DEFINING THE THERAPEUTIC WINDOW IN SPINAL MUSCULAR ATROPHY: TIME POINTS STUDY

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Master of Science

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by

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TIME POINTS STUDY

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

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DEDICATION PAGE

I dedicate this work to my Mother and Father, Debbie and George Robbins, whose wisdom, support and perseverance helped shape my character allowing me to succeed in the pursuit of my dreams.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AchR	Acetylcholine receptor
ALS	Amyotrophic lateral sclerosis
ASO	Antisense oligonucleotide
BBB	Blood-brain barrier
C	Cytosine
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
E	Embryonic day
GFP	Green fluorescent protein
HEK293T	Human embryonic kidney-293T cell line
HDACi	Histone deacetylase inhibitors
hnRNP	Heterogeneous ribonucleoprotein particle
ICV	Intracerebroventricular
IP	Intraperitoneal
ISS-N1	Intronic splicing silencer N-1
IV	Intravenous
mRNA	Messenger RNA
mSmn	Murine *survival motor neuron* gene

NINDS	National Institute of Neurological Disorders and Stroke
P	Postnatal day
PBA	Phenylbutyrate
PEI	Polyethyleneimine
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SACHDNC	Secretary's Advisory Committee on Heritable Diseases in Newborns and Children
SAHA	Suberoylanilide hydroxamic acid
scAAV-SMN	Self-complementary adeno-associated virus expressing full-length SMN
Sm	Smith core protein
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron
SMN2	Severe mouse model with the genotype $Smn^{-/-}$;$SMN2^{+/+}$
SMNΔ7	SMN delta 7; SMN lacking exon 7
SMN^{RT}	SMN Read-Through mouse model
snRNP	Small nuclear ribonucleoprotein
STAT5	Signal transducer and activator of transcription 5
T	Thymine
TSA	Trichostatin A
v.g.	Viral genomes
VPA	Valproic acid

ABSTRACT

Spinal muscular atrophy (SMA) is caused by the loss of a single gene, *survival motor neuron-1* (*SMN1*), which results in the rapid deterioration of motor neuron integrity and function, most often leading to infantile death. Administration of self complementary adeno-associated virus expressing full-length SMN cDNA (scAAV-SMN) has proven an effective means to rescue the SMA phenotype in SMA mice, either by intravenous (IV) or intracerebroventricular (ICV) administration at very early time points. We have recently shown that ICV delivery of scAAV9-SMN is more effective than a similar dose of vector administered via an IV injection, thereby providing an important mechanism to examine a timeline for ameliorating the disease and determining the optimal therapeutic window. SMNΔ7 mice were injected with scAAV9-SMN vector via ICV injection on a single day, from P2 through P8. At each delivery point from P2 through P7, scAAV9-SMN decreased disease severity, ranging from a near complete rescue (P2) to a significant, albeit lesser degree (P7) in which animals lived ~130% longer. Our study demonstrates that a maximal benefit is obtained when treatment is delivered during a specified therapeutic window of the pre-symptomatic stages of SMA in the SMNΔ7 mouse model. Although disease severity can be significantly decreased when SMN levels are increased at later stages of the disease, there is a time (after postnatal day 8) at which therapy is no longer effective.

CHAPTER ONE: INTRODUCTION TO SPINAL MUSCULAR ATROPHY

Spinal Muscular Atrophy Overview

The National Institute of Neurological Disorders and Stroke (NINDS) has classified over 600 neurological disorders affecting millions of people worldwide (50 million in the USA alone), with an annual cost in hundreds of billions of dollars (National Institutes of Health website). Some of the more prevalent neurodegenerative diseases include Amyotrophic Lateral Sclerosis (ALS), Friedreich's Ataxia, Alzheimer's, Huntington's, Parkinson's, Lewy Body Disease, and Spinal Muscular Atrophy (SMA) (NINDS website).

SMA is an autosomal recessive neurodegenerative disorder, and it is caused by the mutation of a single gene, *survival motor neuron-1* (*SMN1*) [1], which codes for the ubiquitously expressed protein, SMN. Humans possess another nearly identical gene, *SMN2*, which can produce fully functioning SMN protein, but only at low levels. This is due to a C to T conversion within the 5′ end of exon 7 resulting in alternatively spliced *SMN2*-derived transcripts. SMA is a motor neuron disease and is characterized by the loss of α-motor neurons in the ventral horn of the spinal cord, which leads to muscle wasting, atrophy, paralysis and death in the more severe cases [1]. SMA is a common genetic cause of infantile death with an incidence of 1:6,000 – 1:10,000 live births and a carrier frequency of 1 in 40 [2-5]. It is still unclear what critical function is disrupted by the severe reduction of SMN protein levels, but this leads to the pathogenesis of SMA.

Strategies to circumvent SMA progression focus on *SMN1* gene replacement or the alteration of SMN2 splicing [6]. Currently, there is no cure for SMA; and although clinical studies have shown that early intervention and nutritional support have positive outcomes for

SMA patients [7], SMA still remains absent from the required newborn screening panel. A pilot study is underway to assess the feasibility of prenatal screening for SMA in all newborns (Newborn Screening Translational Research Network at https://www.nbstrn.org).

Spinal Muscular Atrophy Genetics

SMA was first described by Werdnig and Hoffmann in the 1890s and became known as Werdnig-Hoffmann disease at that time, but today is synonymous with the most severe form of SMA. In their autopsy reports, Werdnig and Hoffmann described loss of α-motor neurons in the ventral horn of the spinal cord as well as atrophy of skeletal muscle [8]. It was not until 1995 that the SMA-determining gene was identified by Lefebvre et al. (Figure 1.1A) [1, 9]. Telomeric *SMN1* and centromeric *SMN2* are nearly identical and are positioned inversely from each other due to an early intrachromosomal duplication event, at the genomic region of 5q13 [1]. *SMN1* produces full-length transcript and functional SMN protein, but the alternatively spliced SMN2 transcript produces approximately 90% truncated protein (Figure 1.1B and 1.1C) [10]. Although *SMN1* and *SMN2* are completely identical in amino acid sequence, there is a crucial translationally silent "C" to "T" transition located in exon 7 at position +6, which results in severe disruption of SMN2 splicing [11]. Importantly, *SMN2* is a disease modifier because there is a strong inverse correlation between *SMN2* copy number and disease severity, where higher *SMN2* copy numbers result in a milder phenotype (Figure 1.2) [12, 13].

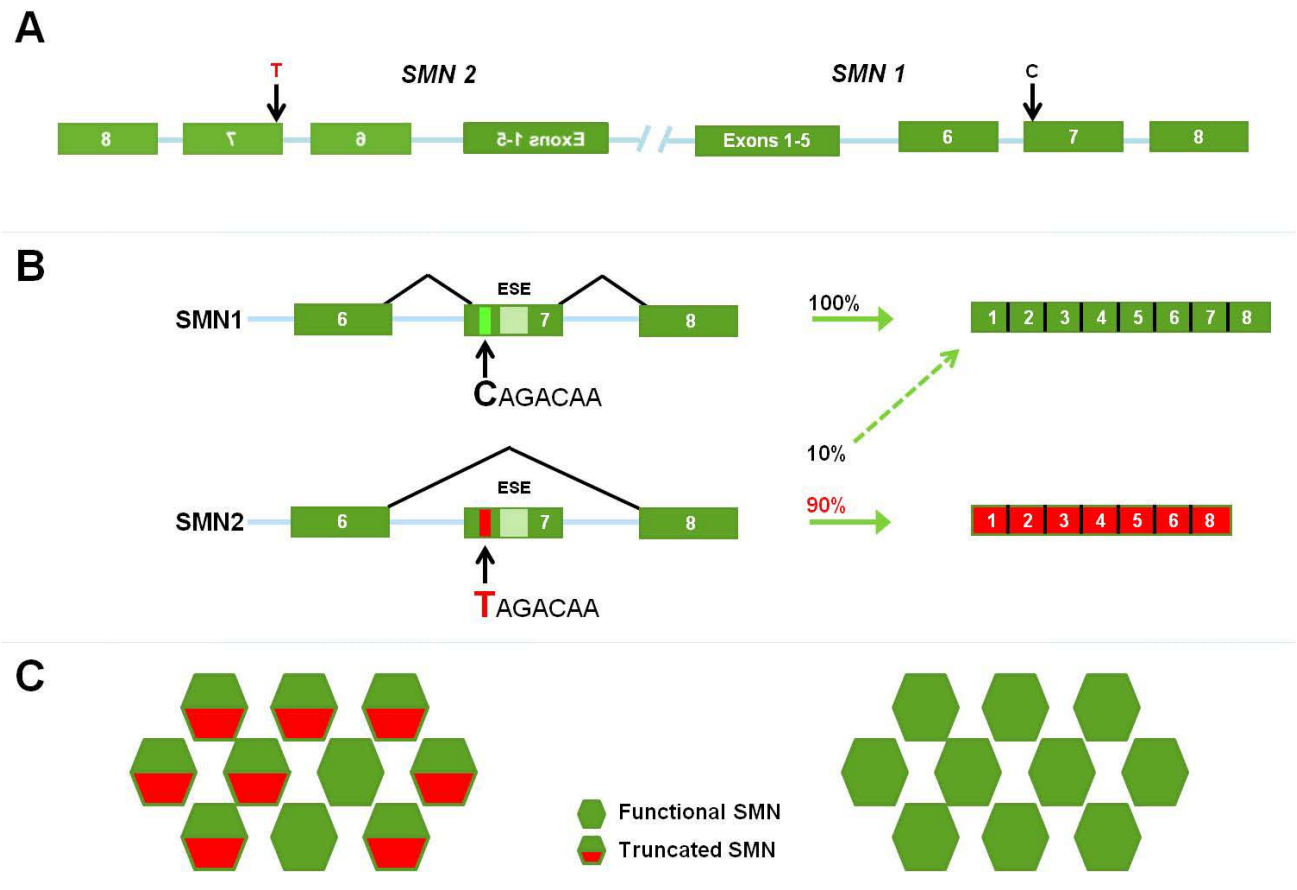

Figure 1.1. Schematic representation of *SMN1* and *SMN2* at the DNA, RNA and protein level. A) *SMN1* and *SMN2* are located on Chromosome 5 in reverse order and differ by a single nucleotide in exon 7. B) SMN2 is alternatively spliced resulting in a greater abundance of transcripts lacking exon 7 (SMNΔ7). C) SMNΔ7 transcripts produce truncated, non-functional protein.

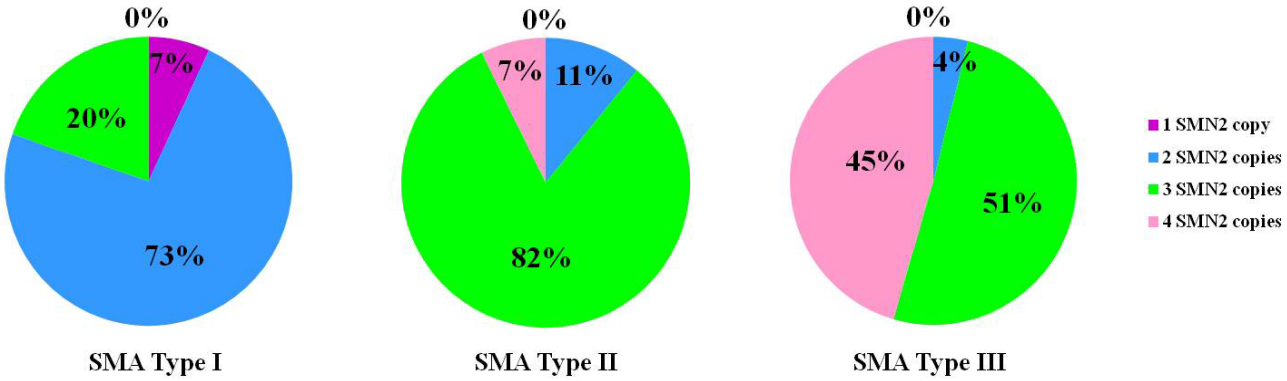

Figure 1.2. Circle graphs depicting SMA Type I, Type II and Type III patients and their corresponding *SMN2* copy number. *SMN2* is an important disease-modifying gene because each copy provides ~10% basal SMN levels which collectively, increases overall SMN protein levels with the addition of each *SMN2* copy. Data adapted from [14].

There are a few nucleotide differences between *SMN1* and *SMN2* but these do not alter protein structure [1, 11]. However, the C to T transition in exon 7 results in a functional difference between the two genes due to aberrant splicing of the SMN2 transcript [11]. Positive splicing regulators, such as SF2/ASF, are prevented from binding the region of exon 7, possibly due to the disruption of an exonic splice enhancer region [15] or from the creation of an exonic splice silencer region [16] following the single nucleotide change. Additionally, factors such as hnRNP-A1, which promote the exclusion of exon 7 [16], recruit negative regulators such as Element 1 [17] and ISS-N1 [18], which act as splicing repressors. This highly dynamic region in SMN2 leads to aberrant splicing ~90% of the time, generating a truncated transcript lacking exon 7 (SMNΔ7) [1, 11, 19]. The SMNΔ7 protein cannot self-associate [20] or form complexes efficiently with RNAs and proteins [21], making it unstable, and thus it is rapidly degraded [22].

