AAV-based approaches to model and treat spinal muscular atrophy

DISSERTATION

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By

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Abstract

The discovery of AAV9’s ability to transduce motor neurons along the entire length of the spinal cord has the potential to revolutionize the field of gene therapy. The most obvious disease to treat with this new technology is spinal muscular atrophy (SMA), a monogenetic disease where spinal motor neurons are the primary site of pathogenesis. Additionally, SMA is a good choice for gene therapy since all patients express at least some SMN, thereby decreasing the concern of anti-transgene immune responses. Currently, there is no therapy for children with SMA besides respiratory support and palliative care, so new therapies for this disease are desperately needed.

After showing remarkable success in treating a severe mouse model of SMA, we have focused on answering the most important questions regarding how to best translate this potential therapy to the clinic. We have determined the age-related window of opportunity for targeting spinal motor neurons and assessed toxicity and safety of the proposed clinical doses in mice and nonhuman primates. In the course of our studies we discovered the surprising finding that SMA mice have a severe heart failure phenotype. Thus increased cardiac surveillance in SMA clinical trial patients is warranted.

While the severe form of SMA is also the most common, we sought to assess an alternate muscle-enhancing therapy that successfully increased strength in a less-severe mouse model of SMA. Finally, we have created a model that accurately models less-severe
SMA by using AAV9 to deliver a mouse-specific shRNA against Smn. This technique has allowed us to control the amount of Smn produced throughout the spinal cord more tightly than has been accomplished before, yielding a reliable model in which the full spectrum of SMA can be accurately modeled. Together, the data we have collected have helped to guide the design of the first-in-human clinical trial of AAV9, which will push the boundary of AAV-based medicine farther than any single trial has before.
Dedication

This document is dedicated to my sweet wife,

Maury,

and to my wonderfully spirited daughters,

Abigail, Molly and Elinor.
Acknowledgments

I give special thanks to my Advisor, Dr. Brian Kaspar, for spending so much of his limited time to mentor me. I will always be grateful for the perspective and focus he helped me gain during my time in his laboratory.

The majority of the work presented herein was performed in very close collaboration with Dr. Kevin Foust. The majority of what I have learned about scientific discovery and communication was taught to me by Dr. Foust during and after his time in the Kaspar Laboratory.

Additionally, I would like to thank Nature Publishing Group and Oxford University Press for allowing me to include data in this dissertation that was originally published in their journals Nature Biotechnology, Human Molecular Genetics, and Molecular Therapy.
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Major Field: Integrated Biomedical Science Program
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Chapter 1: Introduction to spinal muscular atrophy and modern gene therapy

Spinal muscular atrophy

Proximal spinal muscular atrophy (SMA) is a devastating disease affecting approximately 1 in 10,000 newborns worldwide [1]. Patients with this disease most often present with rapid onset of weakness prior to 6 months of age, although there is a spectrum of disease severity [2]. The weakness is due to a loss of innervation of skeletal muscle, with the axial and proximal limb muscles being more severely affected.

Figure 1 Schematic of the long arm of chromosome 5. Region 5q13 is the site of an inverted repeat of ~500 kb. The SMN genes reside within this region. The key difference between SMN2 and SMN1 is a C > T mutation in SMN2, which disrupts correct splicing and decreases the amount of full-length product (FL-SMN) to ~10-20% of what is produced by SMN1.

The gene responsible for SMA was identified in 1995, and it was named the survival motor neuron (SMN) gene [3]. It was discovered that humans have two versions of this gene due to an evolutionarily late duplication on chromosome 5q13. The telomeric copy
(SMN1) and the centromeric copy (SMN2) encode for the same protein, but they vary greatly in the quantity of full-length product that is produced [2, 4]. The SMN1 gene produces almost 100% full-length SMN, but about 80-90% of the SMN2 product is missing exon 7 (Δ7SMN), which is rapidly degraded and is only mildly beneficial [5, 6]. The cause of this discrepancy was mapped to a single, silent C>T mutation in the exon of SMN2. This mutation disrupts an exonic splicing enhancer (ESE) region and creates an exonic splicing silencer (ESS) sequence, resulting in decreased exon recognition by splicing machinery [7-9].

SMA severity is predicted by SMN2 copy number

Patients with homozygous deletions or mutations in SMN1 then rely solely on the remaining SMN2 gene, which produces sufficient SMN for almost all cell types throughout the body. However, for reasons still not understood, lower motor neurons of the spinal cord require more SMN than is normally produced from SMN2 [10]. The most severe presentation of SMA is in patients with severe mutations and only 1 copy of SMN2. This form, known as SMA Type 0, often presents prenatally and patients rarely survive more than a few weeks after birth. Fortunately, this form is also very rare. In the most common form of SMA, patients have 2 copies of SMN2, which leads to onset of severe weakness prior to 6 months of age and almost 100% mortality or ventilator dependence by 2 years old. This common form is termed SMA Type 1, and accounts for ~70% of SMA. About 30% of SMA patients, however, have more copies of SMN2 or less-severe mutations that produce more full-length SMN, and therefore have a milder form of the disease. SMA Type 2 patients typically have 3 SMN2 copies and can sit up on
their own, but will never be able to walk, and Type 3 patients can have 3-5 copies of \( SMN2 \), and may be able to walk until late in childhood or even well into their adult years. More rare forms of SMA include patients with 5 or more copies of \( SMN2 \), known as Type 4 or Type 5 SMA and will not experience weakness until later in adulthood [2, 11-13].

\textit{SMA patients desperately need therapeutic options}

While the genetic cause of SMA was discovered 17 years ago, the only advances in SMA therapy have been in providing respiratory support and combating pulmonary complications associated with weakness [14]. Until 2009, no therapy had been able to alter the course of the disease in patients or in mouse models. The most successful preclinical study used the histone deacetylase (HDAC) inhibitor, trichostatin-A (TSA), to indiscriminately increase transcription with the hope of creating more \( SMN2 \)-derived products, including full-length SMN [15, 16]. When applied to the most widely used mouse model of SMA, mean lifespan was increased from 14 days to 38 days. While the efficacy of TSA treatment was unprecedented, surviving mice developed severe necrosis throughout the body, perhaps by unmasking an underlying unintended phenotype in the mouse model. Regardless, the compound has a very narrow therapeutic window and translation to the clinic was never pursued. Similar compounds such as phenylbutyrate, valproic acid, and hydroxyurea have shown lesser benefit in SMA mice and yielded no significant improvement in SMA patients [14].
Adeno-associated virus (AAV)

Discovery and initial characterization of AAV

In 1965, two groups studying adenovirus independently made an advantageous discovery [17, 18]. They noticed small contaminating DNA viruses in adenovirus preparations that only replicated in the presence of adenovirus. Soon after, AAV was also isolated from human samples, but it only seemed to travel along with adenovirus and herpesvirus, not actually cause any human disease at all [19].

This lack of clinical impact is extremely advantageous to the idea of clinical gene therapy, but in the 1960s, it was likely difficult to imagine how studying a non-pathogenic replication defective AAV particle might be advantageous. This may account for the initial lag in AAV interest [20], but by the 1970s, characterization of AAV was well underway.

The first descriptions of AAV’s molecular genetics came from the research of Lionel Crawford, Jim Rose and Ken Burns, who showed that AAV preparations contained complementary single-stranded DNA that would associate into double stranded molecules when released into target cells [21, 22]. This soon led to the very important discovery of AAV’s inverted terminal repeats (ITRs), their role in self-priming replication, and the helper role adenovirus plays in the AAV lifecycle [23-29]. By mutational analysis, the trans-acting function of the two AAV genes, Rep and Cap, were also elucidated, thereby first defining the natural payload of AAV to be ~4.5Kb, with only the ITRs needed in cis for proper packaging [30-32].
Recombinant AAV can be used a molecular tool

In 1982, the first recombinant adeno-associated viral (AAV) particles were produced, soon followed by the first proof that mammalian cells could be infected by recombinant AAV and express foreign transgenes. These initial vectors were created by cotransfection of kidney blastoma (KB) or human embryonic kidney (HEK)-298 cells with a plasmid containing the ITR sequences flanking the gene of interest and a plasmid containing the AAV Rep and Cap genes in trans, and infecting these cells with wild type adenovirus to activate AAV’s p5, p19 and p40 promoters [33-39].

These initial viral preparations were crude cell lysates that contained contaminating proteins and newly made adenovirus. The adenovirus could be heat inactivated, but further purification was needed [20]. Much of the initial purification was performed using CsCl density gradients, and this is still a common practice today for research grade product [40].

There were drawbacks to CsCl purification methods, however, such as the potential danger of contaminating preparations with Cesium or purified vector losing potency if exposed too long to CsCl [41]. Methods were developed for further purification, including many different varieties of column chromatography, which greatly increased the purity of resulting viral preparations [42, 43]. In 1999, two groups developed an alternative purification strategy using iodixanol, an FDA-approved contrast agent, as a gradient material [41, 44]. This method yielded very clean viral preparations, and it remains a preferred method for clinical virus preparations today.
AAV as a gene therapy vector

The fact that it is not associated with human disease, and other unique aspects of its lifecycle supported the hypothesis that AAV would be effective as a gene therapy vector to treat human disease [45]. The first application to human disease was in cystic fibrosis, the most common genetic disease, in which patients have a mutated cystic fibrosis transmembrane regulator (CFTR) gene, as others had done using other viral vectors such as adenovirus and retrovirus [46, 47]. First, AAV was used to reintroduce the CFTR gene into cell lines from cystic fibrosis patients [48, 49]. Soon thereafter the first in vivo experiment was performed in rabbits, in which the CFTR transgene was successfully expressed for 6 months [50]. This finding quickly launched the field forward, and the first primate safety trial was completed in 1996 [51].

AAV-based human gene therapy was quickly becoming a reality, leading to the first human clinical trial in 1996 in mild cystic fibrosis patients [52]. However, after a patient died in the infamous ornithine transcarbamylase (OTC) trial using adenovirus, gene therapy progress slowed to a crawl until more stringent standards were agreed upon and AAV vectors had more time to be proven safe in pre-clinical studies. But field has recovered quickly. In 2004, the first phase II clinical trial using AAV, again for cystic fibrosis, was underway. At that time, a total of 11 AAV gene therapy protocols had been approved. In 2008, that number increased to 38, and by the beginning of this year (2012) 86 clinical AAV protocols had been approved worldwide [53-55]. Many of these trials have been quite successful, such as the Leiber’s congenital amaurosis trial, and others,
while being somewhat disappointing, serve as important lessons that continue to guide how gene therapy clinical trials should be designed [53-54].

**AAV serotypes expand the capabilities of gene therapy**

Almost all of the initial characterization of AAV was using DNA derived from serotype 2 until the 1990s [20]. However, the subsequent discovery and characterization of other serotypes has extended the possibilities of gene therapy since each serotype has its own tropism and ability to travel through different compartments in the body [56-61]. For example, AAV1 is particularly good at being retrogradely transported by neurons, and AAV8 and AAV9, when delivered intravascularly, are able to escape the vasculature and transduce striated muscle with high efficiency [62-64]. An almost infinite arsenal of naturally-occurring AAV serotypes remains to be characterized, and new useful gene therapy candidates continue to be unveiled.

**The blood-brain barrier stifled early gene therapy in SMA**

The blood-brain-barrier poses a significant challenge to any new therapeutic strategy targeting the central nervous system (CNS), and this barrier has proven itself to be particularly formidable for gene therapy. Direct injections into nervous tissue for widespread targeting, such as would be required to treat SMA, would require multiple injections into very sensitive neuronal tissue, making it an impractical solution. Therefore, other strategies to circumvent the blood-brain-barrier need to be used. One group used a rabies pseudotyped lentiviral vector to introduce the SMN gene via retrograde transport after muscular injection [65, 66]. However, this resulted in only a 3-5 day increase in survival, likely due to either to insufficient numbers of targeted motor
neurons or toxicity of the vector. Others had used various serotypes of adeno-associated virus to target motor neurons by either retrograde delivery or by intravenous injection, but the low percentage of transduced motor neurons across the blood-brain-barrier made success unlikely if applied to SMA [67].

*AAV9 overcomes the blood-brain barrier in mice*

Fortunately for SMA patients and neuroscientists everywhere, our group discovered that a particular serotype of AAV (AAV9) was able to cross the blood-brain barrier after intravenous injection in mice [68]. In particular, a single injection in the peripheral vasculature was sufficient to transduce >70% of motor neurons throughout the entire spinal cord. The most suitable application of this exciting new method of gene delivery was to determine whether postnatal replacement of SMN by systemically delivered AAV9 would be sufficient to rescue a model of SMA.

*Preclinical studies show efficacy and safety of AAV9-based gene therapy*

When I joined the Kaspar laboratory, it had already been shown that AAV9 could deliver transgene to spinal motor neurons in neonatal mice, however the following questions remained:

1. Can AAV9 target sufficient motor neurons to rescue a rapid progressing mouse model of SMA?
2. Can postnatal SMN replacement positively impact SMA pathogenesis in SMA mice?
3. What is the time window for efficient transduction of motor neurons by AAV9 in mice?
3. Can AAV9 similarly infect motor neurons in larger species (i.e. nonhuman primates)?

4. Is the safety profile of high dose of AAV9 that is required for motor neuron transduction sufficient to justify clinical translation?

In the course of answering the above questions, I discovered that SMA mice have a severe cardiac phenotype that was previously uncharacterized. The unexpected findings of this study (described within) provide sufficient rationale for monitoring cardiac status in future SMA clinical trial patients and have helped to guide our clinical trial design.

*AAV9-based approaches for treating and modeling mild SMA*

Realizing that many SMA patients with milder disease (i.e. type 3) might not receive be good candidates for AAV9-SMN therapy, at least not in the initial trial, we hypothesized that enhancing muscle mass and strength may be of benefit to this subset of SMA patients. Previously, our laboratory demonstrated robust muscle enhancement in mice and nonhuman primates from AAV-delivered transgenic follistatin [69]. We similarly applied this approach to a new model of mild SMA, the C/C mouse [70].

While follistatin treatment of C/C mice showed significant benefit, the shortcomings of the C/C model urged us to attempt a unique vector-based strategy to more carefully modulate the levels of Smn to create a more robust model of mild SMA. The resulting mice have remarkably similar deficits to SMA Type 3 patients, and this model may be used for future studies aimed at developing therapies specifically for less-severe patients. These studies outline a clear path forward that has led the cusp of the first in human clinical trial. The unprecedented efficacy and safety of this relatively aggressive gene
therapy approach leaves us confident that AAV9-SMN therapy is likely to be successful in treating Type 1 infants. Furthermore, the widespread impact of AAV9 technology on understanding and treating neurological disease is just beginning to be realized.

References


Chapter 2: Rescue of a Spinal Muscular Atrophy Mouse Model by Early Postnatal scAAV9-SMN Delivery

This chapter has been published in the peer-reviewed journal Nature Biotechnology.

This work is the result of collaboration with the authors listed below. I was involved with designing and performing experiments and writing the manuscript, especially the experiments regarding timing of vector delivery in mice and gene delivery to nonhuman primates.

Authors


Introduction

Proximal spinal muscular atrophy (SMA) results in motor neuron death in the spinal cord. SMA is caused by loss of survival motor neuron gene 1 (SMN1) and retention of SMN2 resulting in reduced levels of SMN, a ubiquitously expressed protein important in the assembly of RNP complexes [1, 5-7]. Neuronal expression of SMN appears essential [8]. Recent work using a mouse model of SMA showed that postnatal lentiviral mediated delivery of SMN to motoneurons produced a 3-5 day increase in survival in a model that normally survives ~13 days [9]. Pharmacological approaches increased survival up to ~40 days [10, 11]. We, and others, recently demonstrated that intravenous injection of
self-complementary adeno-associated virus 9 (scAAV9) into one day old (P1) mice and cats infects ~60% of motoneurons after intravenous injection, indicating the potential for this approach in treating SMA [2, 12]. Here, we report that scAAV9-SMN mediated gene replacement in SMA mice results in an unprecedented improvement in survival and motor function [13]. We also demonstrate that scAAV9-GFP crosses the blood-brain-barrier in non-human primates transducing motoneurons, thus indicating the possibility of translating this treatment option to human patients.
Figure 2 Phenotypic correction within SMA mice. (a) scAAV9-GFP in SMA animals results in GFP expression within dorsal root ganglia and motoneurons (ChAT staining in red) in the lumbar spinal cord 10-days post-injection. (b) Western blots from tissues of control, scAAV9-SMN treated and untreated SMA animals show elevated levels of SMN expression in SMN treated animals compared to control animals, but levels are still below that of control littermates. (c) Righting ability shows SMN treated animals could right themselves similarly to control animals by P13. (d) SMA animals treated with scAAV9-SMN are larger than GFP treated animals. (e) scAAV9-SMN treatment of SMA animals results in greatly extended survival. (f) scAAV9-SMN treated animals have greater body mass versus GFP treatment. Scale bars, 200μm (a); 50μm (a inset)
Results

To determine transduction levels in SMA mice (SMN2+/+; SMNAΔ7+/+; Smn-/-), we injected 5x10^{11} genomes of scAAV9-GFP or -SMN (n=4/group) under control of the chicken-β-actin hybrid promoter into the facial vein at P1. We found 42+/-2% of lumbar spinal motoneurons expressing GFP (**Figure 2a**) 10 days post injection. The levels of SMN in the brain, spinal cord and muscle in scAAV9-SMN-treated animals are shown in (**Figure 2b**). SMN levels were increased in brain, spinal cord and muscle in treated animals, but were still less than controls (SMN2+/+; SMNAΔ7+/+; Smn+/-) in neural tissue (**Figure 3**). Spinal cord immunohistochemistry demonstrated expression of SMN within choline acetyl transferase (ChAT) positive cells after scAAV9-SMN injection.

