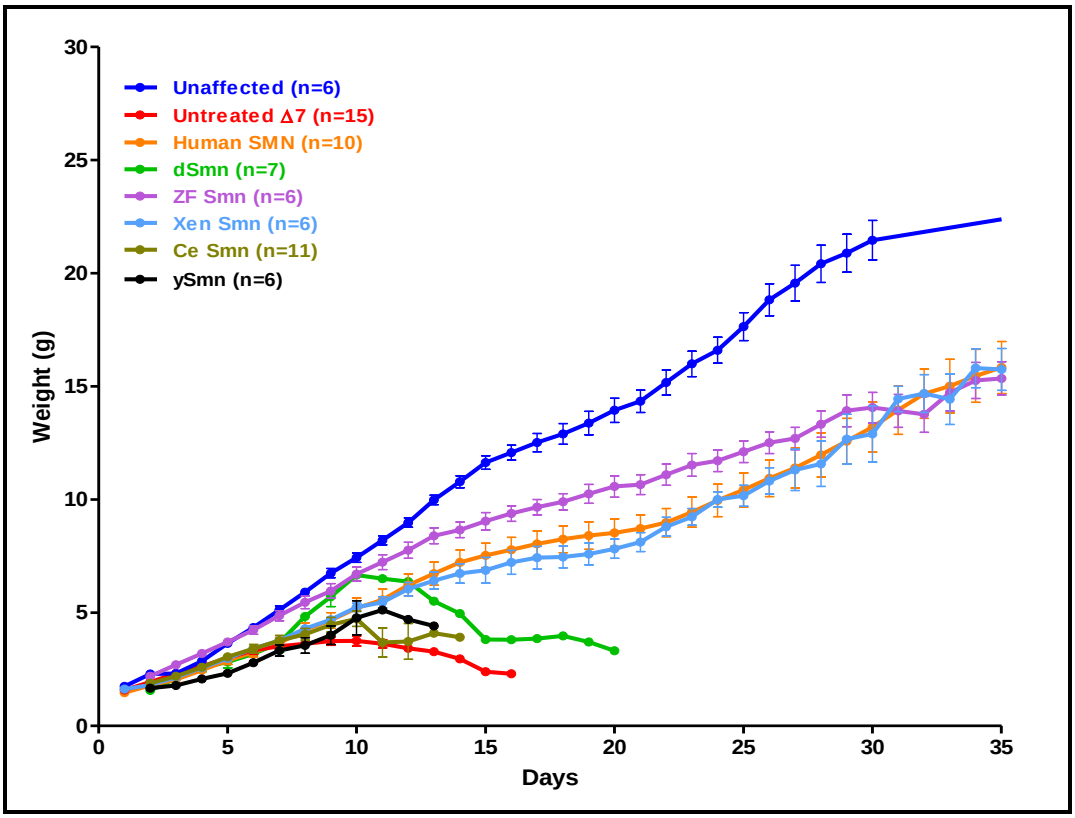

Figure 9: dSmn did not increase SMNΔ7 mice weight gain.

Unaffected, untreated Δ7, human SMN, *Drosophila* (dSmn), zebrafish (ZF), *Xenopus* (Xen), *C. elegans* (Ce), and *S. pombe* (Yeast) Smn treated mice were monitored and weighed daily.

Xenopus Smn, a significant increase in percent weight gain was observed compared to untreated SMA mice (Figure 10). Therefore, *Drosophila*, *C. elegans* and *S. pombe* Smn were unable to rescue the daily and percent weight gain of SMNΔ7 mice, while *Xenopus* and zebrafish Smn did. Further corroborating evolutionary conservation differences arising with *Xenopus* Smn compared with other species.

Discussion

In order to reveal the function of SMN pertaining to SMA disease formation, we thought a good approach to this investigation was to compare SMN from different species to determine the importance of each domain relative to SMA disease manifestation. We began examining the Tudor domain first, because it is a highly conserved domain that binds Sm proteins, and mutations in this domain have been observed in Type I and III SMA patients. The species we examined here included: human, zebrafish, *Xenopus*, *C. elegans*, *Drosophila*, and *S. pombe*. We expected that SMN from lower species would not improve disease phenotypes, since SMN is less conserved with more distance from human SMN. From there we could differentiate the importance of each domain of SMN in SMA. Our results demonstrated that *Xenopus* and zebrafish Smn were able to partially rescue the disease phenotypes in the SMNΔ7 mouse model, while *Drosophila, C. elegans,* and *S. pombe* Smn were not.