SMN Protein Function

SMN is a multifunctional protein known to bind many cellular proteins [23-26] and a few viral proteins [27-29]; and it is found in both the nucleus and cytoplasm of all cell types [30-32]. In the nucleus, SMN binds Gemin proteins forming aggregates called gems [12, 30, 31], which have been used as a cell biomarker because gem numbers correspond to overall SMN levels [23]. There are a multitude of cellular activities in which SMN is involved, however, the best understood function is the role SMN plays in snRNP biogenesis. SMN is crucial in transcriptional activation [23, 27, 33], translational regulation [23, 33, 34] stress response [23, 33, 35, 36] and cell apoptosis [23, 37]. Furthermore, SMN functions in axonal RNA trafficking [23, 33, 38] and RNA splicing [23, 33, 39]. In the context of snRNP biogenesis and assembly,

SMN is essential [23, 33, 40] (Figure 1.3). Pre-mRNA splicing is mediated by the spliceosome, a complex of RNAs and RNA-binding proteins. In eukaryotic cells, these spliceosomal small nuclear ribonucleoproteins (snRNPs) are generated by a master assemblyosome [41], the SMN complex [23, 33, 40], before they organize into the final spliceosome machinery. snRNAs are transcribed in the nucleus and exported to the cytoplasm where the SMN complex (consisting of SMN and Gemins 2-8) [33] transfers Smith core proteins (Sm) onto the appropriate site of the snRNA [42]. The binding of Sm proteins onto the snRNAs is highly specific, and the SMN complex ensures this process is performed correctly [33, 42, 43]. Once the Sm cores have been assembled, the 3’ end of the snRNA processed, and the 5’ cap hypermethylated, the mature snRNP is imported into the nucleus by the SMN complex [44, 45] where it will associate with snRNP-specific proteins to generate the complete spliceosomal complex. When SMN levels are low, a decrease in Sm core assembly is observed which is correlated with SMA severity [33, 42].

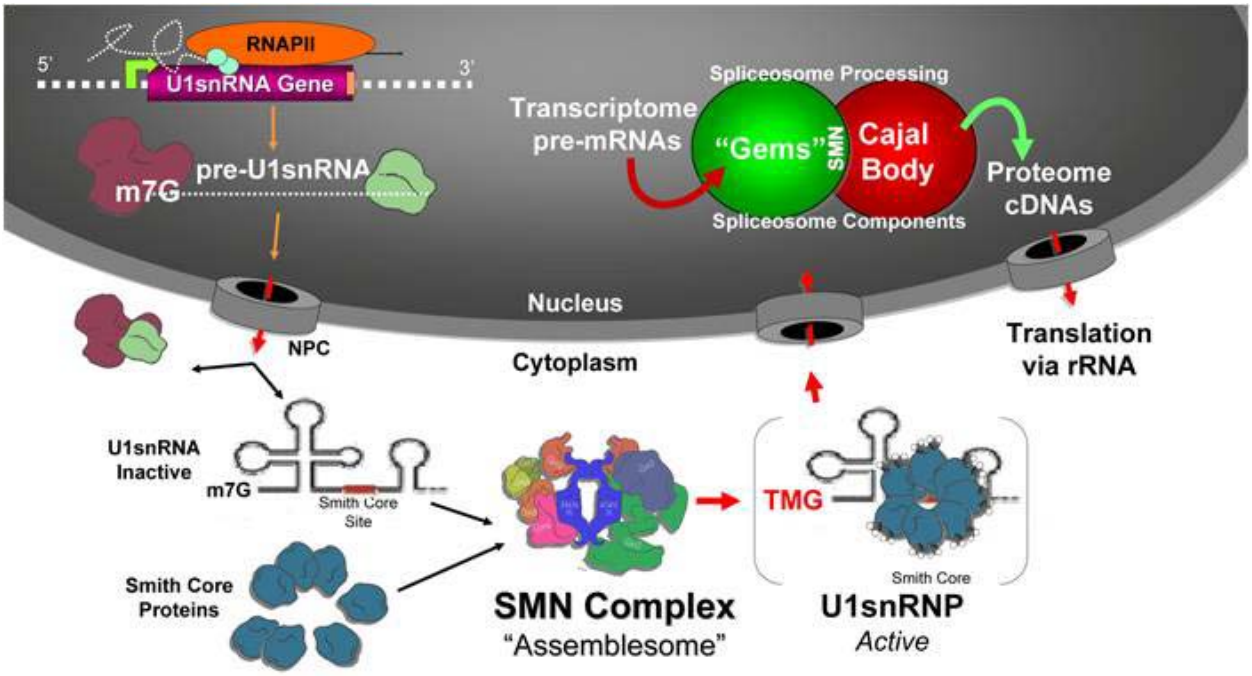

Figure 1.3. Diagram of SMN's role in snRNP biogenesis. In the cytoplasm, the SMN complex assembles Sm proteins into a heptameric ring structure [23, 46]. Following transcription in the nucleus, snRNAs are transported to the cytoplasm where the SMN complex assembles the heptameric ring of Sm core proteins onto the specific binding site of the snRNA [33, 42, 43]. Following maturation, the SMN complex shuttles the snRNP into the nucleus where it will be assembled into the final spliceosomal complex [44, 45].

Clinical Assessment of Spinal Muscular Atrophy

SMA is classified into five subtypes according to disease severity and age of onset. 95% of SMA patients have a deletion in both copies of *SMN1*, while the remainder of the cases occur from frameshift, nonsense and missense mutations [47]. Type 0 SMA is embryonic lethality, and this occurs only if the fetus carries mutations in both the *SMN1* and *SMN2* genes. SMA Type I (Werdnig-Hoffmann disease) is the most severe clinical presentation of the disease and the most prevalent type among patients; 65% of all new cases and 80% of all current cases are Type I [7]. These patients present with symptoms before 6 months of age [48]; they have trouble eating and breathing and they never gain the strength to sit upright or crawl. Children with SMA Type I die by the age of two, usually from respiratory complications [7, 47, 48]. SMA Type II patients become symptomatic within 18 months [48], and are diagnosed once parents notice an extensive delay in motor control; for example the child may have trouble controlling head movements and may not move much or cannot sit upright. These patients are wheelchair bound and their life expectancy is variable, with 70% of patients living into adulthood [6]. Patients with Type III SMA, also known as Kugelberg-Welander disease, exhibit symptoms in adolescence and may eventually become wheelchair-bound [47] although they have a normal life expectancy [48]. Adult onset SMA, Type IV, is the mildest form of the disease and is characterized by mild muscle weakness usually after the age of 25 [47]. The clinical spectrum of SMA is broad and it may be difficult to classify an individual as having one type of SMA or another because often symptoms and disease characteristics overlap [48]. However, appropriate diagnosis and assessment of disease severity and progression are important in order to provide the patient with therapeutic intervention specifically tailored to their needs [7, 49].

In 2006, Pyatt and Prior described a multiplex real-time PCR assay that identifies affected individuals and carriers with 100% sensitivity and specificity [50]. Then in 2008, SMA was nominated to be added to the federally mandated newborn screening panel [7]. The Secretary's Advisory Committee on Heritable Diseases in Newborns and Children (SACHDNC) declined the proposal until further evidence could be collected through population-based screening to determine the efficacy and feasibility of screening all newborns for SMA. This population-based study is underway, involving several medical centers. Current studies are ongoing at the Department of Pediatrics at the University of Utah, School of Medicine and Colorado School of Public Health at the University of Colorado, coordinated under the Newborn Screening Translational Research Network (NBSTRN at https://www.nbstrn.org).

Mouse Models of Spinal Muscular Atrophy

Multiple mouse models have been generated to dissect the biological processes and pathogenesis of SMA and importantly, they are a powerful tool used to investigate preclinical therapeutics in a mammalian context. Models exhibiting disease phenotypes ranging from severe to intermediate have been created to mimic the pathology observed in Type I, and Type II and Type III patients, respectively.

As with all species, except humans, mice have only a single *Smn* gene (equivalent of human *SMN1*) [51, 52] which results in embryonic lethality when lost or mutated [53]. In 2000, the first SMA mouse model ($Smn^{-/-}$;$SMN2^{+/+}$) was generated independently by two laboratories: FVB.Cg-*Smn1*^{tm1Hung} Tg(SMN2)2Hung*Smn1*^{tm1Hung}/J (Jax 005058; [54]) and FVB.Cg-Tg(SMN2)89Ahmb*Smn1*^{tm1Msd}/J (Jax 005024; [55]). Known as the severe "*SMN2*" model, this

transgenic mouse line was developed by inserting 2 copies of the human *SMN2* transgene into the mouse *Smn*-null background (*Smn*$^{-/-}$; [53]). The low levels of SMN provided by the transgene were sufficient to rescue embryonic lethality however; this model is born symptomatic, with an average survival of 5 days and never reaches the same weight as unaffected littermates.

A slightly less severe model (*Smn*$^{-/-}$;*SMN2*$^{+/+}$;SMNΔ7$^{+/+}$) was later developed in 2005 by Le et al., with the addition of an SMN cDNA lacking exon7 (SMNΔ7) onto the background of the severe model (FVB.Cg-Tg(SMN2*delta7)4299AhmbTg(SMN2)89Ahmb*Smn1*^{tm1Msd}/J; Jax 005025) [56]. As this work originated to investigate possible adverse effects incurred by the SMNΔ7 product, this group instead observed that this protein product associates with and stabilizes full-length SMN, as well as extends the survival to an average of 13 days. This model is termed the SMNΔ7 mouse model and it is born presymptomatic. This model has been the workhorse for many investigations in recent years because it becomes symptomatic during the early postnatal stage and lives relatively long enough to evaluate therapeutic intervention.

As severe models are useful for evaluating the accelerated progression of SMA Type I, it is imperative to have intermediate models with a milder disease phenotype available to study the less severe pathogenesis of Types II and III SMA [6]. Therefore, intermediate models have been generated that exhibit a milder disease progression for which subtle differences, once masked by severe pathology, can now be brought to light; as well as, new therapeutics formulated that are more appropriate for Type II and III patients.

Our lab recently generated an intermediate model for SMA called the Read through model SMN^{RT} (*SMN2*$^{+/+}$;SMN^{RT};*Smn*$^{-/-}$) [57]. This mouse line has the same genetics as the SMNΔ7 model, however, the Δ7 transgene is manipulated to produce a more stable isoform than

SMNΔ7 [58], called SMN read through. This model demonstrates a moderate disease severity and these mice live an average lifespan of 30 days.

Another intermediate SMA mouse model, called the *Smn*$^{2B/-}$ model, has also been used for SMA research [59]. These mice possess 15% of normal SMN protein levels and they become symptomatic around postnatal day 10. *Smn*$^{2B/-}$ mice live an average lifespan of 28 days. Intermediate mouse models are advantageous for extracting subtle biological information that may be overlooked by the overt disease phenotype of the severe models. Furthermore, use of these intermediate models will aid in the development of *SMN2*-independent therapies.

Therapeutic Intervention in SMA

Therapeutics aimed to attenuate SMA disease progression have encompassed various strategies ranging from those that focus on gene therapy or boosting *SMN2* productivity, to those that use small molecular compounds to augment pathology through secondary pathways [6].

Many treatments have evolved that target *SMN2* either by activating the promoter or increasing exon 7 inclusion during splicing [6]. Histone deacetylase inhibitors (HDACi) are pharmacologic compounds that aid in the decondensation of chromatin, which promotes gene expression, although non-specifically and at a global level. One such HDACi, sodium butyrate, increased exon 7 inclusion in a cell-based model and increased SMN levels in motor neurons of the spinal cord in SMA mice [60]. Valproic acid (VPA) and phenylbutyrate (PBA) HDAC inhibitors only modestly increased SMN in patient fibroblasts *in vitro*, and the extent of this increase was dependent on *SMN2* copy number [61, 62]. Unfortunately, both of these compounds provided minimal effectiveness in clinical trials [6, 63-65]. HDAC inhibitors, Trichostatin A

(TSA) and suberoylanilide hydroxamic acid (SAHA) both significantly extended survival in SMA mice [66, 67]; and TSA treatment supplemented with nutritional support increased survival even further [68]. In addition, administration of sodium butyrate or SAHA in the drinking water of gestating female mice beginning on embryonic day 15, led to a decrease in disease severity of SMA progeny or amelioration of embryonic lethality, respectively [60, 67]. Interestingly, HDAC inhibitors seem to provide general neuronal protection in a manner independent of SMN, although it is not clear as to how this occurs. However, it has been proposed that this neuroprotection may be due to the inhibition of atrogene pathways which normally mediate the breakdown of muscle proteins in a myogenin-dependent manner [69].