![Graph showing SMN levels in different tissues](image)

**Figure 3** Quantifications of western blots from Figure 2. Bands across treatment groups were averaged and compared with their respective loading controls. The normalized averages were compared to control animals and graphed.
We next evaluated whether scAAV9-SMN treatment of SMA animals improved motor function [14]. SMA animals treated with scAAV9-SMN or -GFP were evaluated for the ability of the animals to right themselves compared to control and untreated animals (n=10/group). We found that control animals could right themselves quickly, whereas the SMN- and GFP-treated SMA animals showed difficulty at P5. However, by P13, 90% of SMN treated animals could right themselves compared to 20% of GFP-treated controls and 0% of untreated SMA animals, suggesting that SMN treated animals improved (Figure 2c). Evaluating animals at P18 showed SMN treated animals were larger than GFP treated but smaller than controls (Figure 2d). Locomotive ability of the SMN-treated animals were nearly identical to controls as assayed by x, y and z plane beam breaks (open field testing) and wheel running (Figure 4). Age-matched untreated SMA animals were not available as controls for open field or running wheel analysis due to their short lifespan.

Figure 4 SMA animals treated with scAAV9-SMN performed similarly to control littermates in open field testing. Total beam breaks in the X (a), Y (b) and Z (c) planes are graphed between 13 and 30 days of age.
We next evaluated survival in SMN-treated SMA animals (n=11) compared to GFP-treated SMA animals (n=11). No GFP treated control animals survived past P22, with a median lifespan of 15.5 days (Fig. 1e). We analyzed body weight in treated SMN- or GFP-treated animals compared to wild-type littermates. The GFP-treated animals’ weight peaked at P10 and then precipitously declined until death. In contrast, SMN treated animals showed a steady weight gain to approximately P40, where the weight stabilized at 17 grams; ½ of the weight of controls (Fig. 1f). The smaller size of corrected animals is likely related to the tropism and incomplete transduction of scAAV9 resulting in a “chimeric” animal, meaning some cells are still diseased. Additionally, the smaller size also suggests an embryonic role for SMN. Surprisingly, no deaths occurred in the SMN-treated group until P97. Furthermore, this death appeared to be unrelated to SMA. The mouse died after trimming of long extensor teeth. We euthanized 4 animals (P90-99) for electrophysiology of neuromuscular junctions (NMJ). The remaining six animals are still alive and surpassing 250 days of age.

A recent report demonstrated that neuromuscular transmission was abnormal in SMA mice [15]. To determine whether the reduction in endplate currents (EPCs) was corrected with scAAV9-SMN, we recorded EPCs from the tibialis anterior (TA) muscle[16]. P9-10 animals were evaluated to ensure the presence of the reported abnormalities within our mice. Control mice had an EPC amplitude of 19.1 ± 0.8 nA versus 6.4 ± 0.8 nA in untreated SMA animals (p=0.001) confirming published results [15]. Interestingly, P10 scAAV9-SMN-treated SMA animals had a significant improvement (8.8 ± 0.8 vs. 6.4 ± 0.8 nA, p<0.05) over age-matched untreated SMA animals. However, gene therapy
treatment had not restored normal EPC at P10 (19.1 ± 0.8 vs. 8.8 ± 0.8 nA, p=0.001). At P90-99, there was no difference in EPC amplitude between controls and SMA mice that had been treated with scAAV-SMN (Figure 5a). Thus, treatment with scAAV9-SMN fully corrected the reduction in synaptic current. Importantly, P90-99 age-matched untreated SMA animals were not available as controls due to their short lifespan.

Figure 5 Adult NMJs were electrophysiologically examined to determine whether SMN-treated SMA mice NMJs gained full maturity. At this point, untreated SMA mice are dead. (a) scAAV9-SMN treatment restores endplate currents (EPC) in ~90 day old SMA animals. In control mice mean EPC amplitude was 82.6 ± 3.5 nA, and in treated SMA mice it was 83.4 ± 4.1 nA (p = .89, n = 4 mice for each group). (b) Affected animals treated with scAAV9-SMN had an increase in miniature endplate currents. (c) Both control and treated SMA endplate currents had a similar degree of depression during 50 Hz nerve stimulation. (d-i) Representative sections from the transverse abdominis (TVA), a proximal muscle with innervation abnormalities in SMA mice, shows normal innervation in both wild type (d-f) and SMN treated (g-i) animals. Scale bars, 10 μm.
The number of synaptic vesicles released following nerve stimulation (quantal content) and the amplitude of the muscle response to the transmitter released from a single vesicle (quantal amplitude) determine the amplitude of EPCs. Untreated SMA mice have a reduction in EPC due primarily to reduced quantal content [15]. In our P9-10 cohort, untreated SMA animals had a reduced quantal content when compared with wild-type controls (5.7 ±0.6 vs. 12.8 ±0.6, p<0.05), but scAAV9-SMN treated animals were again improved over the untreated animals (9.5 ± 0.6 vs. 5.7 ± 0.6, p<0.05), but not to the level of wild-type animals (9.5 ± 0.6 vs. 12.8 ±0.6, p<0.05). At P90-99, when we measured quantal content in treated SMA mice, a mild reduction was present (control = 61.3 ± 3.5, SMA treated = 50.3 ± 2.6, p < 0.05), but was compensated for by a statistically significant increase in quantal amplitude (Figure 5b, control = 1.39 ± .06, SMA treated = 1.74 ± .08, p < 0.05). Quantal amplitudes in young animals had no significant differences (control = 1.6 ± 0.1, untreated SMA= 1.3 ± 0.1, treated SMA = 1.1 ±0.1 nA, p=0.28).

The reduction in vesicle release in untreated SMA mice was due to a decrease in probability of vesicle release, demonstrated by increased facilitation of EPCs during repetitive stimulation [15]. Both control and treated SMA EPCs were reduced by close to 20% by the 10th pulse of a 50 Hz train of stimuli (Figure 5c, 22 ± 3 % reduction in control vs 19 ± 1 % reduction in treated SMA, p = .36). This suggests that the reduction in probability of release was corrected by replacement of SMN. During electrophysiologic recording, no evidence of denervation was noted. Furthermore, all adult NMJs analyzed showed normal morphology and full maturity (Figure 5d-i). P9-10 transverse abdominis immunohistochemistry showed the typical neurofilament
accumulation in untreated SMA NMJs [15, 17-19], whereas treated SMA NMJs showed a marked reduction in neurofilament accumulation (Figure 6).

![Figure 6](image.png)

A recent study using an HDAC inhibitor to extend survival of SMA mice reported necrosis of the extremities and internal tissues [20]. In our study, mice developed necrotic pinna between P45-70 (Figure 7). Pathological examination of the pinna noted vascular necrosis, but necrosis was not found elsewhere. We previously demonstrated that vascular endothelium was among the cell types transduced after systemic scAAV9 delivery [2]. Lack of necrosis in the tail and hind-paws could be due to treatment of
vascular tissue, whereas the development of the pinna after P1 precludes correction of this tissue due to loss of recombinant vector genomes in dividing cells [21-23].

Figure 7 Necrosis of the ear pinna of a scAAV9-SMN treated mouse at P59. (a) SMN treated mice developed areas of necrosis on their ear tips between 40-70 days of age. (b) Biopsy of the affected tissue showed epidermal necrosis extending into the dermis (white arrow) while the intact epidermis is hyperplastic and edematous (black arrows). (c) High magnification examination demonstrated neutrophil infiltration (white arrow) and vascular necrosis (black arrow) with leukocytoclastic vasculitis (d, black arrow) and fibrinoid necrosis (d, white arrow).
To explore the therapeutic window in SMA mice, we performed systemic scAAV9-GFP injections at varying postnatal time points to evaluate the pattern of transduction of motoneurons or astrocytes. scAAV9-GFP systemic injections in P2, P5 and P10 aged mice showed distinct differences observed in the spinal cord. There was a shift from neuronal transduction in P2 treated animals towards predominantly glial transduction in older, P10 animals which is consistent with our previous studies and knowledge of the developing blood-brain-barrier in mice (Figure 8a-i) [2, 24]. To determine the therapeutic effect of SMN delivery at these various time points, small cohorts of SMA affected mice were injected with scAAV9-SMN at P2, P5 and P10 and evaluated for changes in survival and body weight (Figure 8j-k). P2 injected animals were rescued and indistinguishable from P1 scAAV9-SMN injected animals. However, P5 animals showed a more modest increase in survival, with a survival increase of ~15 days, while P10 animals were unchanged from GFP injected SMA pups. These findings support previous studies as to the importance of increasing SMN levels in neurons of SMA mice [8]. Further, these results suggest a finite period in development in which intravenous injection of scAAV9 can target neurons in sufficient numbers for benefit in SMA.

A key question for the translational of these findings to a clinical application is the ability of scAAV9 to traverse the blood-brain-barrier in non-human primates [25]. Therefore, we intravenously injected a male, P1 cynomolgus macaque with 1x10^{14} particles (2.2x10^{11} particles/g of body weight) of scAAV9-GFP and euthanized it 25 days post injection. Examination of the spinal cord revealed robust GFP expression within the dorsal root ganglia and motoneurons along the entire neuraxis (Figure 8l-q), as seen in
P1 injected mice. This crucial finding demonstrates that early systemic delivery of scAAV9 can efficiently target motoneurons in non-human primates.

Figure 8 Systemic injection of scAAV9-GFP into SMA mice of varying ages. Animals injected at P21 (a-c) have a transduction pattern identical to P1 injected animals with motor neuron transduction in lumbar spinal cord. P5 injected animals (d-f) have more glial transduction and less motor neurons (f inset, arrow) transduced than younger animals in lumbar spinal cord analysis. The pattern of increased glial transduction increases in P10 animals (g-i). GFP (green), ChAT (red, a motor neuron marker) and merged (yellow). scAAV9-SMN injection into P2 in SMA animals rescues lifespan and increases body weight (n=6) whereas P5 AAV9- SMN delivery in SMA animals imparts only partial rescue of lifespan and body weight (n=4) compared to control AAV9-GFP treated (n=10) (j-k). No increase in life-span or body weight was seen in Post-natal day 10 AAV9-SMN delivered animals (n=4). Systemic injection of scAAV9-GFP into a P1 cynomolgus macaque results in a similar transduction pattern within the spinal cord as previously demonstrated in P1 mice demonstrating translation in a larger species. GFP (l, o), ChAT (m, p) and merged (n, q) images from thoracic spinal cord demonstrate motoneuron transduction. A representative longitudinal section is shown in (l-n) indicating transduction along the neuraxis. Transverse sections (o-q) mimic the pattern of dorsal root ganglia and motoneuron transduction seen in P1 injected mice. Inset scale bars =50μm, c, f and i =100μm, n and q =200μm.
Conclusion

In conclusion, we report here the most robust, postnatal rescue of SMA mice to date with correction of motor function, neuromuscular electrophysiology, and increased survival following a one-time gene delivery of SMN. Intravenous scAAV9 treats neurons, muscle and vascular endothelium, all of which have been proposed as target cells for treatment [2]. While this study does not attempt to dissect the roles of different cell types in SMA, our P10 data demonstrates that SMN replacement in astrocytes is not effective to delay disease, consistent with transgenic approaches that have tested this [8]. Definitively, we demonstrate the potential efficacy of postnatal therapy. Our results begin to define a crucial window of opportunity for scAAV9 to target motoneurons within neonates. Due
to the accelerated development seen in mice, future studies in non-human primates are warranted to further elucidate the therapeutic window. Novel vector developments such as AAV capsid modification, mutagenesis or shuffling may further expand the opportunity to target neurons in the adult [26-28]. Although SMA children are often asymptomatic at birth, newborn screening that can detect SMA has been developed, thus supporting the ability to deliver scAAV9-SMN in human SMA neonates [29]. Additionally, our non-invasive delivery of AAV9 in non-human primates is the first report of widespread transduction within the spinal cord of a species more closely related to humans. It appears vascular delivery of scAAV9 in the mouse and non-human primate is safe, well tolerated, and that this gene delivery paradigm could be used to target the nervous system for other diseases. We are continuing to advance this delivery system in non-human primates to set the stage for human clinical trials of scAAV9-SMN in SMA.

References


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Methods

All animal procedures were approved by Nationwide Children’s Hospital Institutional Animal Care and Use Committee, Wright State Institutional Animal Care and Use Committee and Ohio State University Animal Care and Use Committee.

Animals

SMA parent mice (Smn +/-, SMN2 +/-, SMNΔ7+/+) were time mated. Cages were monitored 18-21 days after visualization of a vaginal plug for the presence of litters. Once litters were delivered, the mother was separated out, pups were given tattoos for identification and tail samples were collected. Tail samples were incubated in lysis solution (25mM NaOH, 0.2mM EDTA) at 90°C for one hour. After incubation, tubes were placed on ice for ten minutes then received an equal volume of neutralization solution (40mM Tris pH5). After the neutralization buffer, the extracted genomic DNA was added to two different PCR reactions for the mouse Smn allele (Forward 1: 5’-CCAGCTCCGGATATTGGGATTG, Reverse 1: 5’-AGGTCCCACCACCTAAGAAAGCC, Forward 2: 5’-TGCTCTGGGCTGTAGGCATTGC, Reverse 2: 5’-GCTGTGCTTTTTGGCTTATCTG)
and one reaction for the mouse Smn knockout allele (Forward: 5’-
GCCTGCGATGTCGTTTCTGTGAGG, Reverse: 5’-
CAGCGCGGATCGTCAAGC). After analysis of the genotyping PCR, litters were culled to three animals. Affected animals (Smn -/-, SMN2 +/-, SMNΔ7+/+) were injected as previously described with 5x10^{11} particles of self complementary AAV9 SMN or GFP [2].

*Ultrasound-guided intracardiac delivery of AAV9*

In older mice we utilized ultrasound-guided intracardiac injections to efficiently deliver the gene therapy vector. Animals were anesthetized using 1-2.5% isoflurane (in O_2 gas) throughout the procedure. Animals were secured with tape to a heated platform and fitted with a nose cone. A Vevo 2100 ultrasound was used to visualize the animal’s heart and monitor vitals. For AAV9-GFP injections into wildtype mice, p5 animals were injected with 7E+11 vector genomes (vg, in 70ul), p10 animals with 3.5E+11vg (in 70ul), into the left ventricle. Affected mice were similarly injected with AAV9-SMN at p5 and p10 with 3E+11vg (in 60ul). Upon successful vector delivery, the animals were monitored for signs of cardio-pulmonary distress, disconnected from the anesthetic machine and placed it in an oxygen recovery chamber for a short period of time prior to returning it to its cage.

*Non-human Primate Subjects*

The selected experimental subject was a 1-day old Cynomolgus macaque (*Macaca fascicularis*) born in December 2009, at The Mannheimer Foundation, Inc., an AAALAC International-accredited facility. The dam and sire of this neonate were both part of the
Cynomolgus breeding colony at the Foundation, housed in an outdoor enclosure. All Cynomolgus macaques at the Foundation are fed a commercial diet (Harlan / Teklad 2050), supplemented with seeds, fruits/produce and provided with fresh water *ad libitum*. All uses and procedures were approved and in accordance with the Institutional Animal Care and Use Committee (IACUC) of the Mannheimer Foundation, Inc. Both dam and newborn were negative for selected retroviruses (Simian Immunodeficiency Virus, Simian Retrovirus type D and Simian T-Cell Lymphotropic Virus type 1) and B-virus (*Cercopithecine Herpes Virus 1*).

**AAV9 administration to non-human primates**

As soon as the newborn subject was identified and selected in the outdoor enclosure, it was brought into an indoor hospital room on the same birth-day, and pair-housed along with its dam. Within 24 hours of the birth event, and just prior to the AAV9 delivery, both dam and newborn were sedated with Ketamine HCL (at a dose of 10 mg/kg IM) and briefly separated from each other to perform the initial procedures. Baseline blood samples were collected from both dam and newborn via femoral venipunctures. After blood samples were collected, the dam was placed back in its cage; the newborn was positioned on sternal recumbency, and one of its legs shaved/disinfected in preparation for the intravenous injection of the AAV9. A 24-gauge IV catheter was placed into the saphenous vein. Vector solution was drawn into a 12 cc syringe pre-wetted with saline solution (0.9% NaCl). The IV catheter was flushed and its patency verified by using approximately 1 cc of the same saline solution. A total volume of 10 cc of the AAV9 was delivered intravenously (medium dose of 1-5E+12 vector genomes per gram, vg/g) in a
bolus fashion. After injection, the newborn was recovered in a controlled temperature
islette (set at 95°F) and returned to its dam after full recovery was achieved.

Perfusion-Fixation procedures and organ collections

25 days post-AAV9 injection dam and infant were accessed once more by sedation with
Ketamine HCL (at a dose of 10 mg/kg) and Telazol (at a dose of 5 mg/kg) respectively. A
follow-up blood sample was collected from the dam; the infant was separated and taken
to the necropsy room for its terminal collections and procedures. A 24-gauge IV catheter
was placed into a saphenous vein in order to facilitate subsequent doses of the anesthetic
drug and the delivery of the euthanasia solution. Once the infant was confirmed to be in a
deep anesthetic plane, a blood sample was collected and then both thoracic and
abdominal cavities were exposed to proceed with the perfusion process. A 20-gauge
needle was inserted intra-cardially into the left ventricle, the right auricle was sectioned
and the perfusion started first with 0.9% NaCl, and then with 4% paraformaldehyde
solution. A total of approximately 1.5 liters of the solution were perfused via gravity
flow. Upon completion of the perfusion, several sections of major organs were harvested
and fixed by immersion using the same paraformaldehyde solution.