It is important to note the differences in the domains between *Drosophila, C. elegans* and *Xenopus*. The Tudor domain which we originally began exploring, is highly conserved between Smn from these three species (Figure 11). Since our data showed

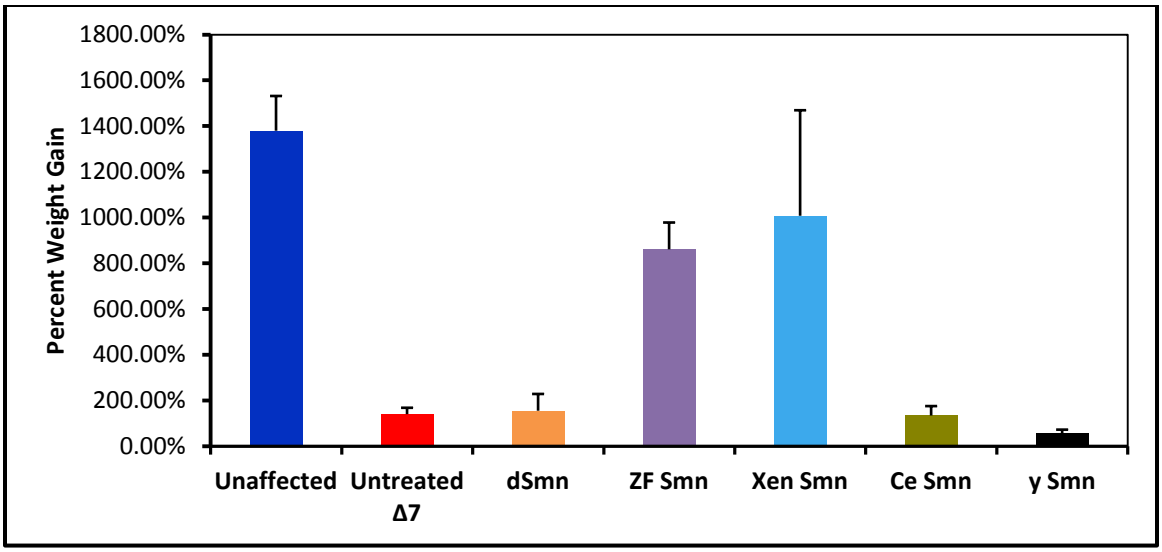

Figure 10: dSmn did not rescue the percent weight gain of SMNΔ7 mice.

Percent weight gain of each group was determined by the equation: ((peak weight - birth weight)/birth weight)*100.

Standard Deviations: unaffected: 1.519, untreated Δ7: 0.280, *Drosophila* Smn (dSmn): 0.7331, zebrafish (ZF) Smn: 1.173, *Xenopus* (Xen) Smn: 4.61, *C. elegans* (Ce) Smn 0.412, *S. pombe* (Yeast) Smn 0.173.

Standard Errors: unaffected: 0.438, untreated Δ7: 0.072, *Drosophila* Smn (dSmn): 0.277, zebrafish (ZF) Smn: 0.478, *Xenopus* (Xen) Smn: 1.883, *C. elegans* (Ce) Smn: 0.124, *S. pombe* (Yeast) Smn: 0.070.

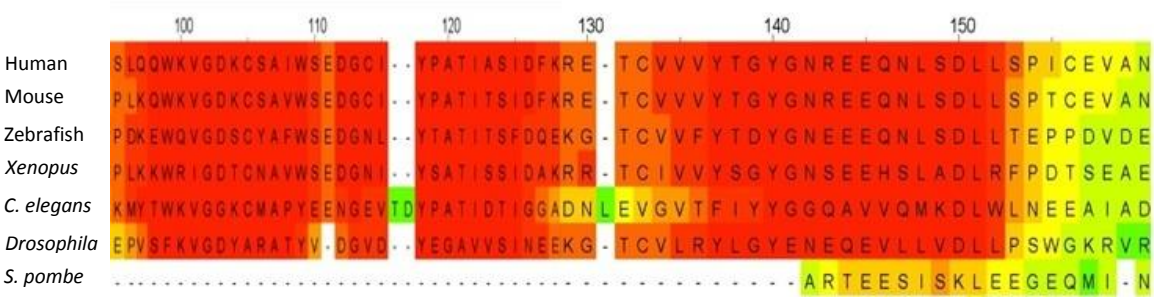

Figure 11: Sequence alignment of the Tudor domain from human SMN, Mouse Smn, Zebrafish Smn, *Xenopus* Smn, *C. elegans* Smn, *Drosophila* Smn, and *S. pombe* Smn.

that *Drosophila* and *C. elegans* Smn failed to rescue disease phenotypes in SMA mice, it indicated that the Tudor domain only was not enough to improve the disease phenotypes. In addition, Exons 1 and 5 are least conserved between all three species, compared to Human SMN. Exon 5 contains the profilin domain as well as an oligomerization domain. However, Exon 1 has not been shown to contain any major domains linked to important functions of SMN. The profilin domain binds and sequesters ATP-actin in an inactive globular form, and the oligomerization domain is responsible for self association [3]. Since both of these domains are important for proper assembly of snRNPs, sequence differences in these two domains may explain why *Drosophila* Smn did not rescue the SMNΔ7 mouse model.