SMN expression has been shown to be in part, regulated via the STAT5 pathway [70, 71]. Prolactin, a known activator of this pathway, is a hormone and canonically penetrates the blood-brain barrier. Farooq et al. (2011) demonstrated that increased SMN expression and protein levels *in vitro* and *in vivo* could be attained, along with a significant extension of survival in SMA mice following prolactin administration. Although prolactin did not provide a robust rescue, it may be possible to use this treatment as a supplemental therapy to maximize effects of other regimens.

Another strategy implemented to modulate *SMN2* splicing is through the use of nucleic acids to promote exon 7 inclusion [6]. Antisense oligonucleotides (ASOs) and bifunctional RNAs [72-79] perform this function in a manner similar to RNA interference; using a short stretch of nucleotides (e.g. 20mer) to bind a specific RNA target sequence with a high degree of specificity. For example, as in the case of SMA, an ASO may be designed to target a splice repressor that normally promotes exon 7 exclusion; in this way, inhibition of a repressor would

favor the retention of exon 7 [77]. ASOs are modified for stability and reduced nuclease activity, affording them an extended half-life [6]. Bifunctional RNAs are used in a similar manner only they perform a dual function; they contain a RNA sequence domain complementary to the RNA target sequence and an additional untethered sequence which serves as a binding domain for specific splicing factors [77]. These recent advances in RNA-based therapeutics have greatly accelerated a new wave of research [6, 80] that can embrace the limitless possibilities and versatility of RNA, making this therapy amenable to the study of innumerable diseases.

A very promising new compound produced by ISIS Pharmaceuticals is currently being investigated in a multiple-dose Phase 2 study (ClinicalTrials.gov, Identifier: NCT01839656); ISIS-SMN_{Rx} is an ASO designed to modulate *SMN2* splicing to promote exon 7 inclusion. This therapeutic is injected intrathecally into the cerebrospinal fluid and remains in the central nervous system (CNS) for an extensive amount of time. However, the current Phase II study, which is expected to be completed in 2014, is addressing the question of whether larger doses are safe and can be well tolerated. A Phase III study is anticipated to begin in 2014.

Gene Therapy for Spinal Muscular Atrophy

Gene therapy provides the most robust rescue in SMA mice to date and has been utilized extensively by many laboratories. SMA is well suited for viral-based gene delivery because the disease is monogenic and the SMN cDNA is small enough to be packaged into a viral vector. Adeno-associated virus (AAV) serotype 9 has broad tissue tropism with a high transduction rate into motor neurons, and as SMA is a motor neuron disease, it is appropriate that this serotype be utilized to specifically target the CNS [81-83].

Using GFP as a reporter, it has been demonstrated that systemic delivery of AAV9-GFP by intravenous injection, results in extensive transduction in motor neurons of neonatal mice (60%), but was limited in adult mice in which astrocytes were the predominant target [84]. This suggests that AAV9 was able to permeate the CNS of neonates where the blood-brain barrier (BBB) is not yet fully formed as opposed to adult mice in which the BBB has fully developed. Two explanations have been proposed to further elucidate this observation [84]. First, is the fact that astrogenesis occurs in mice during the first two weeks of the postnatal period and second; it is possible that astrocytic projections make contact with vascular endothelia and thus, capture most of the virus particles before they are able to make their way past, to the motor neurons. The BBB poses an obstacle for viral gene delivery because the endothelial cells separating the vasculature and cerebrospinal fluid (CSF) form a tight barrier through which many molecules cannot pass. However, with the advent of double-stranded self-complementary AAV vectors (scAAV) in which the second strand synthesis step is bypassed and more specifically, the generation of the scAAV9 vector, it was demonstrated that the efficiency of adult CNS transduction could be increased [82]. Intravenous administration of scAAV9-GFP not only provided robust transduction of neonatal motor neurons (likely due to the accelerated onset of expression obtained with scAAV as opposed to AAV), but also transduced up to 28% of motor neurons in adult mice [82]. Furthermore, following administration in neonates, GFP-positive cells were detected in the CNS as well as non-CNS tissues, in contrast to an intraperitoneal (IP) injection which resulted in only a few transduced cells in the CNS, and to an even lesser extent following intramuscular injection [82]. Once the potential for achieving motor neuron

transduction *in vivo* was demonstrated, multiple laboratories applied this technology to SMA and used this vector to overexpress SMN in SMA mouse models.

Therapeutic Window for SMA Intervention

Route of delivery and timing of administration are two important decisions to consider when applying gene therapy to SMA because variations of both lead to distinguishable results. Initial studies implementing scAAV9 to overexpress SMN, administered the virus by intravenous injection (IV) between the day of birth (P0) and postnatal day 2 (P2). This mode of intervention completely ameliorates disease progression in the SMNΔ7 model but does not have the same effect on the more severe *SMN2* mice, although it does extend their survival significantly. SMNΔ7 mice are born presymptomatic and live on average, 14 days however; when injected with scAAV9-SMN into the facial vein during this early postnatal period, these mice gained significantly more weight that untreated controls and lived a normal lifespan (>200 days) [81, 85]. On the contrary, disease progression could not be ameliorated in the *SMN2* model but the animals did gain significantly more weight than control SMA mice, and they lived on average, 100% longer than controls [86].

Prolonging SMN induction proves to be less efficient. Only a modest benefit was achieved when scAAV9-SMN was administered intravenously on P5, extending survival from 15 to 30 days; significantly less than what the same injection provides on P1 or P2, in which mice lived over 250 days [81]. Further delay of treatment until P10 provided no benefit, indicative of a narrow window of opportunity for therapeutic intervention to have an effect.

In parallel, this decreased therapeutic benefit from prolonging intervention was also described in the case of ASO technology. Porensky et al. (2012) used an ASO that modulates SMN2 splicing to increase exon 7 inclusion [87]. Direct CNS administration on P0 provided a robust rescue in SMNΔ7 mice. Survival was extended from 15 to 100 days, SMN levels were significantly increased and SMN expression was successfully restored to motor neurons with high expression in the cytoplasm, as well as the formation of gems within the nucleus. Similar to previous reports describing scAAV9-SMN intervention, administration at a later time point (P4) decreased the efficiency of this ASO therapeutic, by providing only a modest extension in survival (41 days) when mice were injected by ICV, and significantly less when injected by IV (21 days) [87].

In 2012, a detailed analysis of the differential effects observed between ICV and IV administration was reported for both the symptomatic *SMN2* mice and the presymptomatic SMNΔ7 mice [85, 86]. ICV or IV delivery of $2x10^{11}$ viral genomes (v.g.) into symptomatic mice on P1 significantly extended survival from an average of 5 days to 17 and 10 days, respectively. SMN was significantly increased in the brain and spinal cord of both groups. Muscle fiber area was only slightly increased in the ICV group but not in the IV injected mice [86]. However, a more substantial benefit was observed when intervention was administered before symptoms appeared. In the SMNΔ7 model, delivery of $2x10^{10}$ v.g. on P2 resulted in a complete rescue when delivered either by ICV or IV, with sufficient SMN restoration in the CNS, as well as restoration of motor function [85]. As this treatment did not provide a full rescue in *SMN2* mice as it did in SMNΔ7 mice, this further supports the indication of a therapeutic window in which treatment must be administered before the disease progresses too far. Restoration of SMN once

overt symptoms are occurring may not be the best method for treating the disease at this point, meaning that this therapy may need to be adapted or perhaps a combinatorial therapeutic regimen may prove a more effective means of halting the disease once it has progressed to advanced stages.

Further elucidation of this prospective therapeutic window was provided in reports of which an SMN-inducible mouse model was used. In order to mimic human SMA in mice, Lutz et al. (2011) created a mouse line in which the endogenous *Smn* gene was designed to behave like human *SMN2* by replacing murine *Smn* exon 7 and 8 with a cassette consisting of human *SMN2* exon 7 and 8. Additionally, an inverted and silent copy of mouse *Smn* exon 7 was placed within *SMN2* in the intron between exon 7 and 8. The generated hybrid allele contained also, *loxP* sites flanking both exon 7s. This allowed conditional and irreversible activation by cassette inversion to express functional, full-length protein at wild-type levels within 48 hours of tamoxifen administration. When they turned on SMN expression during embryonic development on embryonic day 6 (E6), they observed a complete restoration to a wild-type phenotype [88]. This is interesting because throughout most studies that utilize the SMNΔ7 model, the treated mice always have a smaller, weaker overt phenotype compared to unaffected mice, although they do often gain significantly more weight than untreated controls. SMN induction following tamoxifen administration on P4 or P6 provided a substantial rescue but P8 induction only slightly enhanced survival while P10 did not provide any benefit [88].

Furthermore, Le et al. (2001) developed another SMA inducible mouse model where SMN is expressed only upon administration of doxycycline. The SMN construct here consists of two minimal cytomegalovirus (CMV) promoters and a tetracycline response element (TRE).

This dual reporter system, upon doxycycline activation, drives SMN and luciferase expression [89]. This inducible model took three days for an increase in SMN to be observed following induction, however, this report provided similar results as the Lutz group [90]. Induction at E13 or P0/P1 provided a complete rescue, with mice living over 200 days however; the benefit was not as substantial as the rescue Lutz et al. (2011) described when induction began on E6. As expected, induction on P2 (SMN increase detectable at P5) had less dramatic effect where all the mice except one, lived on average 25 days, with the exception living to 151 days. Also, mice induced on P0/P1 in which SMN expression was turned off at P28, in general lived only a month following SMN removal, with the exception of one mouse that lived over 8 months. Outliers such as these two above are common occurrences in these types of studies and this may be due to intrinsic genetic or epigenetic factors or overall pathological differences between mice [89].

Overall, early restoration of SMN provides the most beneficial outcome whereas delayed intervention substantially diminishes the efficacy of this therapeutic modality. Route of administration also changes the outcome, where systemic and direct CNS injections provide the best results when implemented in a timely manner.

It is interesting to compare the studies involving direct CNS administration to the report by Hua et al. (2011) who demonstrated that ASO delivery into the periphery alone, increased survival significantly compared to direct CNS administration. This might suggest that peripheral tissues that succumb to SMN depletion may have a negative effect on downstream targets, perhaps some in the CNS, and that is perhaps why Hua et al. (2011) observed these results. However, it is difficult to determine as of now because the systemic dose that was injected was much higher than the dose administered to the CNS [91].

Timing of therapeutic administration for SMA has shown to be of utmost importance, where intervention at the presymptomatic stages has the most beneficial outcome. It is still unclear why this is the case but one explanation highlights the importance of the target cell population. There may be a window in which motor neurons are no longer able to respond to treatment. Perhaps they have degenerated to a point where no matter how much SMN is pumped into them, it may not fix the damage that has already progressed. It is also possible these cells lose specific communication signals and are no longer able to be transduced by the virus. Another limit to this intervention is the mode of administration. As scAAV9 readily transduces cells in the CNS when administered intravenously in neonates, this effect is diminished when intervention is delayed after birth. This may possibly be due to complete closure of the BBB or other hemodynamic alterations in which the virus is not sufficiently transported into the CNS.

Furthermore, there is evidence of viral entry by retrograde transport from the muscle; however, the reports on direct intramuscular injection do not conclusively support this [92]. It is still debated whether systemic or direct CNS injection is the best route of delivery as there are two schools of thought about this. Some researchers hold true to the idea that SMA is a motor neuron disease and that SMN replacement in motor neurons is sufficient to halt the disease whereas; the other side of the argument highlights that motor neuron induction alone, does not completely ameliorate all symptoms [33]. Replacement of SMN solely in motor neurons does not preserve the integrity of all tissues as is evident in the numerous studies that report on the sudden death often observed of a sub-group of treated mice [93]. This suggests that SMA affects multiple organ systems and supports the idea of a multifactorial intervention scheme.

CHAPTER TWO: SPINAL MUSCULAR ATROPHY - DISEASE DEVELOPMENT AND THERAPEUTIC WINDOW USING SCAAV9-SMN IN THE DELTA7 MOUSE MODEL

Introduction

SMA is well suited for therapeutic intervention because the disease is monogenic, the patient population is homogenous and humans possess the important disease-modifying gene, *SMN2*. Strategies to circumvent SMA progression focus on *SMN1* gene replacement or the alteration of SMN2 splicing. Gene therapy using self-complementary adeno-associated virus, serotype 9 (scAAV9) to deliver SMN, either systemically or directly into the CNS, has shown the most promising results for rescuing SMA mice when administered before the therapeutic window closes.