Viral Vector

AAV9 was produced by transient transfection procedures using a double stranded AAV2-
ITR based CB-GFP vector, with a plasmid encoding Rep2Cap9 sequence as previously
described along with a adenoviral helper plasmid; pHelper (Stratagene, La Jolla, CA) in
293 cells. Our serotype 9 sequence was verified by sequencing and identical to that
previously described. Virus was purified by two cesium chloride density gradient
purification steps, dialyzed against phosphate-buffered saline (PBS) and formulated with 0.001% Pluronic-F68 to prevent virus aggregation and stored at 4°C. All vector preparations were titered by quantitative-PCR using Taq-Man technology. Purity of vectors was assessed by 4-12% SDS-Acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA).

Behavior

Pups were weighed daily and tested for righting reflex every other day from P5-P13. Pups were placed on their sides and time to right was recorded, with a maximum of thirty seconds allowed\textsuperscript{14}. Every five days between P15 and P30, animals were tested in an open field analysis (San Diego Instruments, San Diego, CA). Animals were given several minutes within the testing chamber prior to the beginning of testing then activity was monitored for five minutes. Beam breaks were recorded in the X, Y and Z planes, averaged across groups at each time point then graphed.

Immunofluorescence

Whole mount tissue: Whole mount TVA muscle was blocked in 10% Tween-20 (Sigma), 4% goat serum (Sigma), PBS for 30 min. Whole mount tissue was incubated with goat anti-mouse neurofilament 160, (1:500, Chemicon) in 10% Tween-20, 0.4% goat serum, PBS overnight and incubated with Alexa Fluor-488 anti-mouse secondary antibody (1:1000, Molecular Probes) for 2 hours, and Alexa Fluor594 alpha-Bungarotoxin (1:1000, Molecular Probes) for 30 minutes. Tissues were mounted in Vectashield (Vector Labs).
Tissue sections

Whole mount transverse abdominis muscle were post-fixed in 4% paraformaldehyde then stained with chicken anti-mouse neurofilament heavy chain, (1:1000, EnCor Biotechnology Inc.) in 10% Tween-20, 0.4% goat serum, PBS for 2 hours and incubated with Alexa Fluor 488 anti-chicken secondary antibody (1:1000, Molecular Probes) and alpha- bungarotoxin Alexa Fluor 594 (1:1000, Molecular probes) for 30 minutes. Tissue sections were mounted in Vectashield (Vector Labs).

Confocal Microscopy

All images were captured with the Leica TCS_SL scanning confocal microscope system using an inverted Leica DMIRE2 microscope and PMT detectors. Images were captured at room temperature with the following objective: 63x HCX Plan Apo CS oil, NA=1.4.0. A Z-Galvo stage was used to obtain Z-series stacks of approximately 30 images each. Image acquisition, overlays, scale bars, and measurements were produced with the Leica Confocal Software v2.61 and subsequent image processing was performed with Adobe Photoshop CS2.

Cell Counts

For both GFP and Gem quantifications, spinal cords from fixed animals were removed from the carcass. Lumbar enlargements were blocked and sliced into 40µm thick sections on a vibratome (Leica). Sections were collected in order in a 96 well plate. Every 12th section was sampled for a total of eight sections spanning approximately 3.8mm. The sections were fluorescently labeled using immunohistochemistry against choline acetyl transferase, green fluorescent protein or survival motor neuron. Sections
were counted using the 63x objective on a confocal microscope. Greater detail on the extent of CNS transduction by scAAV9 following P1 injection is shown in previous work [2].

**Western Blot**

100 mg of tissue was homogenized in TPER (Pierce, Rockford, IL). The sample was mixed with an equal volume of loading buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue) and run on a 12.5% polyacrylamide gel. Samples were transferred to Immobilon-P (Millipore, Bedford, MA, USA). The blot was blocked in 5% milk powder, 0.5% BSA in PBS–Tween for 1 h, and then incubated for 1 h with a primary antibody cocktail of MANSMA 2, 7, 13 and 19. Bound primary antibody was detected by horseradish peroxidase conjugated secondary antibody followed by chemiluminescence (ECL™ Western Blotting Detection Reagents, Amersham Biosciences). The blots were then stripped and re-probed with a β-actin monoclonal antibody (clone AC-15, Sigma-Aldrich) or a GAPDH monoclonal antibody (Millipore, Billerica, MA) to control for protein loading.

**Electrophysiology**

The recording chamber was continuously perfused with Ringer's solution containing the following (in mmol/l): 118 NaCl, 3.5 KCl, 2 CaCl₂, 0.7 MgSO₄, 26.2 NaHCO₃, 1.7 NaH₂PO₄, and 5.5 glucose, pH 7.3-7.4 (20-22°C, equilibrated with 95% O₂ and 5% CO₂). Endplate recordings were performed as follows. After dissection, the tibialis anterior muscle was partially bisected and folded apart to flatten the muscle. After pinning, muscle strips were stained with 10 µM 4-Di-2ASP [4-(4-diethylaminostyryl)-N-
methylpyridinium iodide] (Molecular Probes) and imaged with an upright epifluorescence microscope. At this concentration, 4-Di-2ASP staining enabled visualization of surface nerve terminals as well as individual surface muscle fibers. All of the endplates were imaged and impaled within 100 µm. We used two-electrode voltage clamp to measure endplate current (EPC) and miniature EPC (MEPC) amplitude. Muscle fibers were crushed away from the endplate band and voltage clamped to -45 mV to avoid movement after nerve stimulation.

Statistics

Statistical analyses were performed using Graph Pad Prizm software. Means were represented with s.e.m. Student t-tests were performed to compare groups using a 95% confidence level. Kaplan Meier Survival analysis was performed.
Chapter 3: Systemic Gene Delivery in Large Species for Targeting Spinal Cord, Brain and Peripheral Tissues for Pediatric Disorders

This chapter has been published in the peer-reviewed journal Molecular Therapy. This work is the result of collaboration with the authors listed below. I helped design and perform experiments and write the manuscript, especially all experiments using nonhuman primates.

Authors


*These authors contributed equally.

Abstract

Adeno-associated virus type 9 (AAV9) is a powerful tool for delivering genes throughout the central nervous system (CNS) following intravenous injection. Preclinical results in pediatric models of spinal muscular atrophy (SMA) and lysosomal storage disorders provide a compelling case for advancing AAV9 to the clinic. An important translational step is to demonstrate efficient CNS targeting in large animals at various ages. In the present study we tested systemically injected AAV9 in cynomolgus macaques, administered at birth through 3 years of age for targeting CNS and peripheral tissues. We show that AAV9 was efficient at crossing the blood-brain-barrier at all time points
investigated. Transgene expression was detected primarily in glial cells throughout the brain, dorsal root ganglia neurons and motor neurons within the spinal cord, providing confidence for translation to SMA patients. Systemic injection also efficiently targeted skeletal muscle and peripheral organs. To specifically target the CNS, we explored AAV9 delivery to cerebrospinal fluid (CSF). CSF injection efficiently targeted motor neurons, and restricted gene expression to the CNS, providing an alternate delivery route and potentially lower manufacturing requirements for older, larger patients. Our findings support the use of AAV9 for gene transfer to the CNS for disorders in pediatric populations.

Introduction
The recent discovery that adeno-associated virus type 9 (AAV9) can cross the blood brain barrier (BBB) and produce extensive transgene expression in the brain and spinal cord suggests a powerful, non-invasive method to deliver genes to the central nervous system (CNS). Pre-clinical results in models of pediatric neurological diseases with widespread pathology indicate systemic gene delivery could have profound therapeutic benefits, such as in spinal muscular atrophy (SMA). SMA is the most common autosomal recessive disease of early childhood with an incidence of 1:6-10,000 live births. In its most common and severe form (type 1), hypotonia and progressive weakness are recognized in the first few months of life, leading to diagnosis by 6 months of age and death due to respiratory failure by age two. There is no treatment available to slow or halt disease progression, but recent preclinical studies utilizing gene delivery in newborn rodent models of SMA suggest gene therapy may hold promise. SMA is an
attractive disease for gene therapy because it is a single gene defect that most frequently results in low amounts of the survival motor neuron (SMN) protein versus a total deficiency [1]. SMN is a ubiquitously expressed protein that is essential in all tissues and is not associated with toxicity when over expressed [2-8]. In addition, disease severity correlates with SMN protein levels suggesting that increasing SMN may be beneficial for patients. Motor neurons are the cell type primarily responsible for pathology of spinal muscular atrophy (SMA), which has precluded the development of an SMA gene therapy due to inefficient targeting of these cells by recombinant vectors [9]. However, the recent discovery that adeno-associated virus type 9 (AAV9) can target ~60% of motor neurons after systemic injection makes efficient delivery to the CNS feasible [10, 11]. A critical step in the translation of an SMA therapy to clinic is the demonstration of (CNS) targeting in non-human primates. AAV9’s crossing of the blood brain barrier has been demonstrated in mice, rats and cats indicating promise for translation to a human population [10-13]. Importantly, we demonstrated that AAV9 can target motor neurons following intravenous injection in a one-day old cynomolgus macaque [3]. However, the data in mice suggests a window of opportunity for targeting motor neurons in young primates may remain as a potential clinical obstacle for advancing AAV9 gene delivery for SMA. When AAV9 is systemically administered to newborn mice, there is extensive neuronal transduction throughout the brain and spinal cord. However when administered to adult mice, the majority of transduced cells are positive for astrocytic markers [11]. Our studies in mice demonstrated that this “switch” in targeted cell types occurred within
the first 10 days of life as demonstrated by a progressive decline in motor neuron transduction between P2 to P10 [3].

For this reason, we examined transgene expression throughout the body of male cynomolgus macaques following vascular delivery at time points from birth through 3 years of age using self-complementary AAV9 delivery of GFP versus PBS controls. In the present work, we expand our earlier findings that systemically administered AAV9 can efficiently target motor neurons in a newborn non-human primate with the addition of successful motor neuron targeting in animals up to three years of age. We also demonstrate extensive transduction of primarily glial cells throughout the brains of all treated animals. We extend our examination to tissues outside of the CNS and report extensive transgene expression within skeletal muscle and multiple organs. Because system-wide transduction may not be desirable in every paradigm, we investigated intrathecal and intracisternal delivery of AAV9 in newborn pigs for its ability to produce CNS transduction [14]. We demonstrate that motor neurons and regions of the brain can be targeted in neonatal pigs using AAV9 delivery to cerebrospinal fluid (CSF), potentially increasing the specificity and lowering the viral dose required for efficacy [15]. Together these findings are supportive of advancing AAV9-based gene delivery to the clinic for treatment of newly diagnosed type 1 SMA patients. In addition to spinal muscular atrophy, the extensive transduction seen in the brain at all time points suggests applications for other pediatric neurological diseases with global pathology such as Rett syndrome and lysosomal storage disorders.
Results

*Systemic injection of AAV9 targets motor neurons in non-human primates through three months*

The observation that, in mice, the cell types targeted by systemic injection of AAV9 shifted from neurons to astrocytes early in postnatal life could impact the potential clinical utility of AAV9. To address whether this pattern was unique to rodents, we sought to define a window of opportunity for targeting motor neurons in non-human primates following systemic delivery of AAV9-GFP. We performed systemic injections of 1-3x10^{14} vg/kg in a maximum volume of 10ml of self-complementary AAV9-GFP with a chicken β-actin hybrid promoter, a CMV immediate early enhancer and an SV40 intron. This is the same promoter construct previously used in our initial AAV9 report as well as in our rescue of the SMA mouse model report [3, 11]. With the exception of P1 animals, serum samples from male cynomolgus macaques were screened for the presence of binding antibodies against AAV9 capsids using an anti-AAV9 ELISA. Animals that were seronegative at 1:50 (the lowest dilution tested) for anti-AAV9 antibodies were injected through the saphenous vein at postnatal day 1, 30 or 90 (P1, P30 or P90 respectively). Animals were euthanized 21-25 days post injection and tissues collected for analysis. We performed *in situ* hybridization on lumbar spinal cord sections of the AAV9 GFP and PBS injected monkeys. Use of an antisense probe against the GFP mRNA detected abundant signal (*Figures 9a, c and e*; dark blue dots) that co-localized with a nuclear counter stain (pink) in the vector treated animals. GFP expression was seen in large neurons of the ventral horn and within small nuclei throughout the grey and
white matter of all the AAV-treated monkeys. Nearly all of the large neuronal-like cells displayed detectable levels of positive signal with additional cells targeted throughout the spinal cord, which likely demonstrates non-neuronal transduction. PBS treated animals had no detectable signal in any of the sections examined (Figure 9g). To confirm that the signal was not the result of non-specific binding of the antisense probe, tissue sections from the same animals were exposed to a sense probe which does not bind mRNA. Importantly, there was no hybridization in sections from either vector or PBS-treated animals when using the sense probe (Figure 9b, d, f and h).
Figure 9 *In situ* hybridization of monkey spinal cords following intravenous injection of either AAV9-GFP or PBS. Regions of lumbar spinal cords were probed with either antisense (a, c, e and g) or sense (b, d, f and h) probes against the vector derived GFP mRNA then counterstained with fast-red as a nuclear label. Within sections incubated with antisense probe, positive labeling is shown in dark blue, and is detected in large ventral neurons (filled arrows) and glia (open arrows) throughout both the grey and white matter of all animals injected with AAV9-GFP. There was no detectable signal when vector treated tissues were incubated with the sense probe indicating a lack of nonspecific probe binding. PBS treated animals had no detectable signal with either the antisense or sense probes.
To identify the GFP positive neurons in the spinal cord we immunolabeled tissue sections for transgene and choline acetyl transferase (ChAT), a motor neuron marker. Examination of labeled sections at all levels of the spinal cord demonstrated extensive GFP labeling in ChAT positive cells in all of the treated monkeys (P1-P90) (Figure 10). GFP positive nerve fibers were also detected coursing through the dorsomedial white matter indicative of transgene expression within the ganglion cells of the dorsal root (Figure 11). As seen with mice, GFP expression within neurons in the parenchyma of the spinal cord was confined to ChAT positive cells. There were also GFP positive cells with glial morphology that were sparsely scattered throughout the sections examined (data not shown). The overall pattern as detected by GFP immunohistochemistry was similar to that seen after neonatal delivery of AAV9 in rodents and cats with expression primarily in neuronal cells that project into the periphery [10, 11]. Importantly, efficient motor neuron targeting with AAV9 persists through at least the first three postnatal months.
Figure 10 Immunofluorescent labeling of GFP and ChAT within motor neurons. Lumbar spinal cord sections from AAV9 or PBS injected animals were labeled with antibodies against the vector derived transgene (GFP; a, d, g and j) and a motor neuron marker (ChAT; b, e, h and k) and are shown in black and white for enhanced contrast. Merged images (c, f, i and l), GFP in green and ChAT in red, indicate extensive transgene expression within motor neurons of the P1, P30 and P90 injected animals. GFP expression was not detected within the spinal cords of PBS injected animals. All scale bars = 200μm.
Figure 11 GFP expression with the dorsal horn of spinal cord. Sections from a P1 AAV9 P1 injected monkey show GFP positive fibers within the dorsomedial white matter and dorsal horn gray matter indicating AAV9 transduction of dorsal root ganglia at the cervical (a), thoracic (b) and lumbar (c) levels.

Intravascular delivery of AAV9 targets motor neurons in a three year old cynomolgus macaque

Due to successful targeting of motor neurons through the first three months of life in non-human primates, we asked whether motor neurons could still be targeted in a three year-old cynomolgus macaque. Utilizing interventional radiological techniques, a catheter was threaded through the brachial artery to the descending aorta while a balloon catheter was simultaneously fed through the femoral artery to the celiac artery and transiently inflated during vector injection. A dose of $2.7 \times 10^{13}$ vg/kg of AAV9-GFP was administered to the descending aorta while partially occluding blood flow to the liver in a procedure designed to give “first-pass” of the virus through the spinal arteries that are responsible for blood flow to the nerve roots of the thoracic cord. Two weeks post injection; the animal was euthanized and examined for GFP expression using in situ hybridization and GFP immunofluorescence. As with the P1-P90 animals, antisense
probed sections of spinal cord indicated GFP expression within both neuronal and glial cells of the spinal cord, while sense probed sections showed no signal (Figure 12a-b). GFP and ChAT immunofluorescent examination of cervical, thoracic and lumbar spinal cord sections revealed GFP positive motor neurons at all levels though less frequently than in younger animals (Figure 12c-e). The apparent decrease in motor neuron transduction may be due to 1/10 the dose given to the 3 year-old animal compared to that of the P1-P90 group. Nevertheless, this is proof that motor neurons can still be targeted utilizing systemic AAV9 delivery in a juvenile cynomolgus macaque even at significantly lower doses.