Exon 7 is important for SMN stability and contains the 'QNQKE' motif for cytoplasmic localization. However, this motif is less conserved in *Xenopus, C. elegans* and *Drosophila* [116-122]. Specifically, the first glutamine in the motif is only observed in *Xenopus*, but not *C. elegans* or *Drosophila* Smn. Therefore, this amino acid may have increased importance for stability of SMN, thereby providing one reason that *Xenopus* Smn is able to partially rescue the survival of the SMNΔ7 mice. It would be interesting to determine the effect of swapping certain domains of Smn from different species, and examining whether this could rescue the disease phenotypes of SMA mice. This would shed light on which function of SMN is crucial to SMA disease manifestation.

Chapter 5: CONCLUSION

Overall View

Spinal Muscular Atrophy is a neurodegenerative disease resulting from the loss of full length SMN protein due to the complete loss of *SMN1*. Luckily, *SMN2* an almost identical copy of *SMN1* produces about 10% of full length SMN. However, this is still not enough to prevent SMA onset. SMN protein is multi-functional, but it is unclear which functions attribute to SMA pathology. The role of SMN with pre-mRNA splicing has been clearly defined, however it is not clear how a reduction of SMN necessary for snRNP assembly correlates to neurodegeneration. Many investigations have been geared to clarify this point. One study showed that knockdown of SMN in HeLa cells reduced U snRNP assembly, and purified U snRNPs rescued developmental arrest in SMN deficient *X. laevis* embryos [34]. Furthermore, motor axon degeneration in zebrafish seen with MO knockdown of SMN, was also observed with Gemin 2 and pICln MO knockdown [34].

Another study addressed the question of why SMN defects with snRNP assembly are not seen in all cell types. They found that SMN activity in snRNP assembly is higher in the CNS than other organs during embryonic development [123]. Additional studies have shown that snRNP assembly is reduced in the spinal cords of severe SMA mice, suggesting that the CNS is preferentially affected by the loss of SMN protein [23, 124].

SMN has also been identified to function in axonal transport, separate from snRNP assembly. Specifically SMN transports β-actin mRNA and other proteins to the growth cone for efficient NMJ formation, since reduced SMN in the axon correlates to a reduction in β-actin mRNA and poorly developed NMJs [25, 28, 125]. Studies have also shown that SMN may play a role with vesicle trafficking in axons, since it binds and moves with the COP1, a Golgi- associated protein [105]. However, it is unclear if the loss of snRNP assembly or axonal transport due to the loss of SMN contributes more to disease severity.

Different domains of SMN carry out different functions, and we wanted to determine which domains are attributed to functions important for SMN activity. To explore this question further, we wanted to investigate SMNrp1. It is homologous to SMN1 at the Tudor domain, which is responsible for binding Sm proteins, which are necessary for snRNP assembly [91]. The homology to SMN also makes it a good candidate as a potential modifier of the SMA phenotype. Because point mutations in this domain have been identified in SMA patients, dysfunction of this domain may contribute to disease onset [96, 115]. However, our data showed that overexpression of SMNrp1 was not able to compensate for the loss of functional Tudor domain in SMN protein. This would suggest that the Tudor domain along with additional domains is required for fully functional SMN protein. It would be interesting to see if overexpression of SMNrp1 in a more severe mouse model may give better insight as to why point mutations are seen in Type I and III patients. Furthermore, this would help determine if these point mutations are a significant contribution to disease severity.

Overexpression of other domains necessary for proper SMN function, in addition to SMNrp1 would help elucidate which domains of SMN are important for disease onset.

In order to investigate more divergent domains, that would either stop working or rescue the SMA phenotype, we investigated how SMN from different species affected the severe mouse model. Our findings suggested that evolutionary differences became apparent in *Xenopus* Smn, while the less conserved *Drosophila* Smn did not rescue the SMA phenotype. Furthermore, it is thought that both the profilin and oligomerization domain could contribute to disease manifestation. Mutations or deletions inhibiting SMN oligomerization reduce the half life of full length SMN, suggesting that SMN self-association is needed for stability [116]. Detecting differences in both domains is of interest in the future, as well as swapping domains to identify which ones have an effect on the phenotype. It can be concluded that the functions carried out by these identified domains is needed to prevent SMA, thereby making them targets for drug therapies in the future.

Separately, we wanted investigate a group of molecules, modifiers that most likely affect the phenotype independent of SMN. We addressed this by looking at one specific modifier of the SMA phenotype, α-synuclein, to see if it could compensate for the loss of SMN protein. Alpha-synuclein, a synaptic vesicle associated protein, thus far has proven to be a substantial modifier to the SMA intermediate mouse model. Due to the drastic improvement seen in intermediate mice with a high dosage, it would be interesting to determine the least amount of α-synuclein required to see an improvement in both survival and weight gain in a dose dependent study. Because low

levels of α-synuclein protein and mRNA have been observed in patient fibroblasts with low SMN protein, overexpression of α-synuclein could potentially be used as a therapeutic in the future [106]. It would also be interesting to investigate the level of α-synuclein protein in severe and mild SMA mouse models, to determine if the amount of full length SMN produced has an effect on α-synuclein protein. Furthermore, determining if it could rescue the phenotype in both models would shed light onto the function of α-synuclein as it relates to SMN. These findings would help further the investigation of the role of SMN in SMA, in order to provide the best therapy for patients.

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