Previously, it has been demonstrated in a severe SMA mouse model, that SMN induction as early as E6 completely ameliorates the disease phenotype [88]. However, administration of scAAV9-SMN between P0 – P2 provides a near complete rescue but does not correct vascular side effects such as tail and ear necrosis; and additionally, peripheral organ defects are often observed [85, 88]. Furthermore, slight delay of therapeutic intervention results in only a modest rescue, and an extended delay provides no benefit. Utilizing the well characterized SMNΔ7 model, we performed a systematic analysis to determine the effects of delaying scAAV9-SMN administration. We injected each mouse with a single injection of $1x10^{11}$ viral genomes (v.g.) scAAV9-SMN at a single time point, P2 through P8. Early SMN induction provided the most benefit, whereas, injection at later time points decreased the efficacy of this therapy. All treated mice lived significantly longer, but SMN induction at the earliest time point (P2) provided the most robust rescue with the fewest early deaths. Treated mice gained weight, demonstrated

restored motor function and exhibited SMN protein induction in the CNS and periphery. Treatment at the various time points resulted in a range of phenotypes observed within the mice, but is was evident that the earliest administration provided the best rescue, while delaying intervention decreased the effectiveness.

Materials and Methods

Animal Handling and Genotyping

All animals were housed and treated with respect to the guidelines of the Animal Care and Use Committee at the University of Missouri and in accordance with the regulations defined in the "Guide for the Care and Use of Laboratory Animals," (National Research Council, 2011).

The SMN delta7 (SMNΔ7) mouse model was utilized for these experiments. SMNΔ7 mice are null for mouse Smn and contain two copies of both transgenes, human *SMN2* and the cDNA coding sequence for SMNΔ7. Mice heterozygous for mSmn ($Smn^{+/-}$;$SMN2^{+/+}$;$SMN\Delta7^{+/+}$) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA; stock 005025) and were bred to generate mSmn knock-out mice ($Smn^{-/-}$;$SMN2^{+/+}$;$SMN\Delta7^{+/+}$) used in these experiments. Heterozygous ($Smn^{+/-}$;$SMN2^{+/+}$;$SMN\Delta7^{+/+}$) mice are used throughout all the experiments as unaffected positive controls and untreated SMA ($Smn^{-/-}$;$SMN2^{+/+}$;$SMN\Delta7^{+/+}$) mice were used as negative controls. On their day of birth, postnatal day 1 (P1), each mouse was numbered using a non-toxic, permanent marker for individual identification, and remarked each day after, until the presence of fur in which they were then marked permanently with fuchsine dye. The distal tip of the tail was removed, ~1 mm, with sharp, heated scissors to aseptically remove the tail tissue and cauterize the wound. Genotyping was carried out by lysing the tail tissue in 75 µl of alkaline solution (25 mM NaOH, 0.2 mM EDTA) for 30 minutes at 95°C followed by neutralization in 75 µl of a TRIZMA-HCl solution (40 mM TRIZMA-HCl) and incubation on ice. Polymerase chain reaction (PCR) conditions were as follows: 95°C 2 minutes, 30 cycles (95°C 15 seconds, 68°C 90 seconds), 68°C 10 minutes. Primers were used to amplify the *mSmn* wild-type allele, forward

(5’-TCTGTGTTCGTGCGTGGTGACTTT-3’) and reverse (5’-CCCACCACCTAAGAAAGCCTCAAT-3’) and the *lacZ mSmn* knockout allele, forward (5’-CCAACTTAATCGCCTTGCAGCACA-3’) and reverse (5’AAGCGAGTGGCAACATGGAAATCG-3’).

Virus Production

scAAV9-SMN viral vector was produced as described previously [86]. HEK293T cells were triple transfected in the presence of polyethyleneimine (PEI) (1mg/ml), pH 5.0 [94]. The scAAV plasmid was constructed to express the open reading frame of human SMN1 cDNA (NCBI accession number NM_000344) under the control of the chicken β-actin promoter. Following media change and cell collection at 24 hours and 48 hours post-transfection, respectively, scAAV9-SMN viral vector was purified using three cesium chloride density-gradient centrifugation steps and dialyzed with HEPES buffer.

Real-time PCR (qPCR)

Quantification of viral genomes was performed using SYBR® Green and primers to amplify the chicken β-actin promoter region, forward (5’-CCGGTGGTGGTGCAAATCAAAGAA-3’) and reverse (5’-AGCAGAAGTAACACTTCCGTACAGGC-3’). The absolute quantitation method using a standard curve was utilized on the Applied Biosystems® 7500 Real-Time PCR System using Applied Biosystems 7500 Sequence Detection Software version 1.3. Viral fractions were diluted 1:1000 and the PCR cycle was as follows: 50°C 2 minutes, 95°C 10 minutes, 40 cycles (95°C 15 seconds), 60°C 1 minute. A standard curve was obtained using serial dilutions of the transgene-containing plasmid (10^{10}-10^{5}) to calculate melting curves of each sample. The viral

fractions containing the highest titer were dialyzed with HEPES buffer (100 mM NaCl, 20 mM HEPES). Following dialysis, qPCR was performed to obtain the final titer of the virus-containing solution to be used for injections into the mice.

Intracerebroventricular Injection of scAAV9-SMN or scAAV9-GFP

Intracerebroventricular (ICV) injection was performed on neonates to administer scAAV9-SMN therapy directly into the CNS. Mice were administered a single injection of $1x10^{11}$ v.g., on one of the following days, P2 through P8. Briefly, a glass-pulled needle was inserted through the frontal plate (lateral to the metopic suture and rostral to the coronal suture) into the left or right ventricle of the neonatal mouse to deliver a 5 µl bolus of viral vector. Unaffected animals were injected with scAAV9-GFP on P2 or P7 and non-injected mice were used as a negative control.

Phenotypic Assessment

All treated and untreated mice were monitored daily to evaluate survival, weight gain and motor function as assessed by the time to right test. The time to right test consists of placing a mouse on its back and determining the time it takes for them to right themselves onto all four paws. A mouse unable to right within 30 seconds is considered to have failed the test for that day.

Tissue Collection

Tissues were collected on P11 for all mice treated with scAAV9-SMN and on P6 or P11 for those injected with scAAV9-GFP. To harvest the tissues, mice were anesthetized using isofluorine followed by cervical dislocation. Brain, spinal cord, skeletal muscle, heart, kidney, liver, and spleen were collected and immediately flash-frozen in liquid nitrogen for those tissues used for protein analysis or submerged in 4% paraformaldehyde if used for histological examination of GFP.

Western Blot Analysis

For the SMNΔ7 mouse Western blots, tissues were collected at selected time points and immediately frozen in liquid nitrogen. Tissue samples were placed at -80^{o}C until ready for analysis. Roughly 100 mg of tissue was homogenized in JLB buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM NaH2(PO4), 25 mM NaF, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and protease inhibitors (Roche, Indianapolis, IN, USA). Equal amounts of protein were separated on 12% SDS-PAGE gels. SMN immunoblots were performed using a mouse SMN specific monoclonal antibody (BD Biosciences #610647, San Jose, CA, USA) diluted 1:2000 in 1% dry milk in TBST (Tris-buffered Saline Tween20 (10mM Tris-HCl, pH7.5, 150mM NaCl, 0.2% Tween20) and a secondary anti-mouse HRP-conjugated secondary antibody (1:10,000). Blots were visualized by chemiluminescence on a Fujifilm imager LAS-3000 and the corresponding software. To verify equal loading, the membranes were then stripped using β-mercaptoethanol for 30 minutes at 50°C and re-probed with anti-β-actin rabbit antibody (Sigma #A5060, St. Louis, MO, USA) diluted 1:2000 and anti-rabbit HRP secondary antibody (1:10,000). Western

blot analysis was performed in triplicate and representative blots are shown. Probes were visualized by chemiluminescence using the Pierce SuperSignal Pico reagents.

Intracerebroventricular Injection of scAAV9-SMN Increases Survival of SMNΔ7 Mice

On average, mice from all treatment groups lived significantly longer than non-injected animals however; there was variability within groups (Figure 2.1, 2.2, 2.3). Mice injected on P2 experience no early deaths (all mice lived past 130 days) and they lived the longest with an average lifespan of 187 days and a median survival of 204 days. The early deaths of the P3 and P4 injected groups occurred on days 47 and 50, respectively. The P3 injected group lost another subset of the population between days 60 and 100 but the remaining three mice lived past 150 days (maximum 187 days). The average survival for the P3 group was 102 days with a median lifespan of 75 days. Mice in the P4 injected group steadily decreased in numbers with the oldest animal living 211 days; and the average for the group was 141 days with a median lifespan of 167 days. In the P5 injected group, seventy percent of the treated mice died between days 22 and 42, with the remaining three mice living 152, 165 and 211 days. The average and median survival for the P5 group was 76 and 37 days, respectively. Sixty-two percent of the mice in the P6 group died between 20 and 35 days while the remaining three mice lived 50, 184 and 211 days; the average and median for the group were 73 and 34 days, respectively. Within the P7 injected group, eighty percent of the animals died between days 21 and 34, while the oldest animal survived 70 days. Mice from the P7 injected group lived on average, 30 days with a median survival of 28 days. The P8 injected group had a significant extension in survival, compared to non-injected animals, although this was not as substantial as the survival extension exhibited by the earlier-injected mice (Figure 2.3). Both the average and median survival values for mice in the P8 group were 18 days. A high degree of variability is observed with the survival data of each group treated at median time points P3 through P7 (Figure 2.4). Overall, mice

injected with scAAV9-SMN at earlier time points had a greater extension in survival compared to mice injected at later time points; and mice from all groups lived significantly longer than non-injected controls which lived on average, 13 days with a median survival of 14 days.

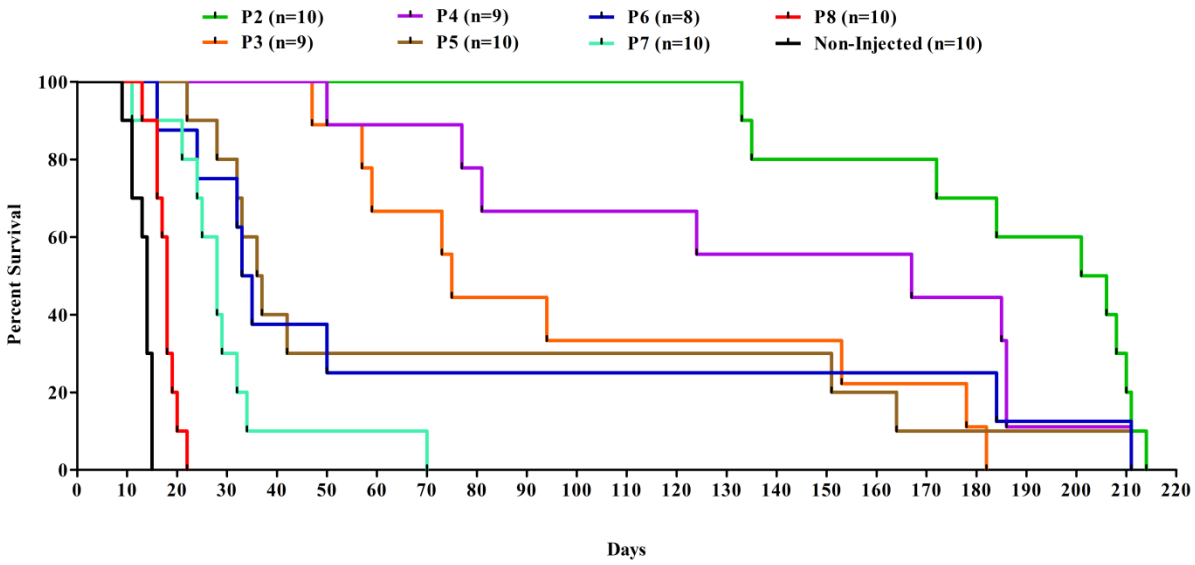

Figure 2.1. ICV injection of scAAV9-SMN increases the survival of SMNΔ7 mice when administered at early time points. Kaplan-Meier survival curve of untreated SMNΔ7 mice and those injected with $1x10^{11}$ v.g. of scAAV9-SMN on a single day P2-P8.