Figure 12 GFP expression in a three year old monkey spinal cord. In situ hybridization again reveals GFP expression in neurons (black arrows) and glia (white arrows) specifically in antisense (a), but not sense (b), probed spinal cord sections from an AAV9 injected animal. GFP (c, black and white) expression was confirmed in motor neurons (d, ChAT, black and white) by co-localization (Merged, GFP in green and ChAT in red e). Scale bar = 100µm.
Intravascular AAV9 produces extensive glial transduction throughout the brain

We next examined brain transduction following systemic delivery of AAV9. Indeed in mice, intravenous injection of AAV9 produced high levels of neuronal transduction in the brain, therefore we examined the brains of the treated monkeys for GFP expression utilizing immunohistochemistry [11]. As performed with the spinal cords, whole mount brains were sectioned in a serial manner and evaluated for GFP expression. Representative sections using high resolution slide scanning are shown in Figure 13 (P1-P90) and Figure 14 (3yr). Slide scanning technology captures images of the entire microscope slide with up to 40x resolution [16]. All GFP injected animals had extensive transgene expression throughout the entire brain. The overall pattern of expression was again similar to that seen in mice with the most abundant number of GFP expressing cells in all cortical regions (Figure 13a, b, f-j, k and o), lateral geniculate (Figure 13a and d), midbrain, pons and medulla. Sub-cortical structures such as thalamus (Figure 13a and c) and putamen (Figure 13k and l) were also GFP positive but at a lower cell density. In contrast to the mice, primate brains of all ages had primarily glial transduction with microglia and astrocytes being the most prominent cell types targeted (Figure 15) as determined by co-labeling with Iba-1 and GFAP (microglia and astrocyte marker, respectively) with neurons interspersed throughout. The scarcity of neuronal transduction was most striking in the hippocampus (Figure 13k and n) and dentate gyrus of the non-human primates because, in mice, neurons of these regions were highly transduced in both neonate and adult treated animals [11]. Transduction was not restricted to grey matter (Figure 13k and m) and included scattered oligodendrocytes
(data not shown). Surprisingly, widespread GFP expression was seen throughout the brain of the three year old animal that received 1/10 the dose compared to the P1-P90 animals. The brain regions transduced in the older animal were consistent with the younger primates with cortical and pontine regions having the highest prevalence of transduced cells. GFP positive cells were predominantly astrocytes and microglia (data not shown) though neurons of cranial nerve nuclei also expressed GFP (Figure 14b).
Figure 13 Whole slide scans of AAV9 and PBS injected monkey brains. Representative sections through similar regions of brains from systemically injected monkey were immunolabeled with anti-GFP antibodies. Panels a, f and k show uniform labeling throughout the sections of AAV9 injected animals but not the PBS injected animals (p). Boxes and arrows indicate the approximate regions from where the high magnification images were acquired. Cortex (a, b, f-j, k and o) consistently had the highest density of GFP expressing cells at all time points as did the lateral geniculate (a and d). Subcortical structures such as thalamus (a and c) and putamen (l and k) were well transduced but at a lower density than cortex. GFP positive cells were also seen within white matter of the pons (a, f and e) and cortex (k and m), as well as within the hippocampus (k and n). The majority of GFP positive cells in all regions had glial morphology though individual neurons could be detected throughout the brain. Brains from PBS injected animals were negative for GFP signal (p-t).
Figure 14 GFP immunohistochemistry from a 3 year old monkey. A scanned section at the level of the oculomotor nucleus (a) from the AAV9 injected three year old monkey. There is extensive GFP expression throughout the section that is primarily glial. Interestingly, neurons of the third cranial nerve (b) and glial within the pontine grey (c) were highly transduced.
Figure 15 GFP immunofluorescence from AAV9 injected monkey brain. Representative brain section from the cortex of an AAV9 GFP P1 injected monkey indicates primarily glial transduction in the monkey brain following systemic injection of AAV9. Immunolabeling for GFP (a and d) Iba-1 (b, microglia) or GFAP (e, astrocytes) indicates that both cell types are targeted in the brains of AAV9 injected monkeys (Merged images, c and f respectively). Open arrows indicate GFP positive microglia while filled arrows indicate GFP positive astrocytes. Scale Bars = 100μm.

AAV9 transduces skeletal muscle and peripheral organs

Numerous groups have demonstrated the ability of AAV to efficiently target skeletal muscle in mice, dogs and primates [17-21]. Therefore we examined skeletal muscle of the AAV9 injected non-human primates for GFP expression. In the young monkeys (P1-P90), all skeletal muscles examined were positive for GFP using immunofluorescent detection (Figure 16) including triceps, diaphragm, transverse abdominus, quadriiceps,
gastrocnemius, tibialis anterior and tongue. Skeletal muscles sampled were from the brachial and pelvic limbs, head and trunk indicating a body-wide distribution. GFP expression was less abundant in the skeletal muscles of the lower dosed, older monkey though still detectable in most muscles (data not shown). Peripheral organs may also be a target of a systemically injected vector, therefore we collected organs from injected animals to look for GFP expression within tissues (Figure 17). While we could detect native GFP expression, we utilized an antibody against GFP to enhance detection levels. As expected, liver had the highest levels of GFP expression, followed by the adrenal medulla (P1 injected animal). Previous studies demonstrated that the heart, while well targeted in mice by AAV9, is not as efficiently transduced in dogs [18, 22]. Our findings in the AAV9 treated monkeys agree with those in dog, in that levels of GFP expression in the heart were lower relative to skeletal muscle. GFP signal was also found in the germinal centers of the spleen in all of the treated animals as well as smooth muscle that lines the intestines. GFP expression was also detected in the leydig cells of the testes of all treated male monkeys. Finally occasional positive cells were identified in the lungs and kidneys of AAV9 GFP treated animals. These results demonstrate that systemic gene delivery targets multiple organ systems with a biodistribution of expression similar to that found in the rodent with the exception of cardiac tissue.
Figure 16 GFP expression within non-human primate skeletal muscle. GFP immunofluorescence from AAV9 injected and PBS injected monkeys demonstrates extensive transgene expression in skeletal muscles of all AAV9 injected animals. GFP expression was detected in the brachial limbs (triceps brachii a-c), trunk (diaphragm e-g and transverse abdominus (TVA) i-k), pelvic limbs (quadriceps m-o, gastrocnemius q-s and tibialis anterior u-w) and head (tongue x-z) of AAV9 systemically injected animals. No GFP signal was detected in any of the muscle from the PBS injected animals (d, h, l, p, t and aa).
Figure 17 GFP expression within assorted organs. Of the tissues examined, GFP expression was most abundant in the livers (a-c) and adrenal medulla (u) of all AAV9 injected monkeys. Detectable GFP expression was also seen in the kidney (e-g), spleen (i-k), heart (m-o), lung (q-s), smooth muscle of the intestines (v-x) and testes (z-bb) of AAV injected animals. GFP was not detected in the tissues collected from PBS injected animals (d, h, l, p, t and y).
AAV9 injection into cerebral spinal fluid of young pigs efficiently targets motor neurons

While some neurological disorders are caused by defects in ubiquitously expressed proteins, in other disorders gene expression in the CNS alone may have a substantial impact [23-25]. Gene delivery to the CSF could produce transduction along the neuraxis with the added benefit of potentially lowering the required dose. In order to examine more localized CNS delivery we performed intrathecal and/or intracisternal injections of 5.2x10^{12} vg/kg of AAV9 GFP into 5-day-old pigs (n=3 each) and examined their brains and spinal cords for GFP expression. In all animals, GFP expression was seen in the dorsal root ganglia as well as the spinal cord grey and white matter. Importantly, AAV9 GFP injection into either the cisternal space at the base of the skull or the intrathecal space at L5 resulted in extensive motor neuron transduction at all levels of the spinal cord (Figure 18a-d) as examined by in situ hybridization. Large ventral horn neurons were also positive for GFP expression by immunohistochemistry at all levels of spinal cord (Figs 18e-l). Immunofluorescence confirmed that the GFP positive cells expressed the motor neuron marker ChAT (Figs 18m-r).
Figure 18 Pig spinal cord after AAV9 injection. *In situ* hybridization of AAV9 (a-b) or PBS (c-d) injected spinal cords reveals GFP signal within motor neurons (filled arrows) and glia (open arrows) of antisense probed sections of AAV injected pigs (a) but not PBS injected (c). Sense probed sections from both treated and control animals had no detectable signal (b and d). Immunohistochemical detection of GFP following intracisternal (e-h) or intrathecal (i-l) injected spinal cords indicates extensive labeling of large ventral horn neurons within AAV9 injected (e-g and i-k) but not PBS injected animals (h and l). Immunofluorescent colabeling of GFP (n and q) and ChAT (o and r) in spinal cord sections from intracisternal or intrathecal AAV9 injected pigs shows that the transduced cells are motor neurons (Merged, m and p). C.sc, cervical spinal cord; T.sc, thoracic spinal cord; L.sc, lumbar spinal cord
AAV9 injection into the cerebrospinal fluid produces transgene expression in the brain

Finally, to further characterize the pattern of expression following cisternal or intrathecal injection of AAV9-GFP into 5-day-old pigs, we examined brains for transgene expression again using GFP immunofluorescence (Figure 19). The regions with the highest levels of GFP expression were cerebellar purkinje cells, nerve fibers within the medulla as well as discrete nuclei, such as the olivary nucleus. Expression within the rest of the brain was restricted to scattered cells near the meningeal surfaces (data not shown). Examination of GFP expression in peripheral organs yielded no visible GFP expression indicating that the majority of the virus was localized to the CNS.
Figure 19 AAV9-GFP mediates transgene expression in the brain after intrathecal delivery. Representative coronal brain section (a) showing GFP expression after intrathecal injection of AAV9-GFP within the hindbrain, fibers of the trigeminal nerve (b) and Purkinje cells (c). (d-f) GFP expression was also detected in ChAT-positive neuron of the olivary nucleus.Cb, cerebellum; NCH, nuclei cochleares; NSV, Nucleus tractus spinalis nervi trigemini; TSV, tractus spinalis nervi trigemini.

Discussion

The recent emergence of AAV9 and its ability to cross the BBB represents a potentially valuable therapeutic and basic science tool. The consistency of performance across species only adds to AAV9’s value. Our previous work and the data presented here are among the first to demonstrate transgene expression within the entire CNS following a peripheral systemic injection in non-human primates [3, 12]. That a similar pattern of
expression is generated in animals of multiple ages is excellent proof of concept that this delivery modality can be advanced to the clinic, especially for pediatric diseases with broad targeting needs.

A recent study also looked at systemic injection of AAV9 for its ability to cross the blood brain barrier in non-human primates [12]. Both the current and previous studies report transgene expression along the entire neuraxis of non-human primates. What is further encouraging about the two data sets is that Gray et al. worked exclusively in 3-4 year old male rhesus macaques at 1/10\(^{\text{th}}\) (\(\sim 1 \times 10^{13}\) vg/kg versus \(\sim 1 \times 10^{14}\) vg/kg) the dose used in the present study for the P1-P90 animals and comparable to our 3 year old animal (2.7\(\times 10^{13}\) vg/kg). The overlap in the 3-year-old age group between the two studies calls into question whether the interventional radiological techniques employed in the current study conferred an advantage with respect to first pass CNS transduction. The occlusion of blood flow to the liver may be beneficial in improving transduction to other tissues, however, further studies are needed to assess the efficacy of procedurally detargeting the liver. Furthermore, emerging techniques in capsid evolution for enhancements of vectors have also shown effectiveness in detargeting the liver [26] and requires additional study for systemic gene delivery to the brain. Nonetheless, together the data suggest that CNS targeting following systemic injection of AAV9 is feasible in animals from birth through 4 years of age over a range of doses. A noteworthy finding between the two studies is the consistent targeting of motor neurons in all of the animals examined regardless of age. These findings will certainly have impact on the development of gene delivery protocols for SMA. While type 1 SMA patients are the most severe and most common, a
significant patient population exists with type 2 and type 3 disease that manifests later in life and produces milder though still debilitating symptoms [27]. The field’s knowledge of the molecular pathology of mild SMA is hampered by the fact that lifespan is often not affected in these patients and a robust animal model is not available for study [28]. The preclinical SMN gene delivery studies were performed in a mouse model that closely resembles type 1 patients and therefore supports a trial within those patients [2-4]. However, because gene delivery to motor neurons can be accomplished at later time points in higher species, it gives hope for later trials in less severe patients. An important question remains though, as to whether type 2 and type 3 patients would benefit from SMN gene restoration within motor neurons at their stage of disease. Outside of motor neurons, the fact that lower doses in larger animals produced appreciable transgene expression within the CNS supports the development of therapies for diseases with secreted transgenes such as lysosomal storage disorders [29].

The studies by Gray et al in 3-4-year-old NHPs show that poor expression was seen in the presence of pre-existing antibodies following systemic delivery [12]. In human clinical trials, transgene expression can be achieved following direct injection into muscle or brain despite pre-existing neutralizing antibodies [30, 31]. While more information is needed regarding the prevalence of neutralizing antibodies to AAV9 within the adult population, the epidemiological data suggests that the overwhelming majority of the adult population is seronegative for neutralizing antibodies against newer AAV serotypes [32-34]. For example, AAV2 is the serotype with the highest prevalence of neutralizing antibodies ranging from 20-60% of the population at a 1:20 dilution, and the prevalence
of individuals with neutralizing antibodies to emerging serotypes such as AAV8 was among the lowest tested (19-32% at a 1:20 dilution) across multiple continents [33]. Of interest, in a French adult population less than 50% of individuals from 25 to 64 years old were seropositive for AAV9 binding antibodies, of which only about 33% inhibited *in vitro* infection. Among those seropositive for anti-AAV9 antibodies, approximately 70% had very low antibody titers of 1:20 which was the lowest dilution tested [32]. Studies in children report that the prevalence of anti-AAV antibodies in children is less than that of adults, indicating the age of acquisition of infection may also factor favorably into delivery into a pediatric population [35, 36]. It is also possible to transiently reduce or eliminate neutralizing antibodies via apheresis which may allow gene targeting even in seropositive patients [37].

An important goal of this study was to address the existence of a window of opportunity for motor neuron targeting in non-human primates. In mice, systemic injection of AAV9 into older animals (P10 and older) led to a dramatic increase in the number of glia targeted within the spinal grey matter when compared to P1 injected mice [11]. Concomitant with the increase in glial targeting was a decrease in motor neuron targeting which could complicate patient selection in a clinical trial for SMA [3, 11]. The successful transduction of motor neurons in all the treated monkeys suggests that the hypothetical window closes much later, if at all, in non-human primates. The data from both the spinal cord *in situ* hybridization and the monkey brain histology demonstrate glial cells are well transduced by AAV9 at all ages studied, suggesting that astrocytes may still be a viable target for AAV9 delivery under appropriate circumstances.
Another departure from the data generated in mice is the types of cells targeted in the brains of monkeys after systemic AAV9 injection. Experiments in mice targeted predominantly neurons throughout the brains of neonate injected animals, while in monkeys the cells targeted were mostly astrocytes and microglia. This discrepancy is likely due to the different timing of gliogenesis between the two species [38]. Therefore, vector escaping from the vasculature in the primate brain would likely encounter glial endfeet that ensheathe the endothelial cells prior to meeting neurons. Nonetheless, the number of neurological diseases that have implicated glial cells as contributing to pathology continues to grow [39]. The abundance and distribution of cells targeted throughout the primate brains of all the treated animals suggests AAV9 gene delivery could have immense impact on diseases throughout the nervous system, such as amyotrophic lateral sclerosis, Rett syndrome, and lysosomal storage diseases [29, 40, 41].

The high amount of GFP expression throughout all the skeletal muscles examined is consistent with data from numerous groups in mice and dogs [18, 22]. Head to head comparisons would be required in non-human primates to determine if the hierarchy of muscle transducing serotypes from mice is consistent in monkeys. Transduction of peripheral organs is an expected side effect of systemic delivery. Indeed, the persistence of expression in the liver of the younger primates is noteworthy when compared to data in mice in which episomal viral genomes are lost through the rapid cell divisions of the developing mouse liver. However the slower development of primates compared to rodents likely explains why we saw GFP persistence in the liver at the time of sacrifice; 3 weeks post-injection. For example, in the P1 animal there was a difference of only 4g
between birth and sacrifice three weeks later. Similar vector doses in P1 mice also show the clearance of transgene expression from liver as a factor of time post injection (data not shown). Therefore, we predict the expression of transgene in non-human primate liver would diminish over time, due to hepatocyte division. Depending on the paradigm, transduction of organs can be potentially beneficial or harmful, and techniques exist to restrict the viral expression to target cells such as motor neurons if needed [42-45]. As for the tissues targeted in this study, our data is in agreement with data generated in cats that showed skeletal muscle, liver and adrenal medulla as highly transduced sites following systemic injection of AAV9 [10].

Anticipating the potential requirement to avoid off target expression of corrective transgenes outside the CNS, we have also demonstrated that AAV9 delivery into the cerebrospinal fluid can deliver transgene throughout the CNS with limited peripheral expression. Recent studies utilizing gene transfer for SMA into the lateral ventricles and lumbar spinal cord of neonatal SMA mice had a profound effect on survival, though it is not clear how favorably it compares with systemic administration [24, 25]. Development of an intrathecal delivery paradigm would reduce viral load that in turn could reduce the risk of an immune response as well as decrease viral production requirements particularly for older, presumably larger patients. Another complicating factor, at least in the context of SMA, is the involvement of non-neuronal tissues. Our group and others recently reported cardiac deficits in multiple SMA mouse models that could suggest the need for SMN protein in cells besides motor neurons [46-48]. The relevance of similar heart findings in humans also remains to be elucidated. Designing studies to examine the
contribution of cell types other than motor neurons to SMA pathology is difficult due to the models available due in part to early pathology and the small size of SMA mice. The data presented here give increasing confidence that systemic and intrathecal delivery of AAV9 will likely be successful when applied to pediatric neurological disease, potentially even in older children. The published preclinical efficacy data in a model of SMA and the effectiveness of motor neuron targeting within large animals in the current report highlight the need for safety studies utilizing SMN as the next step in advancing SMA gene delivery to clinical trial. The demonstration of motor neuron and glial targeting in large species opens the possibilities to the creation of large animal models of disease to address questions of subcellular localization of proteins or the importance of aging to the manifestation of a disease phenotype. The repertoire of AAV9 and emerging serotypes are just beginning to be explored, yet is demonstrating significant potential in basic and translational science [49].