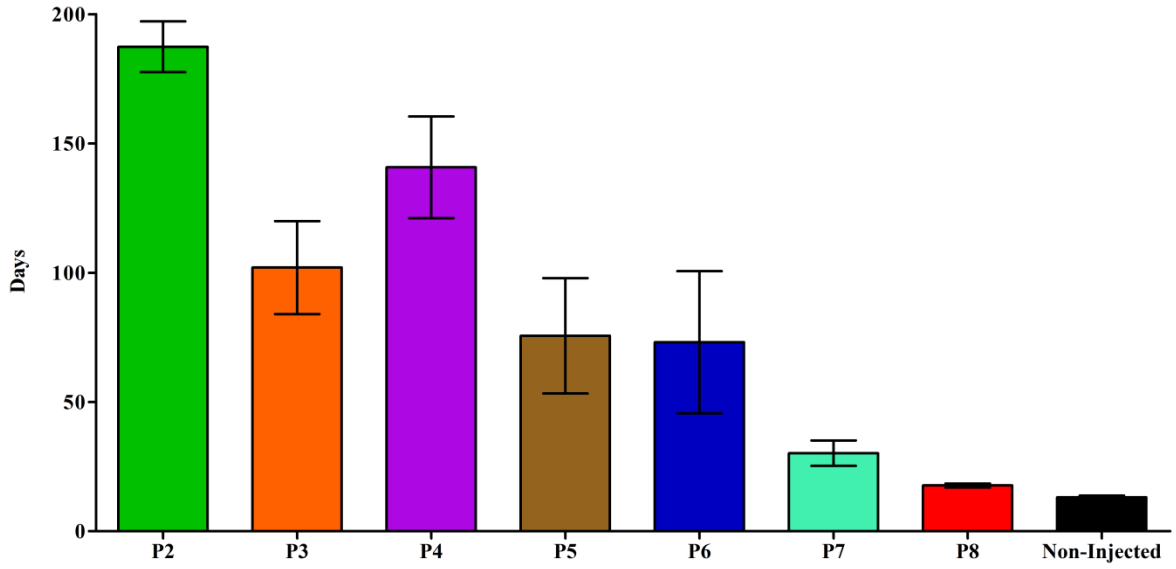

Figure 2.2. SMNΔ7 mice injected at early time points live longer on average compared to mice injected at later time points. All treated SMA mice lived significantly longer than non-injected controls (Refer to *p*-table, Figure 2.3).

	P2	P3	P4	P5	P6	P7	P8	NI
P2		0.0000	0.1090	0.0136	0.0402	0.0000	0.0000	0.0000
P3	***		0.0432	0.0000	0.7188	0.0000	0.0000	0.0000
P4	n.s.	*		0.0755	0.1348	0.0000	0.0000	0.0000
P5	*	n.s.	n.s.		0.9950	0.0181	0.0000	0.0000
P6	*	n.s.	n.s.	n.s.		0.0811	0.0004	0.0000
P7	****	****	****	*	n.s.		0.0002	0.0001
P8	****	****	****	****	***	***		0.0001
NI	****	****	****	****	****	****	****	

Figure 2.3. P-table demonstrating statistically significant differences in average survival of mice in the treatment and non-treatment groups. All treated mice lived significantly longer than non-injected controls. *P*-values were calculated using the logrank Mantel-Cox test and the table illustrates the significance in survival between groups. NI = non-injected. (*n.s.* no significance, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

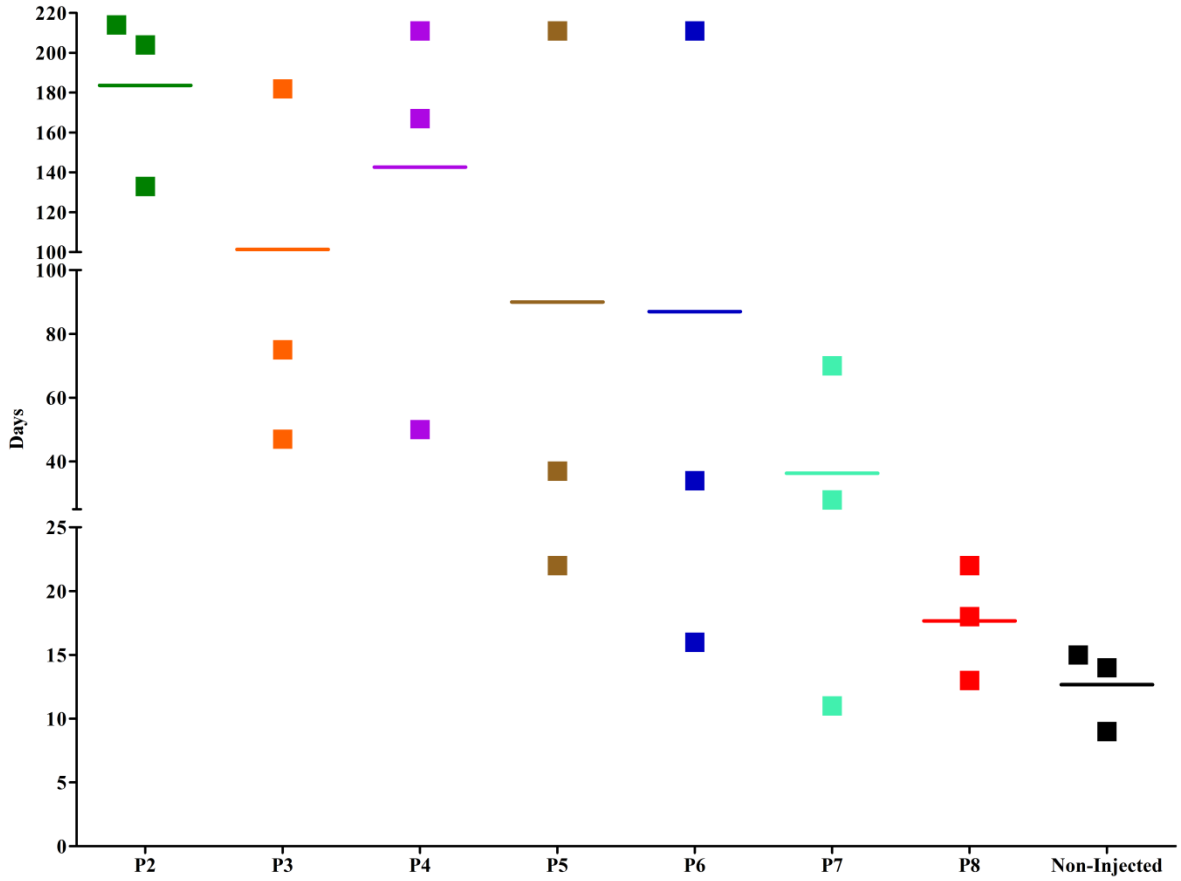

Figure 2.4. There is a high degree of variability in survival observed within each group of mice injected at the median time points. For each treatment group, the line represents the average survival for the group and the data points represent the minimum, median and maximum survival within each group. There is a higher degree of variability observed within groups P3 through P7, while the P2, P8 and the non-injected groups display less variation in survival.

Weight Increase Observed in All Treated Groups

Weight gain was analyzed as the average weight for a given group across days (Figure 2.5) and also as the average percent weight gained from birth to peak weight (Figure 2.6, 2.7). At birth, all SMA pups and unaffected pups are similar in weight and there are no obvious phenotypic differences until around day 7 (Figure 2.8). However, by day 11 all treated mice weighed significantly more than non-injected animals. Even the late injected mice (P7 and P8) weighed significantly more, although this difference was not as substantial as the difference observed between early injected mice and the non-injected controls. Compared to non-injected animals, mice injected on P3 weighed significantly more by day 7, those of P4, P5 and P8 were significantly higher by day nine, group P2 by day 10 and groups P6 and P7 were significantly higher than non-treated animals by day 11. Around day 10, the P8 and non-injected mice reach a plateau and their weight declines from thereon. By day 14 the P8 injected group weighed significantly less than mice from all other treatment groups. At day 17 an emergence of two groups within the weight observations can be observed. The first group consisting of P2, P3 and P4 have similar weight gain and as a group diverge from a second cluster consisting of P5, P6 and P7.

All treated animals exhibited a significantly higher percent weight gain from birth to their peak weight, compared with non-treated animals; the significance between these groups varied (Figure 2.6 and 2.7). Animals injected on P2, P3 or P4 displayed similar weight gain from birth to peak, and there was no significance when compared with each other. Similarly, animals injected on P5, P6 or P7 had comparable percent weight gain and also did not show significance when compared to one another. Comparison of either group P2, P3 or P4 with any of group P5,

P6 or P7 was significant; for all comparisons $p \leq 0.0001$ except P4 compared with P5 or P6 ($p \leq 0.001$). Mice in the P8 group gained significantly less weight than mice in groups P2 through P6, but not P7. All treated mice gained weight following treatment with scAAV9-SMN.

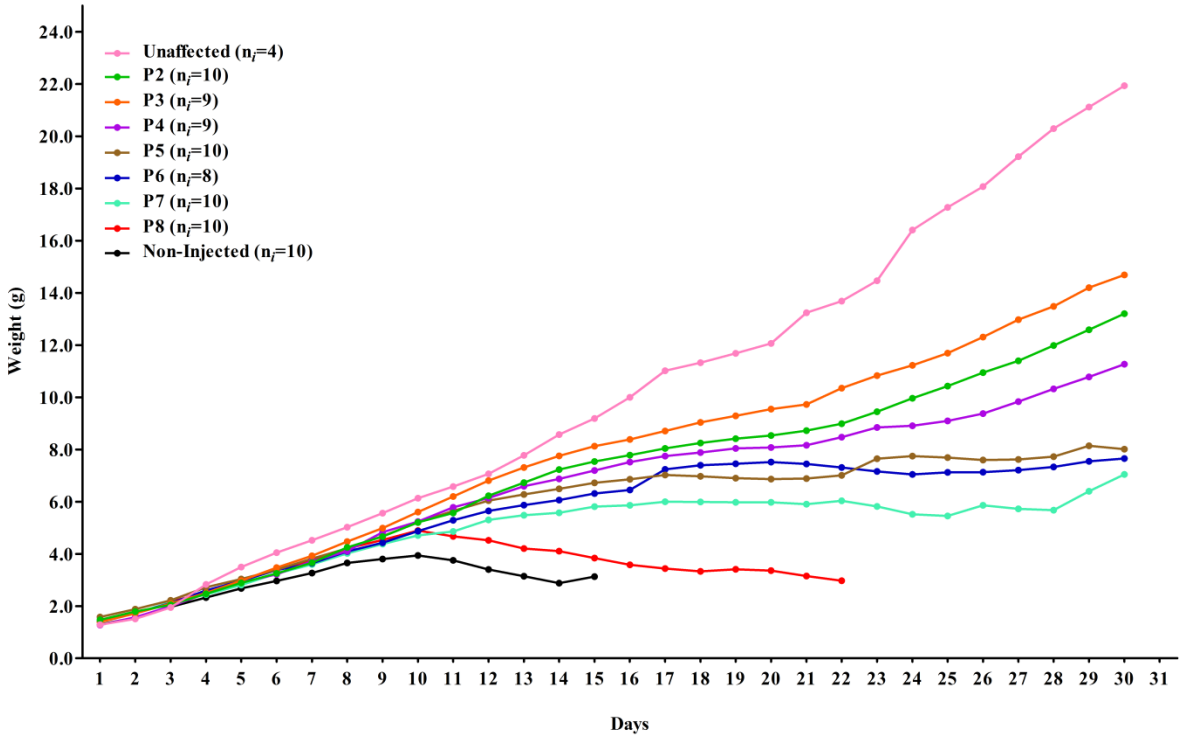

Figure 2.5. SMNΔ7 mice treated with scAAV9-SMN gain weight throughout their lifespan. The average weight per group is plotted across days for the surviving animals in each cohort. n_i = initial number of animals in each group.