Methods

Viral production

Self-complementery AAV9 was produced by transient transfection procedures using a double-stranded AAV2-ITR–based CB-GFP vector, with a plasmid encoding Rep2Cap9 sequence as previously described along with an adenoviral helper plasmid pHelper (Stratagene) in 293 cells [50]. Our serotype 9 sequence was verified by sequencing and was identical to that previously described. Virus was produced in three separate batches for the experiments by a contract manufacturing company (Virapur) and purified by two cesium chloride density gradient purification steps, dialyzed against PBS and formulated
with 0.001% Pluronic-F68 to prevent virus aggregation and stored at 4°C. All vector preparations were titered by quantitative PCR using Taq-Man technology. Purity of vectors was assessed by 4–12% SDS-acrylamide gel electrophoresis and silver staining (Invitrogen).

Animal care and use

All procedures performed were in accordance to either the Mannheimer Foundation, the Research Institute at Nationwide Children’s Hospital or The Ohio State University Institutional Animal Care and Use Committees.

Non-human primate intravascular vector delivery

The breeding, housing and procedures performed on the young, male cynomolgus macaques (Macaca fasciculata, age P1-P90) were carried out at the Mannheimer Foundation (Homestead, FL). Briefly, veterinary staff anesthetized the subject and placed a catheter into the saphenous vein, through which either a suspension of 1-3x10¹⁴ vg/kg AAV9.CBA.GFP or PBS was infused over a period of 5-8 minutes. Upon recovery, subjects were returned to their mother and housed under routine conditions for the duration of the study.

At Nationwide Children’s Hospital, the 3 year-old subject was infused with 2.7x10¹³ vg/kg using interventional radiological techniques to target delivery to the radicular arteries of the thoracic cord. Briefly, the subject was anesthetized and a catheter was introduced percutaneously into the brachial artery and guided to the proximal portion of the descending aorta. A second catheter delivered an occlusive balloon to the distal portion of the descending aorta at the level of the celiac trunk. Proper placement was
confirmed by fluoroscopy and injection of a radiopaque dye via the proximal catheter. Prior to injection, the distal balloon was inflated to occlude blood flow distal to and including the celiac trunk. The viral suspension was delivered over approximately 1 minute, and the balloon was left inflated for another 2 minutes post-infusion. After recovery, the animal was released back to its regular environment for the duration of the study.

Seronegativity for anti-AAV9 antibodies was confirmed in all subjects by ELISA. Briefly, a $2 \times 10^{10}$ vg/ml solution of empty AAV9 capsids was made with a carbonate coating buffer and applied to a 96 well plate and incubated over night at $4^\circ$C. The following day, the plate was washed and blocked with a 5% milk solution in PBS with 0.1% tween-20. Serums were diluted from 1:50 -1:6400 and incubated at room temperature for an hour. The wells were washed with PBS-T and then incubated with an HRP conjugated anti-monkey secondary (Sigma-Aldrich) for one hour at room temperature. The wells were washed with PBS-T then developed with TMB. The reaction was stopped with the addition of hydrochloric acid and absorbance was read at 650nm on a plate reader.

**Intrathecal Injection**

Farm-bred sows (*Sus scrofa domestica*) were obtained from a regional farm. Five-day old (P5) piglets received 0.5cc/kg ketamine induction anesthesia and then were maintained by mask inhalation of 5% isoflurane in oxygen. Body temperature, electrocardiogram and respiratory rate were monitored throughout the procedure. For lumbar puncture, piglets were placed prone and the spine was flexed in order to widen the intervertebral spaces.
The anterior-superior iliac spines were palpated and a line connecting the two points was visualized. The intervertebral space rostral to this line is approximately L5-L6.

Intraoperative fluoroscopy confirmed rostral-caudal and medio-lateral trajectories. Using sterile technique, a 25-gauge needle attached to a 1ml syringe was inserted. Gentle negative pressure was applied to the syringe as the needle was passed until a clear flash of CSF was visualized. For cisterna puncture, the head of the piglet was flexed while maintaining the integrity of the airway. Fluoroscopy again confirmed adequate trajectory. A 25-gauge needle was passed immediately caudal to the occipital bone, and a flash of clear CSF confirmed entry into the cistern magna.

For reagent delivery, the syringe was removed while the needle was held in place. A second 1 cc syringe containing either viral solution (5.2x10^{12}vg/kg) or PBS was secured and the solution was injected into the intrathecal space at a slow and constant rate. After delivery, approximately 0.25ml of sterile PBS was flushed through the spinal needle so as to ensure full delivery of reagent.

We confirmed rostral and caudal intrathecal flow by injecting a radioopaque agent (Omnipaque) and recording intrathecal spread with real-time continuous fluoroscopy.

**Perfusion and tissue-processing**

All subjects (primate and porcine) were euthanized between 21 and 24 days post-injection. Subjects were deeply anesthetized by intramuscular injection of sodium pentobarbital solution (primates) or Telazol followed by Propofol (piglets). A midventral sternal thoracotomy was performed and a cannula was inserted in the aorta through the left ventricle. The right atrium was opened and 0.5-1L of PBS was injected through the
cannula by gravity flow, followed by perfusion with 1L of 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4). Organs were removed and post-fixed 48 hours in 4% PFA before further processing for histological sectioning or stored long-term in 0.1% NaN₃ PBS solution.

**Histology and Microscopy**

Primate and porcine spinal cord segments were embedded in 3% agarose prior to cutting into 40μm horizontal sections using a Leica VT1200 vibrating microtome (Leica Microsystems, Buffalo Grove, IL). Sections were transferred in TBS and stored at 4°C until processing.

Primate and porcine brains were cryoprotected by successive incubation in 10%, 20% and 30% sucrose solutions. Once sufficiently cryoprotected (having sunk in 30% sucrose solution), brains were frozen and whole-mounted on a modified Leica SM 2000R sliding microtome (Leica Microsystems, Buffalo Grove, IL) in OCT (Tissue-Tek, Torrence, CA) and cut into 40μm coronal sections.

For immunofluorescent determination of cell types transduced, floating sections were submerged in blocking solution (10% donkey serum, 1% Triton-X100 in TBS) for 1 hour followed by overnight incubation in primary antibody solution at 4°C. The following primary antibodies were used in this study: Rabbit-anti-GFP (1:500, Invitrogen, Carlsbad, CA), goat-anti-ChAT (1:100, Millipore, Billerica, MA), guinea pig-anti-GFAP (1:1000, Advanced Immunochemical, Long Beach, CA) and rabbit-anti-Iba1 (1:500, Dako, Carpinteria, CA). Primary antibodies were detected using Fitc-, Cy3- or Cy5-conjugated
secondary antibodies (1:1000, Jackson ImmunoResearch, West Grove, PA) and mounted in PVA-DABCO medium.

For immunohistochemical staining, sections were incubated at room temperature in 0.5% H₂O₂/10% MeOH solution and subsequently blocked and stained as above with rabbit-anti-GFP overnight. Anti-GFP antibodies were detected using biotinylated donkey-anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) and developed using Vector NovaRed per the provided protocol (Vector Labs, Burlingame, CA). Sections were then mounted in Cytoseal 60 medium (Thermo Fisher Scientific, Kalamazoo, MI).

Non-neural tissues were cut to approximately 1cm³ blocks and cryoprotected by overnight incubation in 30% sucrose solution. They were then embedded in gum tragacanths and flash-frozen in liquid nitrogen-cooled isopentane. Samples were cut by cryostat into 10-12μm sections and slides stored at -20°C. GFP expression was detected by a similar immunofluorescent protocol as above with the addition of DAPI in secondary antibody solution (1:1000, Invitrogen, Carlsbad, CA).

Fluorescent images were captured using a Zeiss 710 Meta confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) located at TRINCH and processed with LSM software.

Whole brain sections were scanned to 40x resolution at the Biopathology Center in the Research Informatics Core at the Research Institute at Nationwide Children’s Hospital using an Aperio automated slide scanner (Aperio, Vista, CA) and resulting images were processed with ImageScope software.
In situ hybridization

As described previously [11], we generated antisense and sense DIG-UTP–labeled GFP riboprobes. Probe yield and incorporation of DIG-UTP was confirmed by electrophoresis and dot blot. Sections of spinal cord 10 μm-thick were mounted and prepared by fixation with 4% paraformaldehyde, washed in 0.5x SSC, permeabilized by incubation in proteinase K (2.5 μg/ml), washed in 0.5 × SSC and dehydrated in series of alcohol washes. Prehybridization was performed at 42°C using RiboHybe buffer (Ventana, Tucson, AZ) for 1 h followed by hybridization overnight at 55°C with the respective riboprobes on AAV9-injected and PBS-control-injected cord sections. Stringency washes were performed and immunological detection using anti-Digoxigenin AP antibody (1:500, Roche, Tucson, AZ) and development with NBT/BCIP (Thermo Fisher Scientific, Kalamazoo, MI) and Nuclear Fast Red (Vector Labs, Burlingame, CA).

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Chapter 4: Systemically delivered scAAV9-SMN is safe and well tolerated in mice and non-human primates

This work is the result of collaboration with the authors listed below. I designed and performed experiments and wrote the manuscript.

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Abstract

Spinal muscular atrophy (SMA) is a devastating disease that leads to dysfunction and degeneration of spinal motor neurons followed by muscular atrophy and paralysis. SMA is caused by homozygous mutation or deletion of the survival motor neuron (SMN1) gene, and it is the leading genetic cause of death in infants. Despite much effort, there is still no treatment for SMA besides supportive and palliative care. However, we recently developed a gene therapy approach using systemically-delivered self-complementary adeno-associated virus, serotype 9 to carry the SMN gene to motor neurons throughout the entire spinal cord (scAAV9-SMN). This therapy has been successful in rescuing mouse models of the severest forms of SMA.

An important step in translation to the clinic is the pre-clinical demonstration of safety. To this end we performed 6-month safety studies in 24 mice and 4 non-human primates.
(NHPs). Tissues from both mice and NHPs were assessed for histopathological evidence of tissue damage, but none was found. Serum chemistry and hematology studies were likewise unremarkable. Additionally, we found that NHP subjects mounted appropriate immune responses to capsid, but not to transgene, with very high transgene expression remaining at 6 months post-injection. This study provides strong evidence that systemically-delivered scAAV9-SMN is safe and well tolerated, even at the high doses required for penetration of the blood-brain barrier. Furthermore, this study greatly expands the potential of AAV-based therapies to a level far beyond what has been tested previously.

Introduction

Proximal spinal muscular atrophy (SMA) is a devastating neurological disease, affecting 1 in about 10,000 newborns per year, and it is the most common genetic cause of death in infants. SMA is due to a homozygous deletion or mutation of the SMN1 gene, leaving patients to rely solely on the far less-efficient SMN2 gene. Due to SMN2 copy number variations, SMA presents in a clinical spectrum with even the most severe patients expressing a small amount of full-length SMN. While SMN is ubiquitously expressed, low SMN levels primarily affect only spinal motor neurons. This leads to motor neuron dysfunction and death with subsequent muscular atrophy. The most common form of the disease leads to almost complete mortality or ventilator dependence by 2 years old. Besides respiratory support and palliative care, there remains no therapy for these patients.
However, gene therapy holds great promise for treating SMA as well as many other
diseases. Adeno-associated virus (AAV) is an attractive option for clinical gene transfer,
primarily due to its increasingly positive safety profile. AAV infection is not associated
with any human disease, and most serotypes have very low immunogenicity.
Recombinant AAV also has a very low rate of integration into the host genome, but
expression of the episomal transgene persists indefinitely, increasing its utility even
further for treating post-mitotic cells such as striated muscle and nervous tissue. Over the
last 2 decades, clinical gene transfer via AAV has been repeatedly shown to be safe and
effective [1]. Additionally, the design of self-complementary AAVs (scAAV) has further
increased the efficiency of these vectors [2, 3].
More recently, we and others have shown that intravenously-delivered scAAV serotype 9
(scAAV9) is able to effectively transduce the central nervous system of mice, rats, cats
and non-human primates [4-7]. In particular, spinal motor neurons are very well
transduced, making AAV9-based therapy ideal for treating diseases such as SMA and
amyotrophic lateral sclerosis (ALS). We have recently demonstrated a near-complete
rescue of a mouse model of SMA by introducing the SMN gene via AAV9, driven by the
highly active, ubiquitous chick-beta actin (CB) promoter (scAAV9-SMN) [8]. The aim of
this study is to assess the safety of administering high-dose scAAV9-SMN to guide the
clinical translation of this promising gene therapy approach to young SMA patients.
Results

Mouse vector delivery and in-life observations

To initially assess safety and potential toxicity of scAAV9-SMN, we injected P1 wild type FVB/n mice with either vehicle (PBS) (3 males/6 females) or $3.3 \times 10^{14}$ vg/kg of scAAV9-SMN (6 males/9 females) in a total volume of 50 µl via the temporal vein. This dose was previously shown to be most efficacious in the Δ7 mouse model of SMA [8]. We chose to inject P1 mice to more closely approximate the targeted tissues of infants, the planned population for the first-in-human clinical trial. Baseline body mass was measured at time of injection, and runts were excluded a priori. All mice survived the injection procedure and initial 24-hour observation period without any signs of distress or weight loss. Body mass was measured and hands-on observations were performed at least weekly for the remainder of the study, and neither revealed any difference between control and treated cohorts (Figure 20). Two males (one treated and one control animal) had to be removed from the study due to multiple early flooding events in their home cage.
Figure 20 Average Body Mass of control vs. treated mice. Mice were weighed weekly for the entire 6 month study. All subjects gained and maintained appropriate weight throughout, with no difference between treated vs. control (PBS) injected subjects.

At 60, 90 and 180 days post-injection, blood was collected for hematology studies including complete blood count (CBC) with differential (Figure 21). Results in treated and control animals demonstrated no values of clinical significance, and trends remained comparable between treated and control animals. Blood wasn’t collected until 60 days of age to ensure we could safely collect sufficient blood sample.
Figure 21 Mouse CBC with Differential. Whole blood was collected from treated and control animals at 60, 90 and 180 days post injection for complete blood count (CBC). There were no alterations in hematological parameters including red blood cell counts (RBC), hemoglobin concentration (Hbg), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV) and white blood cell count (WBC). Further white blood cell counts indicate no deviations from control numbers of segmented neutrophils, monocytes, lymphocytes, eosinophils or basophils.

At 90, 120 and 180 days post injection, blood was collected for clinical chemistry evaluation, including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (Alk Phos), creatine, blood urea nitrogen (BUN), sodium, potassium, chloride and creatine kinase (CK) (Figure 22). The only value to differ from controls was creatine kinase (CK) at the initial 90-day time point, but this may have been due to the
route of blood collection (tail vein) as the value was similar to controls at later time points when bled through the submandibular sinus.

Figure 22 Mouse Serum Chemistry. Serum was collected from treated and control animals at 90, 120 and 180 days post injection for analysis of clinical chemistries. Creatine kinase (CK) was elevated in the treated subjects at the first blood draw at 90 days but matched control concentrations at the following timepoints. Fewer timepoints were collected for GGTP, alkaline phosphatase, potassium, sodium and chloride due to insufficient quantity of blood. Abbreviations: aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGTP), alkaline phosphatase (Alk Phos), blood urea nitrogen (BUN).
Mouse pathology

Thirteen out of the total of twenty one (n=21) mice were sacrificed at approximately 120 days of age and a smaller cohort (n=8) was sacrificed at 180 days of age. Organs from mice sacrificed at 180 days were sent for histological pathology assessment by a third party, blinded pathologist. Pathological findings were similar between groups, with no significant lesions found. The results of all findings in control and treated subjects are summarized in Table 1. Of particular note, there were no signs of inflammation in the muscles, liver or heart, all of which are known sites of particularly high AAV9 transduction.
### Table 1 Mouse pathology summary

<table>
<thead>
<tr>
<th>Organ</th>
<th>PBS-Control</th>
<th>3.3 x 10^14 vg/kg scAAV9-SMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain*</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Diaphragm</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Gastrocnemius m.</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Gonad</td>
<td># EX 2</td>
<td># EX 5</td>
</tr>
<tr>
<td>Heart</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Kidney</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Large Intestine</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Liver</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Brain*</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Diaphragm</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Gastrocnemius m.</td>
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<td>Gonad</td>
<td># EX 2</td>
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<tr>
<td>Heart</td>
<td># EX 3</td>
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</tr>
<tr>
<td>Kidney</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Large Intestine</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Liver</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>microgranulomas (without associated pathology)</td>
<td>1</td>
<td>33.3%</td>
</tr>
<tr>
<td>multiple microgranulomas associated with focal coagulative necrosis</td>
<td>2</td>
<td>66.7%</td>
</tr>
<tr>
<td>microgranulomas with brown pigment laden macrophages</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>mild diffuse hepatocellular vacuolation suggestive of glycogen accumulation</td>
<td>1</td>
<td>33.3%</td>
</tr>
<tr>
<td>small clusters of lymphocytes present in sinusoids</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>focal hematomatosis</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>mild multifocal perivascular lymphoid aggregates</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Lung</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>mild multifocal peribronchiolar lymphoid infiltrates</td>
<td>1</td>
<td>33.3%</td>
</tr>
<tr>
<td>Lymph Node*</td>
<td># EX 3</td>
<td># EX 4</td>
</tr>
<tr>
<td>Pancreas</td>
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<td># EX 5</td>
</tr>
<tr>
<td>Small Intestine*</td>
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<td># EX 5</td>
</tr>
<tr>
<td>Spinal Cord*</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Spleen</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>mild diffuse distribution of hemosiderin in macrophages of the red pulp</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Stomach</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>cystic mucosal glands present in glandular portion</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Thymus</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Tibialis Anterior m.</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Triceps m.</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>Urinary Bladder</td>
<td># EX 3</td>
<td># EX 5</td>
</tr>
<tr>
<td>mild focal perivascular lymphoid infiltrate</td>
<td>1</td>
<td>33.3%</td>
</tr>
</tbody>
</table>

* No significant lesions are present in any brain or spinal cord sections. These were frozen sections which are thicker than 5 microns making cellular morphology obscure; therefore subtle changes may not be identified.