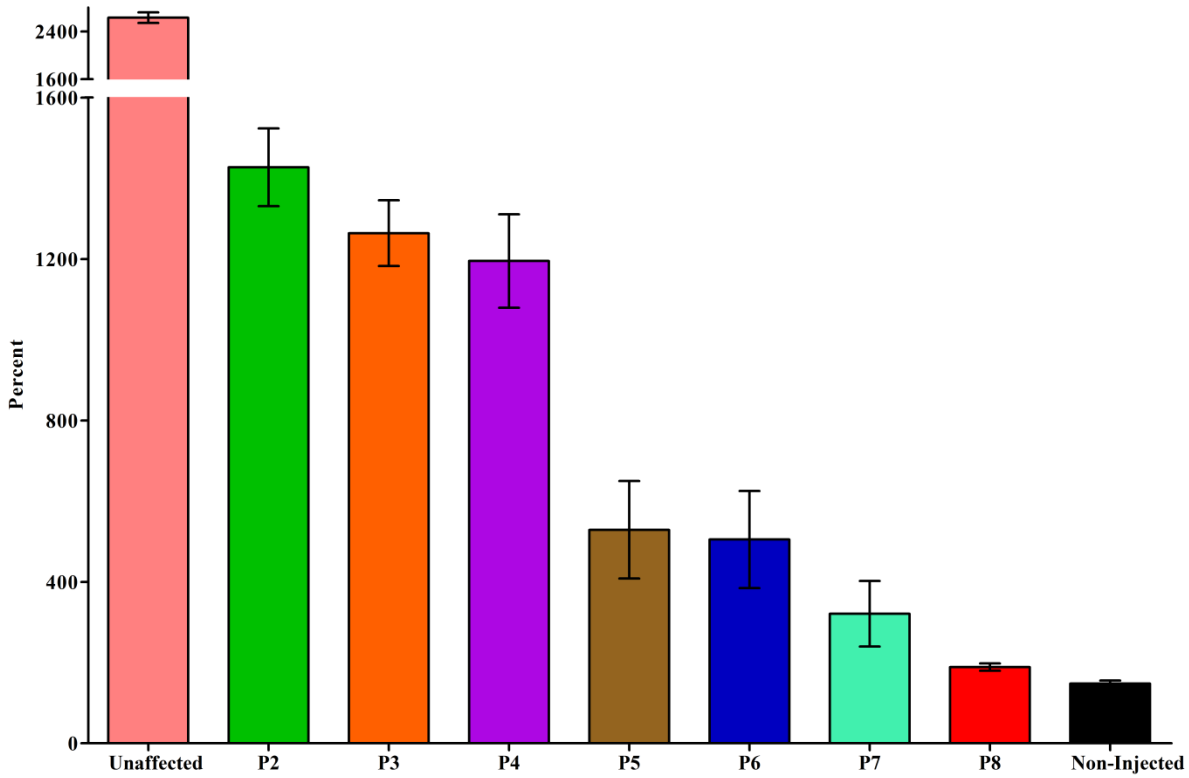

Figure 2.6. Percent weight gained from birth to peak. SMNΔ7 mice (*Smn*$^{-/-}$;SMN2$^{+/+}$;SMNΔ7$^{+/+}$) treated with scAAV9-SMN gain more weight than non-injected controls. Percent weight gained from birth to peak for all treated animals is significantly higher compared to non-injected mice (Refer to *p*-table, Figure 2.7). Weight gain for unaffected heterozygous (*Smn*$^{+/-}$;SMN2$^{+/+}$;SMNΔ7$^{+/+}$) littermates is shown here to provide a visual comparison of treated mice to healthy unaffected individuals; however, these data were not used for statistical comparisons.

	P2	P3	P4	P5	P6	P7	P8	NI
P2		0.2203	0.1395	0.0000	0.0000	0.0000	0.0000	0.0000
P3	n.s.		0.6311	0.0001	0.0001	0.0000	0.0000	0.0000
P4	n.s.	n.s.		0.0010	0.0009	0.0000	0.0000	0.0000
P5	****	****	***		0.8927	0.1701	0.0118	0.0056
P6	****	****	***	n.s.		0.2081	0.0095	0.0042
P7	****	****	****	n.s.	n.s.		0.1236	0.0474
P8	****	****	****	*	**	n.s.		0.0027
NI	****	****	****	**	**	*	**	

Figure 2.7. *P*-table demonstrating statistically significant differences in average weight gained from birth to peak all mice in the treatment and non-treated groups. SMNΔ7 mice treated with scAAV9-SMN gain significantly more weight than untreated littermates. Student's t-tests were performed to compare the average percent weight gained from birth to peak between groups. (*n.s.* no significance, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

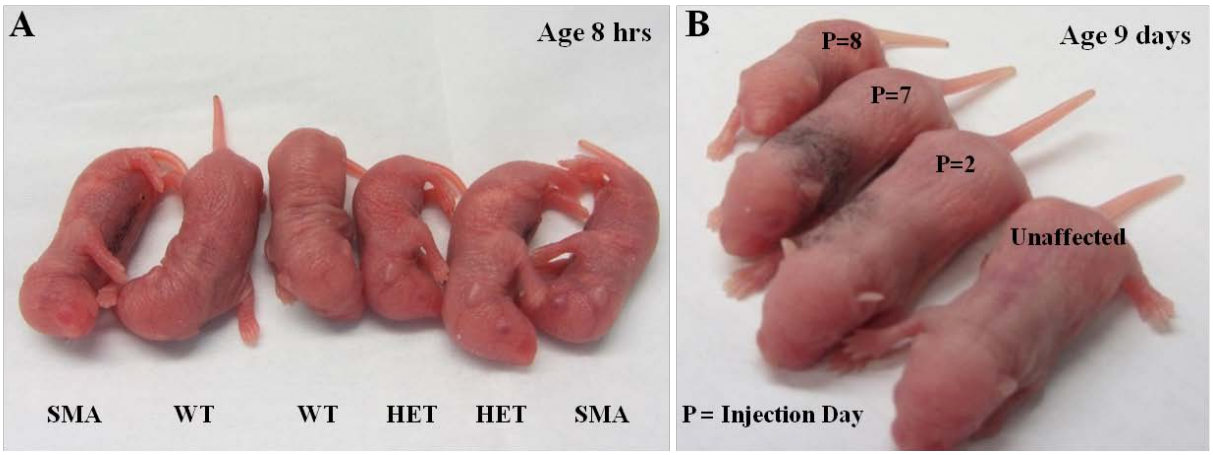

Figure 2.8. Representative images of SMNΔ7 mice. A) Phenotypic differences are not apparent at birth and the pups of the various genotypes cannot be distinguished by observation. WT (*Smn*$^{+/+}$;SMN2$^{+/+}$;SMNΔ7$^{+/+}$), HET (*Smn*$^{+/-}$;SMN2$^{+/+}$;SMNΔ7$^{+/+}$) and SMA (*Smn*$^{-/-}$;SMN2$^{+/+}$;SMNΔ7$^{+/+}$). B) At 9 days old, it can be readily observed that the pup injected at the earliest time point (P2) has gained weight comparable to the unaffected heterozygous littermate; and the pups injected at the latest time points (P7 and P8) are slower to gain weight.

Early Treatment Improves Motor Function in Mice Injected with scAAV9-SMN

Mice have the propensity to right themselves onto all four paws immediately after being placed on their back; and young, healthy neonates achieve this strength within the first week of life [95]. Mice treated with scAAV9-SMN at the different time points exhibited differences in motor function, as assessed by the time to right test. To assess the animals' strength, a mouse was placed on its back and the time it took them to right themselves was measured. A mouse unable to right itself within 30 seconds was recorded as having failed the test for that trial. This test was repeated daily throughout the lifespan of all treated mice. Over time, a greater percentage of animals from groups P2 through P7 gained the ability to right themselves however, this was not the case for the non-injected animals and those injected at the latest time point, P8 (Figure 2.9). Untreated SMNΔ7 mice display weakness beginning around day seven however; some mice are inherently stronger than others. Therefore, it is not uncommon to observe mice from the late injection and non-injected groups performing the time to right test on various days. A high percentage of mice injected on P2, P3, and P4 (80%, 56%, 56%, respectively) gained the ability to right themselves by day eleven, and they maintained this ability throughout the testing period and into adulthood. In contrast, a lower percentage of treated mice from the median time points P5 and P6 (20% and 22%, respectively) are able to right themselves by day 11; but the ability to right does increase for these groups around day 14 (70% and 75%, respectively), and is maintained throughout adulthood. Animals from the P7 group display variability in righting themselves, with few animals turning during the first few days of the testing period. Although these animals do not live until adulthood (Avg. 30 days, Figure 2.2), they all eventually gain and maintain the ability to right throughout their shorter lifespan. Only five of the ten mice in the P8

injected group were ever able to right themselves, and four of them lost this ability 2-3 days prior to death. As expected, mice of the non-injected group performed poorly on this test and only two out of the ten mice gained the ability to right themselves.

Of the mice that are able to right themselves, the early-injected animals tended to turn over faster than those injected at later time points and the mice in the non-injected group. Figure 2.10 displays the average time to right for each group on days 6-24. During the second week of postnatal development P2 and P3 reduce their performance time substantially afterwards which P2 is able to right immediately whereas the P3 group maintains an average time of 4 seconds. The P4 group steadily reduces its average time to right during the second week and is also able to turn over immediately starting on the third week. The P5 and P6 injected mice follow a similar pattern but with less impact; these mice are able to turn over immediately but not until they are into their third week. Mice in the P7 group were slower to gain this ability and did not perform well until into the third week however, they finally decreased their average turning time which they maintained until their death. Only a few animals in the P8 and non-injected groups were able to turn, but they were slow to do so. Motor function varied for mice on a given day, both between groups and within groups (Figure 2.11). However, a trend is apparent between groups of mice injected at different time points, and those injected at earlier time points perform better on the time to right test and are able to maintain this ability throughout their lifespan.

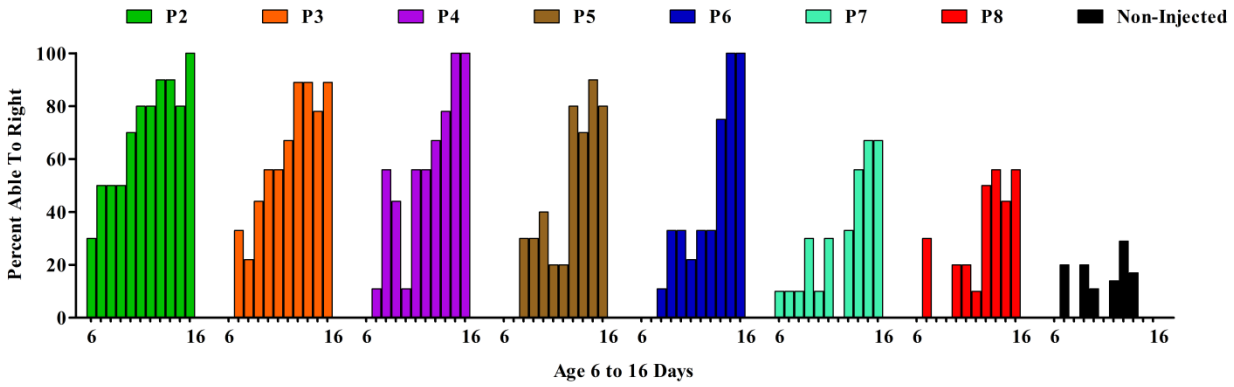

Figure 2.9. Percentage of animals able to right themselves. Assessment of motor function shows that SMNΔ7 mice treated with scAAV9-SMN at earlier time points perform substantially better on the time to right test. Animals treated at early time points gain the ability to right themselves prior to animals injected at later time points.

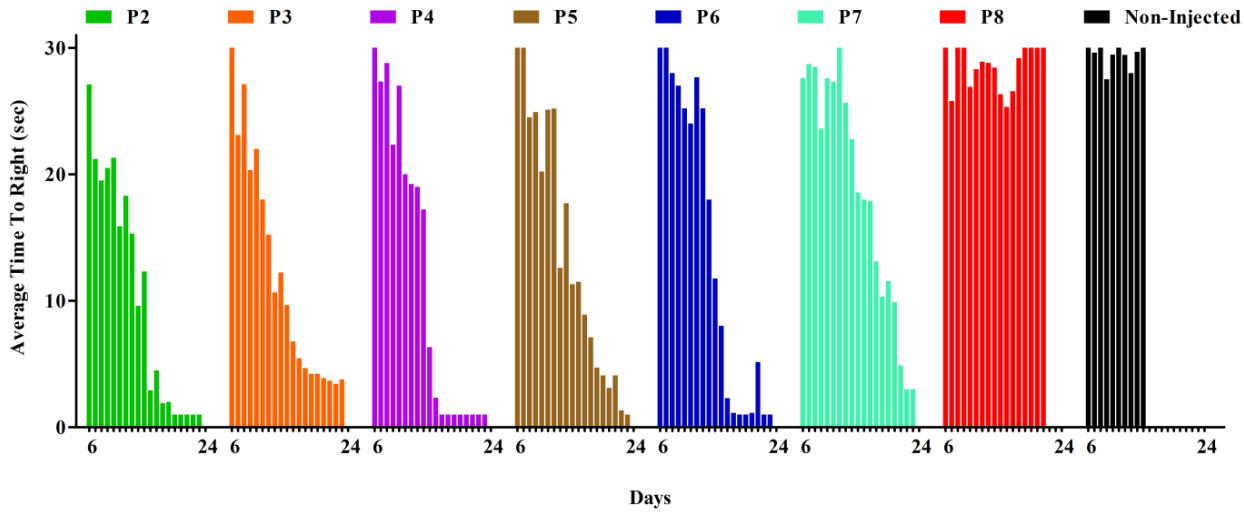

Figure 2.10. Average Time to Right for SMNΔ7 mice treated with scAAV9-SMN. Mice injected at later time points exhibit less muscle control and turn slower than mice injected at earlier time points.