# At least one lymph node was analyzed per animal, dissected from axillary, inguinal and/or popliteal regions

^ While no significant lesions were found, postmortem autolysis precluded satisfactory evaluation of tissue(s) the small intestine of one scAAV9.CB.SMN animal

### Non-human Primate (NHP) Vector delivery and in-life observations

In order to further confirm the safety of systemically-delivered scAAV9-SMN we also assessed the potential toxicity, the biodistribution and level of transgene expression in 4
male cynomolgus macaques (*Macaca fascicularis*). We have chosen to inject at 90 days of age to more closely mimic the likely age of administration in SMA type 1 infants. Furthermore, results from scAAV9-GFP injected animals at P90 show that AAV9 targets a sufficient quantity of motor neurons at this time point. Animals were followed for six months until sacrificed at approximately 9 months of age.

Prior to vector administration, blood was drawn from potential subjects to confirm seronegativity for anti-AAV9 antibodies, and to establish baseline chemistry and hematology values. The scAAV9-SMN vector was administered one time by catheterization of the saphenous vein on Day 0 of the study (postnatal day 90) at a dose of $6.7 \times 10^{13}$/kg. This dose corresponds to the lowest dose tested at which all Δ7 mice showed significant lengthening of survival. Subjects were monitored closely for twenty-four hours post treatment and a hands-on examination by the facility veterinarian was performed weekly thereafter. The veterinary staff found that all subjects were strong, alert and healthy-looking at each assessment. Body mass was also measured weekly, and each animal gained appropriate weight throughout the duration of the study (**Figure 23**).
Figure 23 Body Mass of individual nonhuman primate subjects. Four nonhuman primate subjects (individually identified as 11C2, 11C11, 11C14 and 11C16) were injected with 6.7 x 10^13 vg/Kg at 90 days of age. Weekly body mass measurements show that all subjects appropriately gained weight through the entire 6 months of the study.

Starting at one month post-injection, and monthly thereafter, serum samples from each animal were analyzed for clinical chemistry and hematology. These values were compared to values from non-injected, age-matched control males from the same colony. However, control subjects were housed in an open enclosure and were captured and restrained acutely for each blood collection. Treated subjects were already housed indoors enabling a less-stressful sample collection. Hematology results were very similar to controls (Figure 24), and with the exception of AST and CK, clinical chemistry values were likewise similar (Figure 25). The AST and CK values of treated subjects were well within the normal range for young Cynomolgus macaques, but many of the control values were very high, likely due to the acute stress experienced during collection.
Figure 24 Primate CBC with Differential. Whole blood was collected from each subject prior to scAAV9-SMN injection and monthly thereafter for complete blood count (CBC). The connected values in each graph represent results from each individual subject over time. Control samples were taken from multiple animals from the same colony, and control values are indicated by open circles. There were no significant alterations in any hematological parameters. Abbreviations: Red blood cell count (RBC), hemoglobin concentration (Hbg), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV) and white blood cell count (WBC).
Figure 25 Monkey Serum SuperChem. Serum was collected from each subject prior to scAAV9-SMN injection and monthly thereafter for clinical chemistry analysis. The connected values in each graph represent results from each individual subject over time. Control samples were taken from multiple animals from the same colony, indicated by open circles. Only serum cholesterol differed between treated and control subjects, and the rise in cholesterol only occurred transiently one month after injection. Abbreviations: aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGTP), alkaline phosphatase (Alk Phos), blood urea nitrogen (BUN), and creatine kinase (CK) (CK isoenzymes denoted by CKMM, CKMB, and CKBB).
**NHP Terminal observations**

At approximately 6 months post-injection, a final veterinary evaluation and necropsy was performed on each subject. Again, the veterinary staff found all subjects to be healthy, alert, and active. Immediately prior to necropsy, we collected blood for immunology studies, and immediately following euthanasia we collected samples from major organs for analysis of pathology and biodistribution of expression. During sample collection, veterinary staff performed a preliminary review of internal organs and found all tissues to be normal in appearance with no signs of overt pathology.

**NHP Biodistribution**

RNA was extracted from internal organs from all subjects to quantify the extent and distribution of long-term transgene expression. Quantitative RT-PCR was used to assess vector derived mRNA abundance. Indeed, expression was noted in almost all tissues examined (**Figure 26**). Only the intestines showed little or no expression, consistent with our previous findings by immunofluorescent detection [9]. Human and cynomolgus SMN protein sequences are >90% identical which made transgene expression at the protein level impossible to determine. In order to most closely assess the safety of the construct intended for clinical use, epitope tags to distinguish vector derived from endogenous protein were not used.
Figure 26 Primate Biodistribution of Expression. To determine level of SMN transgene expression, cDNA was synthesized from RNA isolated from the tissues indicated. Quantitative PCR results are reported here on a logarithmic scale for each individual subject. Abbreviations: Spinal cord (SC), transversus abdominus (TVA), quadriceps (Quad), tibialis anterior (TA), reverse transcription (RT), control (CTL).
Histopathology

Histological samples were sent to a third-party pathologist for histopathological analysis. Despite the very high degree of transgene expression is in muscles, liver, heart and adrenal tissues, histopathological findings were minimal, similar to normal findings in non-injected subjects.

*NHP subjects developed appropriate antibody response without lasting cytotoxic T-lymphocyte response*

To assess humoral immune response, we determined endpoint titers of anti-AAV9 and anti-SMN antibodies by ELISA. All subjects were negative for anti-SMN antibodies, which was expected due to ubiquitous expression of host-derived Smn and the high homology between natively expressed Smn and transgenically-derived SMN. However, each subject had high anti-AAV9 titers of at least 1:12,800 which is consistent with our previous findings in AAV9-injected non-human primates. Cellular immune responses to both the SMN transgene and the AAV9 capsid were measured in peripheral blood six months post vector delivery, and there was no detectable cytotoxic T-lymphocyte response to either transgene or AAV9 capsid in any of the four animals (Figure 27). As sufficient blood volume was unavailable for immunological assays prior to the six months time point, we cannot be certain that immunity to these proteins was absent at earlier time points after vector delivery. Regardless, any immune response that did occur did not eliminate transgene expression from transduced tissues. Previous studies have correlated an increase in ALT with an immune response to AAV capsid in the liver of some human subjects following
intraportal delivery of an AAV vector [10]. However, serum levels of AST, ALT, GGTP or CK did not increase at any point during this study.

Figure 27 Cytotoxic T-cell response to capsid and SMN peptide libraries by ELISpot. Circulating cytotoxic T-cells that responded to either AAV9 capsid or SMN were not detected by 6 months post injection in any subject. Abbreviations: interferon-gamma (IFN-γ), spot forming cells (SFC), peripheral blood mononuclear cell (PBMC).

Discussion

The vast majority of gene therapy applications currently being used or tested in the clinical setting are performed as discreet injections of vector to target tissues, though vascular administration of newer AAV serotypes show promise[11]. Recently, systemic delivery of AAV9 has been shown to be of potential benefit in mouse models of mucopolysaccharidosis-III (MPS-III) and SMA [8, 12, 13]. However, the therapeutic doses predicted by studies in SMA mice are ~100 times higher than previous clinical efforts [11]. Therefore the safety profile of systemic scAAV9.CB.SMN in both neonate
rodents and non-human primates shown in this report is exciting due to the unprecedented doses used and the ages of the animals studied.

Spinal muscular atrophy is an attractive candidate for systemic gene therapy for many reasons. First, the disease affects motor neurons throughout the length of the spinal cord, which makes focal injections an impractical solution. Second, SMA is caused by insufficient levels of full-length SMN expression, but not by a complete lack thereof. Therefore, transgenic SMN should be seen as a “self” antigen to the immune system and evade any cellular or humoral immunity because the protein was present in the developing thymus. Third, SMN is ubiquitously expressed, required in every tissue, and overexpression is not associated with toxicity in mice or humans. Off-target expression is not as important a consideration for systemic SMN gene delivery as it may be for other disease paradigms. In fact, we and others have shown that SMA patients may need increased SMN expression in cells other than lower motor neurons [4]. Since SMN is likely not to cause any immune or toxic response itself, this study provides more generalizable data regarding the safety of systemic gene delivery by high-titer AAV9. Remarkably, systemically-delivered AAV9 at very high doses appears to be well-tolerated in all mice assessed, with weight gain, hematology and most serum chemistry values matching between treated and control cohorts. The only serum chemistry value differing between treated and control cohorts was the elevation in 90-day CK in treated mice. Importantly, the CK values equalized between groups at the second and final time point, implying no lasting effects. This transient rise may be due to tissue damage or stress, but CK values are also highly sensitive to how the animal was handled at the time
of collection. The mode of blood collection at 90 days was via the tail vein, but subsequent collection was via mandibular sinus puncture or direct cardiac puncture, and this may have influenced the initial CK values.

Of all hematology and serum chemistry values in primates, serum cholesterol was the only test to show a significant change compared to baseline in 3 of the 4 injected subjects. Importantly, cholesterol levels recovered to baseline by the second month post-injection and remained low for the duration of the study. While this may be a subtle indication of liver stress due to AAV9 infection and/or clearance of viral particles, a transient rise in serum cholesterol without a coinciding rise in AST, ALT or GGTP is of no clinical consequence.

Considering the very high doses used in the mice and non-human primates of this study, the hematology, serum and histopathology results are a remarkable testament to the potential safety of AAV9. This study shows that systemically-delivered AAV9 at these high doses transduces cells throughout the body with particularly high tropism for liver, skeletal and cardiac muscle, confirming previously published data [9]. Despite high transduction and expression in these tissues (Figure 26) lab values that give insight into the health of these systems (especially AST, ALT, GGTP, and CK) show no cause for concern.

Additionally, the remarkable safety profile outlined in this study is likely influenced by other factors such as route of administration, age of subjects at treatment and transduction of key cells within the liver and spleen. By delivering via the vasculature, adverse effects of direct tissue damage, such as inciting more robust anti-capsid immune responses, are
avoided[10]. All the research showing efficacy of AAV9-SMN gene therapy supports early treatment of type 1 SMA patients, ideally prior to 6 months of age. We have modeled this approach by injecting young mice and NHPs, and the absence of persistent T-cell-mediated immunity against transgene and especially capsid may be due in part to the young immune systems in these subjects. Finally, as shown previously [9], when primates are systemically injected with AAV9, hepatic tissue is almost globally transduced and germinal centers within the spleen likewise produce detectable levels of transgene. Based on morphology, these cell types are likely dendritic antigen presenting cells (APCs). A recent study showed multiple mechanisms whereby transduction of liver APCs might lead to immune tolerance to transgene[14]. While immunity to transgene is even less likely when treating SMA patients, this is perhaps an important positive safety consideration when over-expressing transgenic SMN or other genes of interest.

Finally, there have been numerous studies that support not only the safety of increased peripheral SMN expression, but also a potential need to transduce non-motor neuronal tissue[4, 15, 16]. The data included in this report increase our confidence that systemic gene therapy in SMA patients will be safe. When coupled with previous pre-clinical efficacy studies by us and others, our optimism for clinical success is increasingly justified, especially if applied to children early in their disease progression.

Methods

Viral production

The vector, scAAV9.CB.SMN, was produced by transient transfection of 293 cells with a double-stranded AAV2-ITR–based CB-SMN vector, with a plasmid encoding Rep2Cap9
sequence as previously described along with an adenoviral helper plasmid pH Helper (Stratagene). Our serotype 9 sequence was verified by sequencing and was identical to that previously described [5]. Virus was produced by combination of multiple batches and purified by tangential flow filtration, iodixanol centrifugation, dialysis against PBS and formulated with 0.001% Pluronic-F68 to prevent virus aggregation and stored at 4°C. All vector preparations were titered by DNAse-resitant particle assay using Taq-Man technology and a primer-probe set targeting the CMV enhancer sequence. Purity of vectors was assessed by 4–12% SDS-acrylamide gel electrophoresis and silver staining (Invitrogen).

In order to ensure the bioactivity of the scAAV9.CB.SMN test article, we injected a group of Δ7 mice with $3.3 \times 10^{14}$ vg/kg on the day of birth (P1). All mice lived over 21 days, confirming the titer and in vivo potency of the test article.

**Animal care and use**

All procedures performed were in accordance to either the Mannheimer Foundation or the Research Institute at Nationwide Children’s Hospital Institutional Animal Care and Use Committees.

**Mouse intravascular vector delivery**

Newborn wild type FVB/n mice (Jackson labs, Bar Harbor, ME) were injected with $5 \times 10^{11}$ vg in a total volume of 50 ul as previously described [5].

**Non-human primate intravascular vector delivery**

Cynomolgus macaques (*Macaca fasciculata*) were bred, treated and cared for at the Mannheimer Foundation (Homestead, FL). Briefly, veterinary staff anesthetized the
subject and placed a catheter into the saphenous vein, through which $6.7 \times 10^{14}$ vg/kg scAAV9.CB.SMN was infused over a period of 2-3 minutes. Upon recovery, subjects were returned to their mother and housed under routine conditions for the duration of the study.

*Serum Titers*

Seronegativity for anti-AAV9 antibodies was confirmed in all non-human primates by ELISA. Briefly, a $2 \times 10^{10}$ vg/ml solution of empty AAV9 capsids was made with a carbonate coating buffer and applied to a 96 well plate and incubated over night at $4^0$C. The following day, the plate was washed and blocked with a 5% milk solution in PBS with 0.1% tween-20. Sera were diluted from 1:50 - 1:6,400 and incubated at room temperature for an hour. The wells were washed with PBS with 0.001% Tween-20 (PBS-T) and then incubated with an HRP conjugated anti-monkey secondary (Sigma-Aldrich) for one hour at room temperature. The wells were washed with PBS-T then developed with TMB. The reaction was stopped with the addition of hydrochloric acid and absorbance was read at 650nm on a plate reader.

To determine endpoint anti-AAV9 titers, we performed the assay described above, with serial dilutions from 1:50 to 1:409,600.

*Histology and Microscopy*

Postmortem mouse and NMP tissues were post-fixed in 10% neutral buffered formalin, transferred to 70% ethanol overnight and embedded in paraffin. Sections were cut at 4um, deparafinized and stained with hematoxylin and eosin.

*Expression analysis*
NHP tissue samples were collected at necropsy and placed immediately in Trizol reagent and frozen at <70°C until extracted according to standard Trizol protocol. After RNA extraction, cDNA libraries were synthesized by the RT²-first strand kit (Qiagen). SYBR Green-based qPCR was performed on the cDNA from each sample. A vector specific primer pair was designed with the forward primer annealing to vector-derived 5’-UTR and the reverse primer annealing to exon 1 of the SMN transgene.

ELISpot assay.
Peripheral blood T cell responses to SMN and AAV9 capsid were quantified by IFN-γ ELISpot assay. Briefly PBMC isolated on Ficoll hypaque gradients were cultured with synthetic peptides (18 amino acids in length, overlapping by 11 residues) that spanned both the SMN and AAV9 capsid protein. Peptides were organized into pools designated SMN, AAV9-1 and AAV9-2. SMN represented entire SMN protein. AAV9-1 represented the first half of the capsid peptide library and AAV9-2 represented the second half of the capsid peptide library. After incubation at 37°C for 36 hr IFN-γ spot forming cells (SFC) were counted. Fewer than 10 SFC/well were observed in the Negative Control wells where cells were incubated with 10% DMSO and 90% sterile distilled water, the same solution in which the peptides were reconstituted. Responses were considered positive when SFC exceeded 50 per 10⁶ PBMC in duplicate wells.

Acknowledgements
This work was supported by NIH RC2 NS69476-01 (to AHMB and BKK), Families of SMA (to BKK) and the OSU CCTS (to AKB). AKB, KDF, EN, CW, JRM and BKK designed and executed the experiments. AKB and BKK wrote the manuscript. PRM
performed the primate experiments. LB and LS contributed to animal care and tissue analysis. KC performed ELISpot analysis. AM and KRC produced viral vectors.

References


Chapter 5: Early heart failure in the Δ7SMN model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery

This work was published in the peer-reviewed journal Human Molecular Genetics, and it is the result of collaboration with the authors listed below. I designed and performed experiments and wrote the manuscript.

Authors
* These authors contributed equally

Abstract
Proximal spinal muscular atrophy (SMA) is a debilitating neurological disease marked by isolated lower motor neuron death and subsequent atrophy of skeletal muscle. Historically, SMA pathology was thought to be limited to lower motor neurons and the skeletal muscles they control, yet there are several reports describing the coincidence of cardiovascular abnormalities in SMA patients. As new therapies for SMA emerge, it is necessary to determine whether these non-neuromuscular systems need to be targeted. Therefore, we have characterized left ventricular (LV) function of SMA mice (SMN2+/+; SMNΔ7+/+; Smn-/-) and compared it to that of their unaffected littermates at 7 and 14 days of age. Anatomical and physiological measurements made by electrocardiogram
(ECG) and echocardiography show that affected mouse pups have a dramatic decrease in cardiac function. At 14 days of age, SMA mice have bradycardia and develop a marked dilated cardiomyopathy with a concomitant decrease in contractility. Signs of decreased cardiac function are also apparent as early as 7 days of age in SMA animals. Delivery of an SMN1 transgene using a self-complementary adeno-associated virus serotype 9 abolished the symptom of bradycardia and significantly decreased the severity of the heart defect. We conclude that severe SMA animals have compromised cardiac function resulting at least partially from early bradycardia, which is likely attributable to aberrant autonomic signaling. Further cardiographic studies of human SMA patients are needed to clarify the clinical relevance of these findings from this SMA mouse.