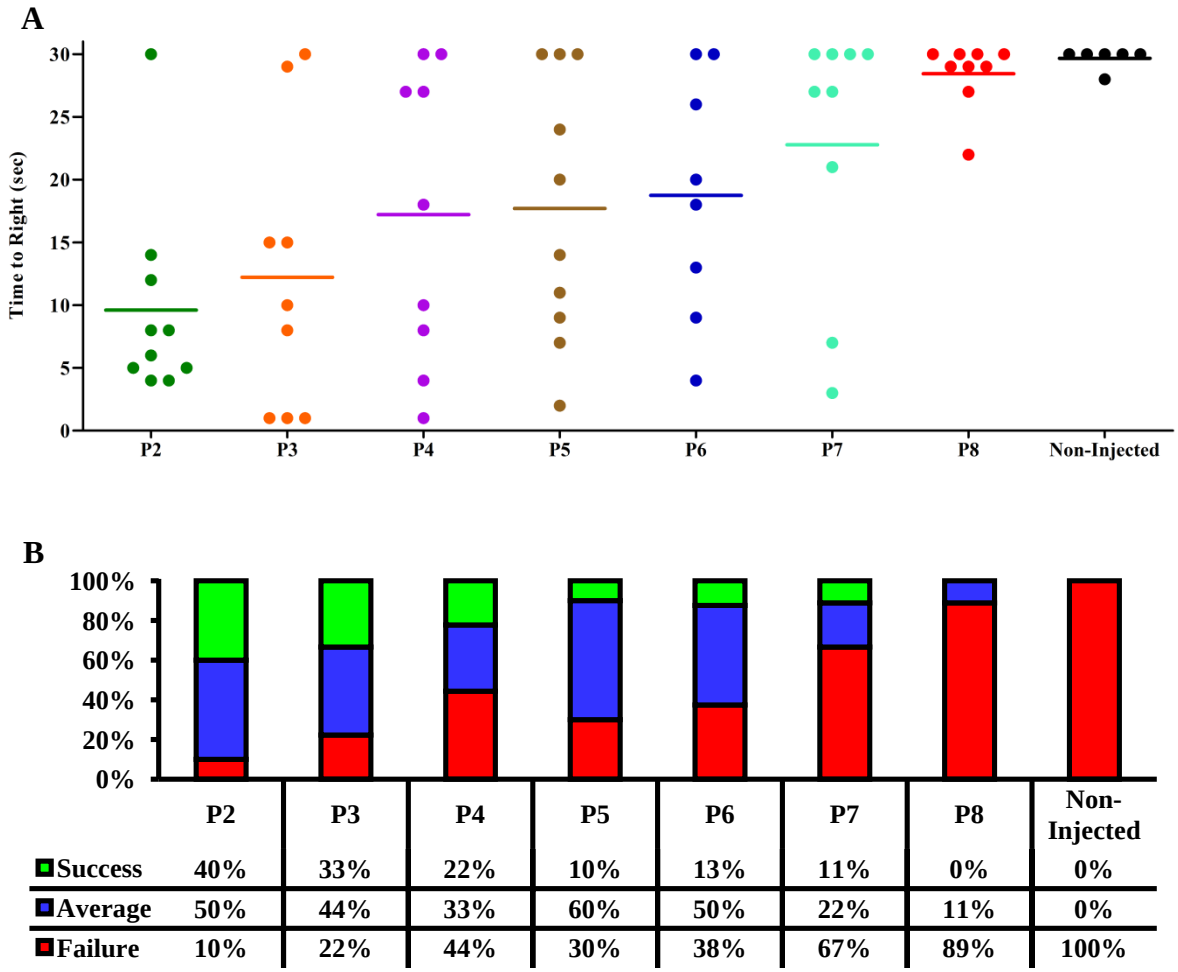

Figure 2.11. Individual performance of the time to right test on day 14. A) At two weeks, earlier treated animals perform better on the time to right test compared to those injected at later time points. B) There is a high degree of variability observed within groups; where some mice are able to right themselves more quickly than others, and some animals never gain the ability to turn. Mice able to right themselves within 0-5 seconds was recorded as a success, 6-24 seconds demonstrated average performance and those from 25-30 seconds were considered failures for this illustration.

SMN Protein Induction

Increased SMN protein was observed in animals treated with scAAV9-SMN (Figure 2.12). SMNΔ7 mice were injected on P2 or P7 and their tissues harvested on day eleven. The increased SMN expression observed in P2 and P7 injected mice compared to unaffected mice was due to the dose of viral particles resulting in higher levels of SMN compared to endogenous expression levels. Furthermore, low levels of SMN observed in the non-injected animals was expected because these mice harbor only two copies of human *SMN2* and only 10% of transcripts from each copy of the gene produce functional SMN protein. Mice injected on P2 exhibited significantly higher SMN levels in the brain compared to P7 injected mice ($p = 0.0037$) and P2 expressed significantly more SMN compared to the non-injected mice ($p = 0.0032$). P2 and P7 injected animals expressed significantly more SMN in the spinal cord compared to non-injected animals ($p = 0.0003$ and $p = 0.0082$, respectively) and the P2 group expressed significantly more protein than P7 ($p = 0.0007$). Robust induction was also observed in peripheral organs such as the heart. In the heart tissue both P2 and P7 injected animals express significantly more SMN than non-injected controls ($p = 0.0006$ and $p = 0.0079$, respectively). Furthermore, scAAV9-SMN administration on P2 resulted in significantly higher SMN levels in the heart compared with the P7 group ($p = 0.0023$). Protein induction on P2 or P7 greatly increased protein levels in the brain, spinal cord and heart.

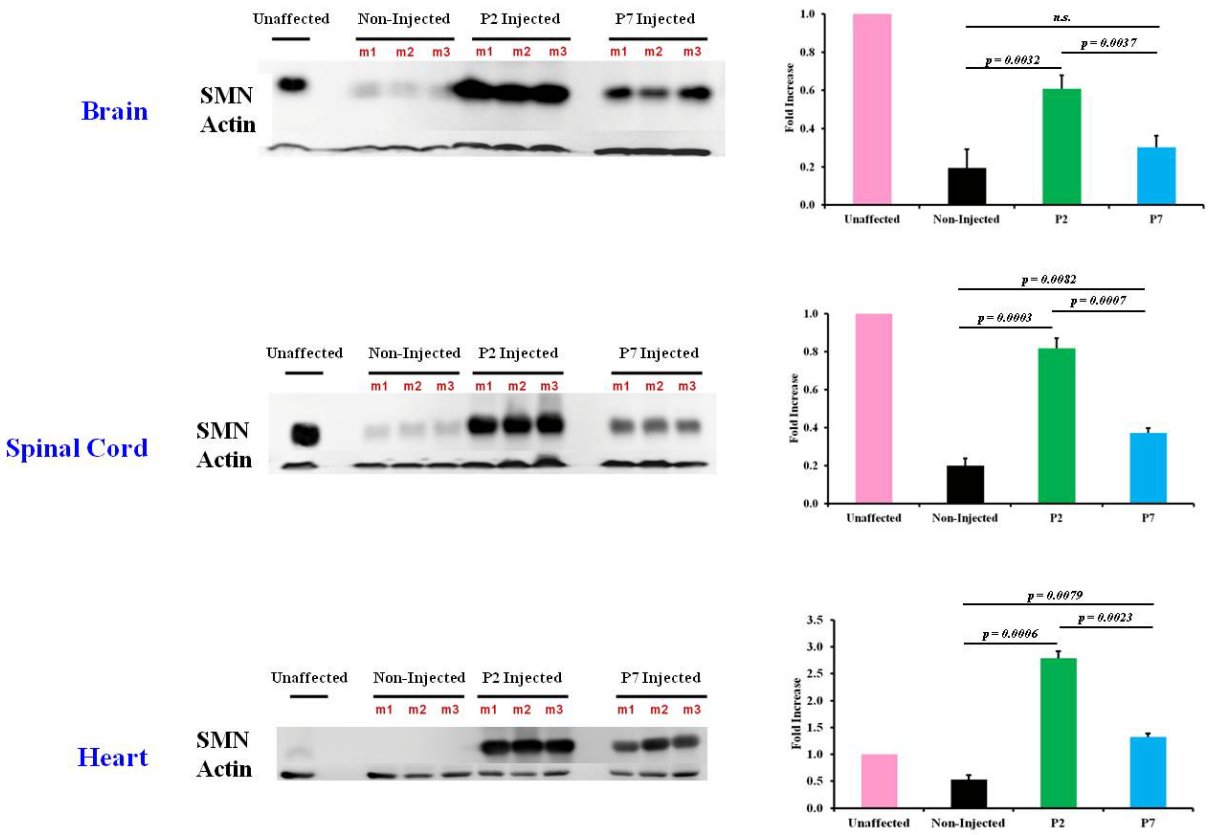

Figure 2.12. SMN protein induction is evident in SMNΔ7 mice injected at early and late time points. In the brain, SMN was upregulated significantly in the P2 mice compared to both the P7 and non-injected groups, but there was no significance observed between P7 and the non-injected group. There was robust expression observed in the spinal cord where both P2 and P7 injected mice expressed significantly more protein than non-injected controls and the P2 group exhibited a significantly higher level of protein than the P7 group. In the heart, both the P2 and P7 injected mice exhibit a significant induction of SMN. The fold difference of the Western blots (n = 3 for each group) is shown as the average SMN/actin ratio after normalization of SMN to β-actin.

To answer the question of whether the robust expression observed in the P2 group compared to P7 was due to the fact that the virus was expressing five days longer in the P2 animals, as tissues from both groups were harvested on day eleven, we also harvested tissues from P2 injected animals on day 6 to account for this discrepancy (Figure 2.13).

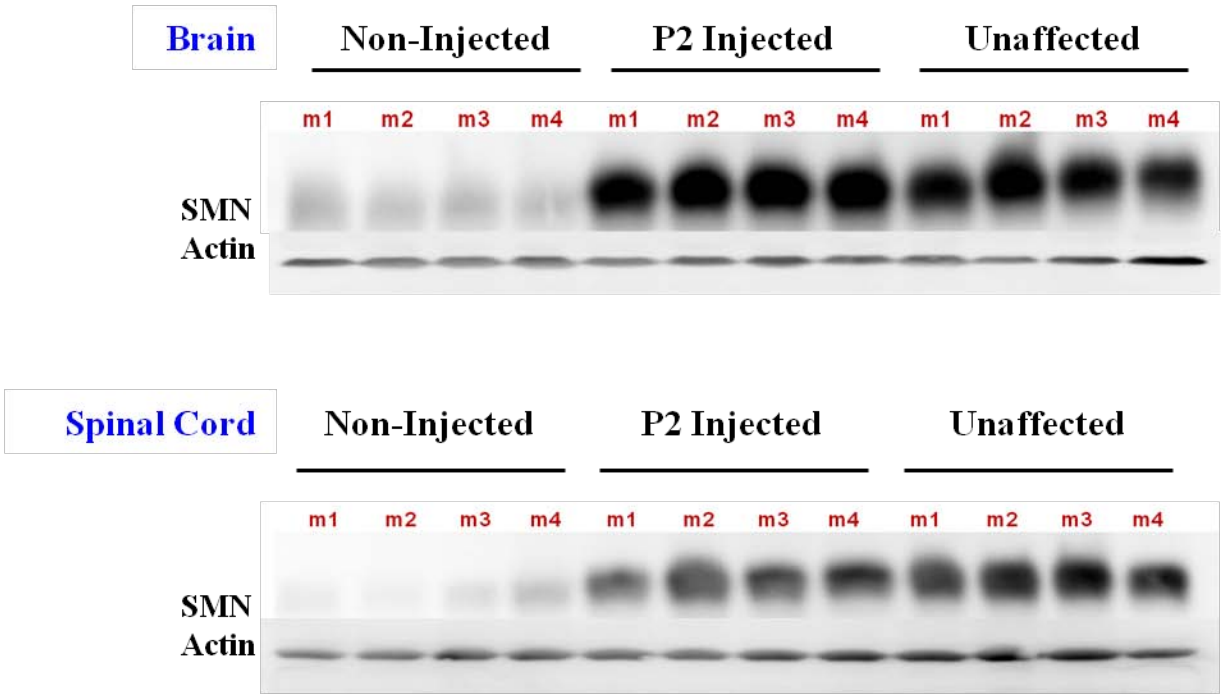

Figure 2.13. Robust SMN expression is observed four days post therapeutic P2 administration, confirming that equal protein induction intensity is consistent with the same time frame as P7 administration.

Peripheral Distribution of scAAV9-SMN and Transduction Analysis

To determine the extent to which scAAV9-SMN traverses the BBB and is distributed throughout the periphery we utilized the scAAV9-GFP vector to observe virus dispersion. Unaffected mice were injected with scAAV9-GFP on P2 or P7 and the heart, liver, spleen and kidney tissues were harvested on day six or day eleven (Figure 2.14). Results confirm that scAAV9 is well distributed throughout the periphery with especially strong GFP expression in the liver and heart tissues.