Introduction

Proximal spinal muscular atrophy (SMA) is a devastating disease that affects 1 in 5,000-10,000 newborns, and is one of the leading genetic causes of infant death in the United States [1]. SMA is caused by a deletion or mutation of the survival motor neuron-1 (SMN1) gene along with retention of the SMN2 gene [2-3]. SMN1 and SMN2 essentially differ by a single nucleotide in exon 7 which disrupts the splicing of SMN2 pre-mRNA such that the majority of the mRNA from SMN2 lacks exon 7 which produces a protein that is ineffective at oligomerization and thus gets rapidly degraded as it does not incorporate into the SMN complex [4-13]. The SMN genes express SMN ubiquitously in all cells, however, high levels of SMN appear to be particularly important for motor neurons as depletion of SMN results in the dysfunction and death of lower motor neurons characteristic of SMA; the exact mechanism or reason for this selectivity is unknown [3].
The copy number of $SMN2$ and the amount of SMN correlates with phenotypic severity with mild patients generally having a high copy number of $SMN2$ and higher levels of SMN protein. [4-5, 14-15]

The most severe form of SMA is type 0 with clear symptoms at birth, followed by type 1 SMA patients who usually die within 2 years. As these patients have the lowest SMN levels as well as $SMN2$ copy number they are the most likely to show defects in cells other than the motor neuron. A few key studies regarding SMA patients have implicated the involvement of cardiovascular and autonomic nervous systems. An early study reported symptomatic cardiac involvement in patients with a milder form of SMA, although the population studied was not genetically defined [16]. A retrospective study of Type 1 SMA patients identified that 15 of 63 SMA patients experienced symptomatic bradycardia [17]. Another report showed an increase in the coincidence of congenital heart defects in very severe type 0 SMA patients. The most common abnormality noted was a septal defect in the atria and/or ventricles [18]. Others performed a battery of autonomic tests on Type 1 SMA patients and reported sympathetic-vagal imbalance, fluctuation of blood pressure, and abnormal skin responses to temperature changes [19]. Whether SMN deficiency is the direct cause for any of these symptoms is still unproven, and further study is required to define the extent of non-motor neuron involvement in SMA.

A number of mouse models of SMA have been developed. The first models had mouse $Smn$ disrupted and contained $SMN2$ [20-21], with low copies of $SMN2$ leading to SMA and high copy number of $SMN2$ rescuing the SMA mice. Subsequently it has been shown
that the transgenic addition of SMN cDNA lacking exon 7 (SMNΔ7, the major gene product of SMN2) extends the life span of mice with low SMN2 copy number and is thus beneficial [22].

We recently demonstrated the efficient transfer of SMN1 in postnatal SMA mice (SMN2+/+, SMNΔ7+/+, Smn-/-, referred to also as the SMNΔ7 mouse) using self-complementary adeno-associated virus, type 9 (scAAV9) [23]. SMA mice of this type die at 15 days of age when not treated. Our treated mice survived to well over 200 days of age and showed no signs of motor neuron disease. Another approach using gene delivery with AAV vectors in severe SMA mice demonstrated similar findings, yet reported potential cardiac manifestations [24]. The treated animals in our study were healthy, but displayed limited necrosis of the eartips which resolved. Other treatments for SMA that extend life to a lesser extent have been reported to result in more widespread necrosis [25]. In addition, some milder SMA mice show signs of distal necrosis [21, 26]. Lastly, some SMA patients are also reported to have decreased blood flow to the extremities leading to discoloration and necrosis, as well as episodes of inappropriate sweating (hyperhydrosis), further implicating dysfunction of the autonomic nervous system [27]. One possible explanation for the abnormal blood flow is altered autonomic tone to the cardiovascular system.

The suggestion of altered blood flow in SMA as well as the presence of bradycardia in some SMA patients prompted us to examine whether a cardiac deficit is present in the SMNΔ7 mouse model of SMA and whether any defect found could be corrected by our scAAV9-SMN postnatal gene delivery treatment. Echocardiographic measurements and
electrocardiographic (ECG) analysis of the heart revealed that SMA animals are bradycardic and develop a dilated cardiomyopathy by 14 days of age, with less severe abnormalities detected as early as 7 days of age. Structural modifications assessed by histological analysis correlated with the in vivo functional findings. Remarkably, scAAV9-SMN therapy improved LV remodeling and fully corrected heart rate. While the mechanisms fully responsible for these cardiac deficits are unknown, neuronal (autonomic) and developmental components may be implicated. Therefore, it is important to investigate whether SMA patients of various severities have or will manifest cardiac deficits.

Results

Echocardiographic analysis of postnatal day 14 SMA mice

Our first step in assessing heart function was to analyze in vivo cardiac structure in p14 SMA mice using echocardiography. We studied three groups of animals: healthy wild type (WT) mice, affected SMA mice and scAAV9-SMN-treated SMA mice. SMA mice have smaller hearts compared to WT, indicated by decreased LV mass (SMA: 27.17 ± 4.46 mg, vs. WT: 67.89 ± 1.47 mg, p<0.001) (Figure 1 A). We also noted that ventricular walls were significantly thinner in these animals (SMA: 0.34 ± 0.02 mm vs. WT: 0.68 ± 0.03 mm, p< 0.001) (Figure 28 B). These findings could partially be due to the small size of SMA mice, so we next compared LV wall thickness (posterior wall dimension, PWD) to the LV diameter in diastole (2xPWD/LVDD) in order to normalize and compare LV structure in each group. This ratio is lower in SMA mice (SMA: 0.24 ± 0.01 vs. WT: 0.45 ± 0.03, p<0.001), suggesting eccentric hypertrophy [28] (Figure 28 C). Interestingly, LV
mass was increased in p14 scAAV9-SMN-treated animals, compared to SMA mice (AAV9: 42.73 ± 2.451mg vs. SMA: 27.17 ± 4.460mg, p=0.01, vs. WT: 67.89 ± 1.47mg, p<0.001), and both the extent of dilation (2xPWD/LVDD – AAV9: 0.39 ± 0.03, vs. SMA: 0.24 ± 0.01, p<0.01, vs. WT: 0.45 ± 0.03, p=0.28) and wall thinning (AAV9: 0.64 ± 0.03mm, vs. SMA: 0.34 ± 0.02 mm, p<0.001, vs. WT: 0.68 ± 0.03mm, p=0.16) was attenuated and returned towards WT values.

Figure 28 Echocardiographic measurements of left ventricular (LV) mass (A), wall thickness (B) and 2xPWD/LVDD (C) show significant decreases in SMA mice compared to wild type (WT) and scAAV9-treated animals, suggesting that SMA mice are undergoing eccentric hypertrophy and are at increased risk for heart failure. While scAAV9-treated mice also have decreased LV mass compared to WT, wall thickness and 2xPWD/LVDD measurements are not significantly changed. Symbols indicate p<0.05 when comparing SMA to WT (*), AAV9 to WT (#) and AAV9 to SMA (+).

It is possible that the abnormal heart dimensions represent a slower growth of the heart in proportion to the stunted growth of SMA animals. Therefore, we compared heart weight to body size post-mortem. We used tibia length as a surrogate measure for body size, since SMA animals experience increased muscular atrophy, thereby making a
comparison to body weight invalid. Tibia lengths (TL) in SMA animals are significantly smaller than those of WT animals at both 7 days (WT: 5.15 ± 0.05 mm vs. SMA: 4.87 ± 0.033 mm, p=0.016) and 14 days of age (WT: 10.65 ± 0.35 mm vs. SMA: 7.33 ± 0.17 mm, p=0.0023) (Figure 29 A-B). Heart weight to tibia length ratios (HW:TL) appear slightly decreased in SMA mice of both 7-day-old (WT: 6.29 ± 0.0028 mg/mm vs. SMA: 5.65 ± 0.21 mg/mm, p= 0.099) and 14-day-old mice (WT: 4.55 ± 0.75 mg/mm vs. SMA: 3.95 ± 0.032 mg/mm, p= 0.36), but these differences were not significant (Figure 29 C-D). This implies that the small cardiac mass of SMA mice is correctly proportional to their small body size and that cardiac dilation (2xPWD/LVDD) is not an artifact of an overall reduction in heart mass or stunted growth. We next confirmed that the small size of the SMA animals did not invalidate other cardiac dimensions observed by echocardiography. To do this, we normalized all wall thickness measurements to the average TL for each group. We found similar differences in wall thickness between SMA and WT animals remains despite normalization to TL in both 7-day (WT: 0.094 ± 0.0028 vs. SMA: 0.074 ± 0.0042, p=0.0017) and 14-day old animals (WT: 0.064 ± 0.0031 vs. SMA: 0.049 ± 0.0028, p=0.0086) (Figure 29 E-F).
Comparisons of heart measures to tibia length. Tibia lengths (TL) were found to be significantly smaller in SMA mice at both 7- (A) and 14-day (B) time points. Heart weight to tibia length ratio (HW:TL) in 7- (C) and 14-day-old (D) mice. HW:TL values are decreased in SMA mice of both 7-day-old (p7 WT: 6.29 ± 0.0028mg/mm vs. p7 SMA: 5.65 ± 0.21, p= 0.099) and 14-day-old (p14 WT: 4.55 ± 0.75mg/mm vs. p14 SMA: 3.95 ± 0.032mg/mm, p= 0.36).

Given that there were significant structural changes occurring in the heart, we next evaluated functional measurements using highly sensitive echocardiography.
Echocardiography allows for precise measurements of cardiac function including heart rate, stroke volume, cardiac output, fractional shortening and Tei index. To perform these studies, we analyzed 5-8 mice in each group using non-invasive measurements with isoflurane gas anesthesia. Anesthesia may affect certain measurements, including heart rate, but given our previous experience in collecting cardiovascular measurements with this protocol along with adequate control mice that were anesthetized, our results are reliable measurements for cardiac function in vivo. As expected based on echocardiographic visualization of the heart, LV function in SMA mice was severely impaired at 14 days of age (Figure 30). We found that SMA mice presented with a strikingly severe sinus bradycardia, a slowing of heart rate (SMA: 320 ± 19bpm vs. WT: 452 ± 10bpm; p<0.001). We next tested SMA mice treated with scAAV9-SMN to determine whether restoring SMN levels could improve the heart rate. Indeed, scAAV9-SMN delivery, which targets neurons within the central and peripheral nervous system as well as the cardiac tissues, completely restore heart rates in treated animals to comparable levels of WT controls (AAV9: 448 ± 11bpm, vs. SMA: 320 ± 19bpm, p<0.001, vs. WT: 452 ± 10bpm; p=0.97) (Figure 30 A). scAAV9-GFP delivery had no negative or beneficial cardiac effects on SMA treated animals for any measurements discussed below (data not shown) and therefore, we analyzed non-injected SMA controls for all remaining studies. Stroke volume (SV), the volume of blood pumped with each contraction, was also severely decreased in SMA mice (SMA: 13.08 ± 2.00μl, vs. WT 22.94 ± 5.35μl, p=0.0025) and delivery of scAAV9-SMN failed to increase levels to that of WT animals (AAV9: 12.85 ± 3.24μl, vs. SMA: 13.08 ± 2.00μl, p=0.891, vs. WT: 22.94 ± 5.35μl,
Cardiac output (heart rate x SV) is significantly decreased in SMA animals (SMA: 4.16 ± 0.30ml/min, vs. WT: 10.29 ± 0.70ml/min, p<0.001). As expected from the above data, scAAV9-SMN improved, but did not restore, cardiac output compared to WT mice (AAV9: 5.75 ± 0.62ml/min, vs. SMA: 4.16 ± 0.30ml/min, p=0.059, vs. WT: 10.29 ± 0.70ml/min, p<0.001;), mainly due to the improved heart rate (Figure 30 C). While the cause of death of SMA mice has been speculated to be due to respiratory failure, animals in our study displayed a high risk for developing a dilated cardiomyopathy leading to a fatal congestive heart failure as evidenced by the decreased stroke volume and eccentric hypertrophy.
Figure 30 Echocardiographic measurements of cardiac function in p14 mice. Cardiac function of the SMA mice was significantly lower than that of WT mice when we assessed heart rate (A), stroke volume (B), cardiac output (C) and fractional shortening (D). scAAV9-treated animals have heart rates indistinguishable from WT (A), but stroke volume (B) is similar to SMA animals, thereby decreasing overall cardiac output as well (C). Fractional shortening (FS), a measure of contractility, is decreased in both SMA and AAV9 mice, though scAAV9-treated mice contract significantly better than untreated SMA mice (D). Tei index is increased in SMA mice, consistent with worse combined systolic and diastolic function, whereas scAAV9-treated mice have similar values to WT, indicating preserved function (E). Symbols indicate p<0.05 when comparing SMA to WT (*), AAV9 to WT (#) and AAV9 to SMA (+).

To assess contractility, m-mode measurements of the changing ventricular diameter over the cardiac cycle were used to calculate fractional shortening (FS%). We found that SMA mice showed a significant decrease in FS% (SMA: 20.82 ± 2.05% vs. WT: 39.19 ± 0.47%, p< 0.001), consistent with the lower stroke volumes already observed.
Contractility was increased by scAAV9-SMN treatment, yet it did not completely restore fractional shortening to WT levels (AAV9: 27.29 ± 1.07%, vs. SMA: 20.82 ± 2.05%, p<0.01, vs. WT: 39.19 ± 0.47%, p<0.001) (Figure 30 D). Additionally we assessed the LV Tei index, which accounts for aspects of both systolic and diastolic function [(isovolumic contraction time + isovolumic relaxation time)/aortic ejection time] [29-31]. SMA mice, as expected, had a significantly higher LV Tei index than WT (SMA: 0.79 ± 0.09 vs. WT: 0.40 ± 0.02, p<0.001), indicating an overall decrease in function. Treatment with scAAV9-SMN however restored the LV Tei index to levels that were nearly identical to WT mice indicating that gene delivery of SMN postnatally could restore an important measure of cardiac function to normal levels (AAV9: 0.38 ± 0.05, vs. SMA: 0.79 ± 0.09, p<0.01, vs. WT: 0.40 ± 0.02, p=0.73) (Figure 30 E).

Echocardiography of p7 animals

Given that we found such dramatic cardiac deficits in structure and function of SMA mice at 14 days of age, we next investigated whether we could detect cardiac abnormalities earlier in the disease (Figure 31). Indeed, SMA mice at 7 days of age presented with early anatomical differences in both left and right ventricles compared to WT animals. LV mass was decreased significantly in SMA mice (SMA: 17.94 ± 2.03mg, vs. WT: 33.50 ± 1.03mg, p<0.001) and treatment with scAAV9-SMN in postnatal day 1 mice led to an increase in mass, yet did not restore mass to WT levels (AAV9: 23.36 ± 1.65mg, vs. SMA: 17.94 ± 2.03mg, p=0.077, vs. WT: 33.50 ± 1.03mg, p<0.001) (Figure 31 A). Wall thickness was significantly decreased in SMA mice (SMA: 0.36 ± 0.02mm vs. WT: 0.47 ± 0.02mm, p<0.01), yet scAAV9-SMN delivery completely preserved wall
thickness in treated animals to similar levels as WT controls (AAV9: 0.43 ± 0.02 mm, vs. WT: 0.47 ± 0.02 mm, p=0.15) (Figure 31 B). We next used the same measure of eccentric dilation as described above (2xPWD/LVDD), and found that the ratio was smaller in SMA mice (SMA: 0.30 ± 0.02 vs. WT: 0.40 ± 0.02, p<0.01), yet was partially corrected in scAAV9-treated mice to levels similar to WT controls (AAV9: 0.35 ± 0.02, vs. SMA: 0.30 ± 0.02, p=0.078, vs. WT: 0.40 ± 0.02, p=0.09) (Figure 31 C).

Figure 31 Echocardiographic measurements of cardiac function in p7 mice. As with p14 animals, measures of LV mass (A), wall thickness (B), dilation (C), heart rate (D), fractional shortening (E), and Tei index (F) all indicate decreased function in SMA mice, while functional measures in scAAV9-treated animals are relatively preserved. Symbols indicate p<0.05 when comparing SMA to WT (*), AAV9 to WT (#) and AAV9 to SMA (+).
In the course of our functional measurements, the most striking cardiac finding in postnatal day 7 SMA animals was significantly lower heart rates compared to WT animals (SMA: 354 ± 18 bpm vs. WT: 444 ± 15 bpm, p<0.001). Similar to postnatal day 14 animals, delivery of scAAV9-SMN at 1 day of age increased heart rates to levels similar to controls (AAV9: 439 ± 18 bpm, vs. SMA: 354 ± 18 bpm, p<0.01, vs. WT: 444 ± 15 bpm, p=0.83) (Figure 3D). We found as early as 7 days of age that SMA mice had a significant decrease in fractional shortening (SMA: 17.20 ± 2.23% vs. WT: 37.3 ± 0.29%, p<0.001) as assessed by m-mode echocardiography. Delivery of scAAV9-SMN significantly increased this measure of contractility. However, scAAV9-treated mice still had much lower fractional shortening compared to WT (AAV9: 25.84 ± 1.6%, vs. SMA: 17.20 ± 2.23% p< 0.01, vs. WT: 37.3 ± 0.29%, p<0.001) (Figure 3E). Tei index in SMA mice was increased at this early timepoint (SMA: 0.54 ± 0.04 vs. WT: 0.33 ± 0.02, p<0.01), however this increase was smaller than that seen in 14-day-old animals (i.e. a difference between SMA and WT of 0.22 in 7-day-olds vs. 0.39 in 14-day-olds), suggesting a gradual progression of heart failure. Tei index was partially preserved in scAAV9-treated mice at 7 days of age, demonstrating an early effect of scAAV9-SMN treatment (AAV9: 0.44 ± 0.04, vs. SMA: 0.54 ± 0.04, p=0.119, vs. WT: 0.33 ± 0.02, p<0.05) (Figure 3F).