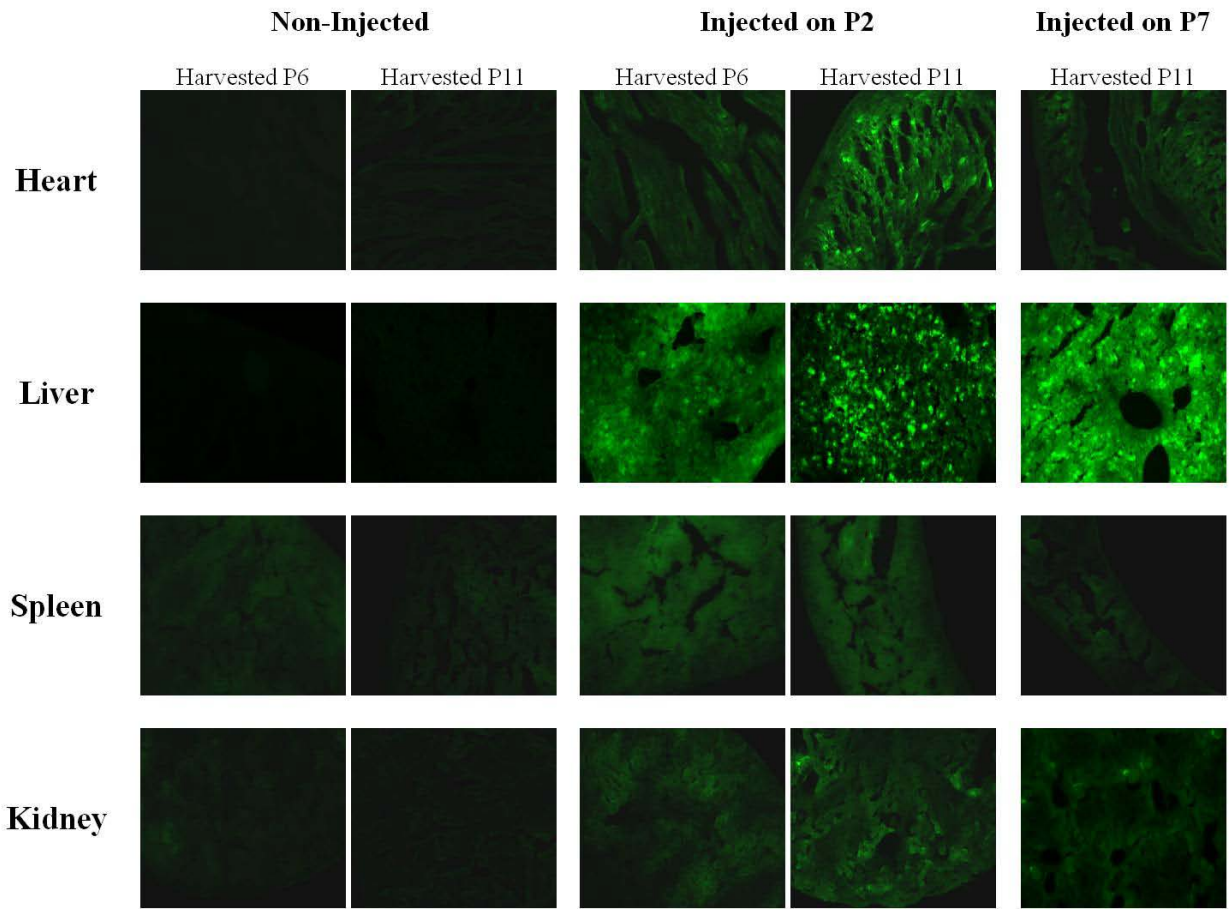

Figure 2.14. Unaffected mice injected with scAAV9-GFP exhibit substantial GFP expression in the periphery. In animals injected on P2, a stronger visualization was present throughout all peripheral tissues whereas, in P7 injected animals GFP was expressed the strongest in the liver.

Discussion

Timing of therapeutic administration is critical for many diseases, and especially for SMA. The majority of infants not diagnosed at birth present with acute respiratory failure within the first year. By this time, the patients are severely compromised, whereas, an early diagnosis would lead to proper nutritional, therapeutic and even respiratory support, which would improve the quality of life for SMA children. Multiple reports have demonstrated that early intervention provides maximal benefit regardless of the mode of therapy: SMN replacement or modulation of SMN2 splicing. Restoring SMN pre- and post-symptomatically has led to complete or partial rescue of SMA mice when SMN is reinstated during embryonic or early postnatal development.

Gene therapy using self-complementary adeno-associated virus, serotype 9 (scAAV9-SMN), to replace SMN, has shown promising results in that it rescues the SMNΔ7 mouse model when administered within the correct time frame [81, 85]. As SMA is namely a motor neuron disease, the scAAV9 vector is efficient at transducing a large proportion of the target cell population. scAAV9-SMN administration to SMNΔ7 mice intravenously on P1 or P2 resulted in a full rescue (survival >250d) but delaying delivery until P5 decreased survival (median ~25d), and treatment on P10 had no effect [81]. Comparison of IV and ICV delivery show that route of administration plays a role in suppressing disease progression. In the severe *SMN2* mouse model, ICV injection significantly extended the survival when compared with IV administration. In the SMNΔ7 model both injection routes resulted in a complete rescue but the ICV group experienced fewer early deaths [85, 86].

Transgenic mice harboring an inducible *Smn* allele have been generated to analyze temporal requirements of wild-type SMN levels. Early induction provides a more substantial

rescue compared to delayed induction, however, reinstatement during embryonic development (E6) completely ameliorates the disease phenotype.

Antisense technology is utilized in SMA research to increase SMN protein levels by modulating SMN2 splicing. For example, use of antisense oligomers (ASOs) to block an intron splice silencer, ISS-N1, has been demonstrated to significantly increase full-length *SMN2* transcript levels and SMN protein in brain and spinal cord of the SMNΔ7 model [77, 87]. When the ISS-N1 ASO was administered by ICV injection on P0, the survival was extended from 15 to 100 days. However, this group found that delaying delivery until P4 decreased the efficacy of the antisense therapy. Furthermore, IV administration on P0 resulted in survival comparable to the ICV group however, when delivery was delayed until P4, the IV injected group exhibited decreased survival.

Taken together, these developments all support the idea that early intervention provides the maximal benefit for these modes of therapy. Here we have provided an in-depth analysis to investigate temporal requirements for SMN protein. Multiple time points during the postnatal period were chosen for analysis in which SMNΔ7 mice were injected with $1x10^{11}$ v.g. scAAV9-SMN by ICV injection on a single day, P2 through P8.

We observed that survival, weight gain and muscle strength correlated with timing of administration, and that mice injected at earlier time points lived longer, gained more weight and performed better on the motor function test than mice injected at later time points (Figure 2.15). All groups lived significantly longer than the P8 injected group and non-injected controls. Interestingly, mice injected on P2, P3 and P4 lived significantly longer (median: 157, 102, 142 days, respectively) than those injected on P7 (median: 30 days). Despite the more modest effect

observed with the P5 and P6 groups, each group had one mouse that lived past 200 days. Furthermore, groups P2, P3 and P4 did not differ significantly from each other in average weight gained, but mice from these earliest time points gained significantly more weight than all other treated mice, and all groups gained significantly more weight than mice from the non-injected group. Mice in all treatment groups (excluding P7) experienced significant weight gain compared to P8 injected mice.

We have performed a systematic analysis to investigate the effect of delaying therapeutic intervention using scAAV9-SMN with direct CNS administration. We utilized the SMNΔ7 mouse model because it exhibits a severe SMA phenotype but lives 13 days [56], allowing enough time for investigation. Also, we used the self-complementary AAV vector because it transduces cells efficiently [82, 83], especially motor neurons [84], and induces expression relatively quickly (within 48 hours) [81]. SMN under the control of the chicken-β-actin promoter results in ubiquitous expression in the CNS and peripheral tissues [96]. In the investigation described here we assessed the phenotype of scAAV9-SMN treated mice using assays widely accepted in the field.

While others have demonstrated that IV injection of scAAV9-SMN provides a complete rescue in SMNΔ7 mice [81, 85], here we have performed ICV injection for direct delivery into the CNS, which provided the same result. However, delaying administration by the intravenous route provided a less substantial rescue [81] than when ICV administration was used, as we have described here. Furthermore, ICV delivery requires less virus to be used, which is translationally important for clinical consideration because virus production is costly. Perhaps in the case of SMA, a combination of virus delivery through both injection routes may demonstrate to be a

more efficient means of gene therapy since peripheral tissues are proposed to be affected by low levels of SMN as well as motor neurons.

The idea that early intervention provides maximal benefit was further supported by the performance of these mice on the time-to-right test. By day 12, over 50% of mice in groups P2, P3 and P4 were able to right themselves whereas, only 10-30% of mice in the late injection groups could perform this test on day 12. Protein induction observed in CNS (robust) and peripheral (modest) tissues of treated mice lends us to suggest that the resulting extent of rescue observed in these mice is due, in part, to increased SMN levels in the CNS. Collectively these data support the importance of early therapeutic intervention and demonstrate that a narrow therapeutic window exists for which therapy must be applied to provide maximal benefit.

This systematic investigation into the effect of delaying therapeutic intervention using scAAV9-SMN in the SMNΔ7 mouse model, has laid the foundation for others to elucidate the correlation between our observations of the overt phenotype of these mice with the molecular attributes involved in SMA pathogenesis. The results from this study support the idea that early intervention provides the maximal benefit for ameliorating SMA disease pathogenesis and this is clinically relevant as the field moves forward with designing therapeutics suitable for SMA patients in the near future.

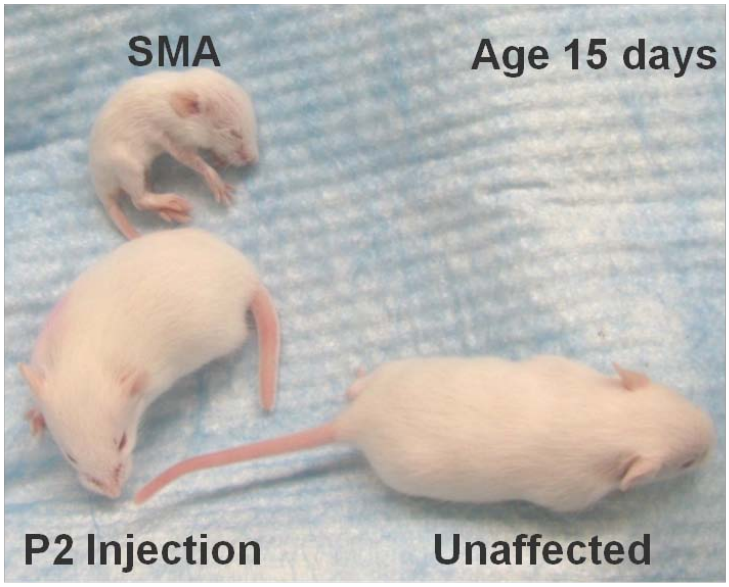

Figure 2.15. Overt phenotype of P2 treated animal with scAAV9-SMN compared to unaffected and non-injected littermates.

CHAPTER THREE: CONCLUSIONS

As described throughout the literature and clinical assessments, early therapeutic intervention provides maximum benefit in SMA patients and animal models. It is still unclear as to the direct progression of SMA disease pathology as to whether direct motor neuron degeneration causes SMA entirely or whether low SMN levels results in decline of peripheral organs and tissues which has a negative impact on motor neurons. Differential strategies have been implemented to further elucidate the biomolecular mechanisms occurring during SMA pathogenesis and each provides insight to answer these questions.

We have provided a systematic and detailed analysis to determine the timing in which the therapeutic window of providing maximal benefit closes, in the context of SMA. While intervention at the earliest stages of postnatal development provides the most robust rescue with a significant extension of survival, we also observed a reduced degree of rescue in the mice injected at the later time points. While the majority of mice injected on P2 lived a full lifespan, only a small cohort of mice from treatment groups P3 through P6 exhibited a full extension of survival; with administration at P7 only providing a modest extension and P8 only provided minimal benefit. Furthermore, the variability observed in weight gain and motor function following treatment at the various time points suggests there is a progressive decline in these animals following initiation of symptoms and that in order for therapeutic intervention to provide a maximal benefit, intervention must be applied before disease symptoms progress too far. Whether the target cell population is lost or too impaired to respond to treatment during late

stages of the disease has yet to determined, but with the advancement in gene therapy and now, RNA-based therapy (ASOs), we will gain a better understanding of SMA disease progression.

Endeavors focused to understand how low SMN levels translate into SMA disease pathology will be a critical component of future research in the SMA field. Development of intermediate SMA mouse models will aid in the elucidation of important biochemical and pathological aspects of SMA disease pathology. These studies can lead to reevaluation of the timing effects and advance the investigations into the impact of delayed therapeutic intervention. Furthermore, a combination of pharmacologics and gene therapy may provide a more substantial effect upon therapeutic intervention [33].

Importantly, investigation into the genetic and molecular basis of SMA has provided further insight on disease progression for other neurodegenerative disorders such as ALS and Alzheimer's, Huntington's and Parkinson's disease. Furthermore, the field of SMA research has widely contributed to, as well as gained insight from, the multitude of other neuromuscular diseases such as Muscular Dystrophy.

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