Histopathological analysis of p14 and p7 animals

We next evaluated the hearts by histological analysis to determine the pathological nature of the cardiac deficits occurring early in SMA mice. Gross analysis of the heart confirmed the echocardiographic findings of dilated ventricles and thinning walls on both
p7 and p14 SMA mice. Pathological analysis of sections from the hearts of SMA mice using hematoxylin and eosin (H&E) staining showed no signs of inflammation, ischemia, or cellular disorder (Figure 32 A-D). We next performed stains to determine if there was early damage leading to collagen deposition in the heart. Indeed, in models of eccentric dilation, a change in the percentage of collagen volume has been observed and is used as an additional marker for cardiac tissue damage. To assess our SMA mice we quantified the amount of collagen present in SMA hearts. We found no increase in collagen in 14 day old SMA mice compared to WT animals using Mason’s trichrome and picrosirius red (PSR) stains, indicating no fibrosis occurring at these time points.
Figure 32 Cardiac histology of p14 mice. H&E mid-ventricular sections show the typical thinning heart walls and dilated ventricles of SMA mice (B) compared to WT (A) (tiled images, scale = 500μm). Higher-power images (40x magnification, scale = 30μm) of WT (C) and SMA mice (D) both show absence of inflammation or other signs of overt tissue pathology at p14. Transmission electron micrographs (TEM) of WT mice (E) show normal mitochondria (M) and myofibers with well-organized fibrils and well-defined sarcomeres (asterisk = Z-line) (scale = 1μm). TEM sections of SMA heart tissue (F) show swollen myofibers with disorganized Z-lines (asterisk) and no clearly defined I bands, A bands or H zones (scale = 1μm). Mitochondria in SMA myocytes are swollen with evidence of crystolysis (mitochondria marked by ‘M’) and in many fields these organelles occupy most of the sarcoplasm. The inset (F) shows a clearer view of a degenerating mitochondria with myelin figure formation (evidence of advanced crystolysis and membrane degradation) (arrowhead) (scale = 0.5μm).

While there were significant cardiac functional deficits with no associated pathological abnormalities of cardiomyocytes based on histological evaluation, we next wished to
analyze cardiomyocytes of SMA animals by transmission electron microscopy (TEM) in order to further examine the ultrastructure of cardiomyocytes. Analysis of SMA hearts surprisingly revealed significant ultrastructural changes in cardiomyocytes (Figure 32 E-F). In 14-day-old SMA cardiomyocytes, mitochondria were found to be swollen in size. In some instances, the myocyte sarcoplasm was nearly filled with mitochondria. Numerous mitochondria also showed degenerative changes such as the fragmentation of cristae along with associated vacuolar degeneration (Figure 32 F, inset). There was marked disorganization and degeneration of myofibers with focal areas of vacuolar change and generalized loss of sarcomeric detail, where only Z lines remain clearly defined (Figure 32 F, astrisk). The presence of myelin bodies throughout the tissue indicates increased organelle and/or membrane turnover, implying significant cardiac dysfunction. We also found that cardiac myocyte diameter was similar between SMA and WT mice at 14 days (WT: 9.63 ± 0.38 μm, vs. SMA: 11.83 ± 1.50 μm, p= 0.34) (Figure 33), which is contrary to what is seen in skeletal muscle of SMA mouse models and patients. In fact, there was a slight increase in cardiomyocyte diameter that could be due to the changes noted by TEM of swollen myofibers and mitochondria, although this increase was not statistically significant.
Cardiomyocyte diameter in 14-day-old mice. Cardiac myocyte diameter was mildly, but not significantly increased in 14-day-old (A) SMA mice (WT: 9.63 ± 0.38μm, vs. p14 SMA: 11.83 ± 1.50μm, p=0.34).

**Dobutamine Stress Challenge**

In order to determine whether the functional and ultrastructural changes were due to decreased sensitivity of the heart to autonomic stimulation, we next recorded our animals’ responses to dobutamine (Figure 34). Dobutamine is a sympathomimetic which should increase both heart rate and contractility in hearts that express the correct complement of adrenergic receptors. As expected, both WT and SMA animals responded appropriately by increasing heart rate 4 minutes after dobutamine injection (percent increase over baseline heart rate - SMA: 24.93 ± 4.92% vs. WT: 27.87 ± 6.18%, p=0.717) (Figure 34 A). Similarly, contractility (as indicated by FS%) increased significantly in WT and SMA groups after injection, though SMA animals responded to a lower degree (percent increase over baseline FS% - SMA: 14.58 ± 4.16% vs. WT: 45.36 ± 4.59%,
p<0.001) (Figure 34 B), likely due to decreased contractile reserve, which is common in heart failure [32].

![Graph showing change in heart rate and fractional shortening between WT and SMA mice.](image)

Figure 34 Dobutamine stress challenge in p14 WT and SMA mice. Heart rate, as expected, increases upon dobutamine administration in WT and SMA mice (A). Fractional shortening (FS) also increases upon dobutamine administration in both groups, whereas in SMA mice, FS increases to a significantly lesser degree (B). In panel B, * denotes significance (p<0.05) compared to WT.

*Autonomic Targets of scAAV9*

The autonomic nervous system helps to maintain blood pressure in part by altering pacemaker activity and signal conduction in the heart (Figure 35 A). The paraventricular nucleus of the hypothalamus (PVN) primarily sets the tone of the sympathetic nervous system by positively stimulating neurons in the nucleus of the tractus solitarius (NTS). NTS neurons integrate input from the PVN and peripheral baroreceptors and send stimulatory projections to the intermediolateral column of the spinal cord (IML), which project to the paravertebral sympathetic ganglia (SG) and on to the heart, stimulating increased heart rate and contractility through activation of noradrenergic receptors. The
vagal nuclei [motor nucleus of the vagal nerve (MNX) and nucleus ambiguus (NA)] are primarily responsible for decreasing heart rate. Vagal fibers project to the cardiac ganglia (CG), which then decreases heart rate primarily by slowing nodal conduction. Due to the remarkable rescue of the heart rate in scAAV9-treated animals, we sought to determine which neurological structures relating to cardiac pacing were transduced following scAAV9-SMN administration. To do this, we injected neonatal WT animals with scAAV9 carrying a GFP transgene to visualize which tissues were transduced [33]. We visualized all the nuclei and ganglia mentioned above, and high-power images of these autonomic loci are shown in Figure 35 B-I. Of these structures, the MNX, NA, CG and the SG are highly transduced and quantification of these autonomic nuclei using sections of brain, spinal cord and heart revealed 90%, 100%, 60% and 50% trasduction respectively), while the PVN, NTS, and IML revealed no significant transgene expression. Besides these neuronal targets, cardiac myocytes of the ventricles and atria are also very well transduced (over 80%) which has been previously reported [34-35] demonstrating that vascularly delivered scAAV9 can target most of the cardiac tissues as well as the innervating autonomic nervous system that assists in the regulation of cardiac function.
Figure 35  A schematic overview of the key neuronal structures controlling heart rate (A). The sympathetic structures observed were the paraventricular nucleus (PVN), the nucleus of tractus solitarius (NTS), the intermediolateral column of the spinal cord (IML) and the sympathetic ganglia (SG). The parasympathetic structures observed include the vagal motor nucleus (MNX), the nucleus ambiguus (NA), the NTS, and the cardiac ganglia (CG). scAAV9 transduction can be assessed by visualizing the presence of a GFP transgene (B-I). The MNX (D), NA (E), CG (F) and SG (H) were all highly transduced, while we found no evidence of transduction of the PVN (B), NTS (C) and IML (G) neurons. Cardiac myocytes were also found to be mostly positive for the GFP transgene (I). Green = GFP in B-I. Red = tyrosine hydroxylase in B and H, neuronal nuclei (NeuN) in C, choline acetyl-transferase (ChAT) in D-E and G, neurofilament-160 (NF-160) in F, and dystrophin in I. Scale = 50µm in panels B-F, H-I, and 20µm in panel G.
Discussion

While the number of case reports presenting cardiac abnormalities in SMA patients is increasing, there have been few to no highly-powered and controlled studies regarding cardiovascular anomalies in SMA. Despite the existence of mouse models for SMA, to date there have been no reports of cardiac dysfunction in these animals. In this study, we specifically evaluated the potential for cardiac manifestation in a severe model of SMA that is routinely used for drug and therapeutic-based screening. In this new report, we have shown that SMN deficiency leads to early and persistent cardiac dysfunction in mice.

SMA mice display many symptoms of cardiac involvement consistent with what is found in the limited reports on human SMA patients. These similarities include a notable bradycardia similar to what has been reported in over 20% of type 1 SMA patients [17-18]. Interestingly, our results using light and electron microscopy are consistent with an early analysis of an endomyocardial biopsy from a type 3 SMA patient [36]. We found that myocytes in SMA mice have disorganized myofibers, swollen, disorganized mitochondria and evidence of organelle and/or membrane turnover. In the mouse we found that >90% of myocytes exhibited ultrastructural pathology, where only 2-4% of myocytes in the reported type 3 SMA patient demonstrated pathology. This is not surprising, though, given that the SMNΔ7 animals model a significantly more severe disease than what is seen in type 3 SMA patients. To our knowledge, no studies have evaluated cardiac ultrastructural pathology in type 1 SMA patients. The exact cause of mitochondrial swelling seen in SMA mice or its relationship to Smn deficiency is
unknown, however, mitochondrial swelling has been reported in ischemia/reperfusion cardiac injury due to potential increased energy demand on the heart [37]. The increased size of mitochondria in the failing SMA myocardium therefore may be explained as a result of compensatory mechanisms due to increased energy demand. This may also be due to a maladaptive response leading to the release of oxidative cytochromes, and/or the onset of mitochondrial permeability transition, both of which are detrimental to cardiac pump function [38].

Contrary to human case reports, we did not observe any septal defects using echocardiography. This is not surprising as these defects were only reported in very severe type 0 SMA patients [18], and the SMA mice used in this study are thought to model a more moderate phenotype (i.e. type 1 or 2 SMA) [22]. In contrast to the overt, progressive development of DCM in all SMA mice examined in this study, there are few reports of human SMA patients developing DCM [16, 39], and this is mostly reported in patients with less severe forms of SMA. However, this may due to the fact that most type 1 patients die before thorough cardiac evaluation and should be a subject of future study.

Heart rate is regulated by neuronal, hormonal and local mechanisms within the heart. Possible reasons for bradycardia include increased vagal (parasympathetic) tone, decreased sympathetic tone, and/or alteration of the nodal and conduction systems in the heart itself. The limited response to the dobutamine stress challenge is typical of heart failure patients [40], but it demonstrates that the hearts of SMA mice can, at least partially, respond to sympathetic stimulation by increasing heart rate. This evidence, together with the fact that scAAV9-SMN is able to transduce 4 out of 7 key autonomic
loci and rescue the heart rate deficit leads us to believe that autonomic dysfunction may be the primary cause of bradycardia in SMA mice. Correct regulation of heart rate is extremely important – not only to ensure proper perfusion throughout the body, but also to protect the heart against undue stress. Neonatal bradycardia has been linked to dilated cardiomyopathies (DCM) in experimental rodents [41] and in human patients [42-43]. It is not surprising, then, that SMA mice develop a very early DCM, especially since we observed slower heart rates in affected animals as early as 3 days after birth (data not shown). Furthermore, hearts with DCM are more susceptible to increased wall stress, leading to decreased function and remodeling of heart tissue. Progressive remodeling can cause development of both systolic and diastolic dysfunction, consistent with an increased Tei index as seen in our p7 and p14 animals. Independent of neuromuscular function, SMA mice are at high risk for early congestive heart failure which has been under-appreciated as a potential confounding factor in therapeutic studies to date.

It appears that the success of scAAV9-SMN treatment is not solely determined by its ability to transduce spinal motor neurons, but also its ability to target the autonomic nervous system. Early postnatal delivery of scAAV9-SMN was able to treat bradycardia and prevent the early development of DCM. Despite the highly successful rescue of heart rate, the relatively normal wall dimensions and efficient transduction of cardiomyocytes, the hearts of treated animals show decreased contractility compared to WT hearts. These results suggest that additional mechanisms also contribute to SMA associated contractile dysfunction. We cannot rule out the possibility that myocyte stress is inflicted in utero.
by autonomic dysfunction prior to treatment with scAAV9-SMN, or that SMN plays a key role in cardiac development that we are unable to treat postnatally. SMA mice can also be malnourished, and anorexia is already a known cause of cardiac dysfunction [43]. Furthermore, the disordered metabolic state caused by muscular atrophy and respiratory distress further complicate the identification of the mechanisms contributing to SMA-associated contractile dysfunction. Future studies should investigate these potential factors. Additionally, it should be determined whether the deficits are unique to the mouse, and correlation between disease severity and extent of cardiac involvement should be assessed.

As the overall lifespan of SMA patients has increased over the past decade and as new drugs and therapies that extend survival of SMA patients emerge, clinicians should be acutely aware of potential heart dysfunction in a subset of SMA patients. SMA patients already have compromised lung function, so potential causes of any additional pulmonary complication (i.e. from congestive heart failure) should be anticipated and closely monitored. Additionally, SMA patients are commonly intubated for respiratory support and can become autonomically unstable due to vagal irritation. The fragility of these patients and the increasing reports of autonomic dysfunction together with our current findings warrant increased attention to the cardiac status of SMA patients, and it potentially highlights the need to investigate cardiac interventions alongside neuromuscular treatments.
Materials and methods

**AAV9 Injection of Neonatal Mice.**

Animals were injected as previously described (23,33) with $5 \times 10^{11}$ particles of scAAV9-SMN or scAAV9-GFP.

**Anesthetic Protocol**

Anesthesia was induced by placing 7 and 14 day mouse neonates in an induction chamber supplied with 4% isoflurane anesthesia delivered in 100% oxygen at a flow rate of 1L/min. Sedated mice were transferred to a heated pad set to maintain body temperature at 37°C and paws were placed on ECG leads. A nosecone was placed over the nose and 2% isoflurane was administered in 100% oxygen at a flow rate of 1L/min. Excess gases were removed using a vacuum scavenging system.

**Echocardiographic Analysis**

A Visual Sonics 2100 Ultra High Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada) was used to perform echocardiographic studies. Three measures at different cardiac cycles were used for analysis according to standards set forth by the American Society for Echocardiography leading edge method. Examples of how images were analyzed are included in **Figure 37**.
Figure 36 Examples of echocardiographic images and analysis from p14 wild type, SMA and scAAV9-treated mice.

Each individual was analyzed in ~20min. A scan head with a center frequency of 40MHz and lateral resolution of 80µM was used for all measurements. Pre-warmed ultrasound
transmission gel (Aquasonic, Parker Laboratory, Fairfield, NJ) was placed on the scan head. Two dimensional (2-D) long axis images were visualized in the left parasternal position in order to measure the left ventricular outflow tract (LVOT) and calculate the left ventricular mass (LV Mass).

\[
LV \text{ Mass (mg)} = 1.05 \times \{(5/6 \times \text{Epicardial Area};d \times (\text{Epicardial Major};d + T)] - \{5/6 \times \text{Epicardial Area};d \times (\text{Epicardial Minor};d + T)]\}
\]

To determine LV stroke volume (SV) color Doppler was used as an overlay to determine the point of fastest flow in the aorta. Pulse Doppler echocardiographic analysis with a sampling size of 0.6mm was used to measure the flow at this point and the velocity time interval (VTI) was obtained. Stroke volume was calculated using the following formula.

\[
SV (\mu l) = 7.85 \times LVOT^2 \times Ao VTI
\]

Cardiac output was calculated from the measurement of SV.

\[
CO (ml/min) = (SV \times HR)
\]

From the four-chamber view pulse Doppler images were obtained with the sample volume placed just to the left of center of the mitral orifice and at the tips of the mitral leaflets. At this point we obtained images with both the mitral inflow and the LV outflow providing us with the isovolumetric relaxation (IVRT), isovolumetric contraction (IVCT), and aortic ejection time (ET). Using these values we calculated the Tei index.

\[
Tei = (IVRT + IVCT) / ET
\]

M-mode images were obtained at the level of the papillary muscles in order to assess left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD) and LV wall thicknesses. Eccentric hypertophy was was calculated as (2 x
posterior wall dimension/LVEDD). Systolic function was assessed using m-mode calculations of fractional shortening.

\[ FS = \frac{LVEDD - LVESD}{LVEDD} \times 100 \]

**Dobutamine Stress Echocardiography**

After baseline images were acquired, 0.5mg/kg dobutamine was injected intraperitoneally. Heart rate was monitored until a stable peak was reached after ~4 min. M-mode images were acquired at the level of the papillary muscles and FS was calculated.

**Mouse Breeding and Genotyping.** All breeding and subsequent use of animals in this study were approved by the IACUCs of the Research Institute at Nationwide Children’s Hospital in and/or the Ohio State University Columbus, OH. The genotype of all breeding pairs used is human SMN2+/+, human SMNΔ7+/+, and mouse Smn+/-. Upon discovery of new birth, all newborn mice were briefly removed from their mother’s cage, tattooed for identification, and a small piece of tail was taken for genotype analysis. Genomic DNA was extracted and added to two different PCR reactions for the mouse Smn allele (Forward 1: 5′-TCCAGCTCCGGATATTGGGATTG, Reverse 1: 5′-AGGTCCCCACCACCTAAGGAAGCC, Forward 2: 5′-GTTGTCTGGGCTGTAGGCATTGC, Reverse 2: 5′-GCTGTGCCTTTTGGCTTATCTG) and one reaction for the mouse Smn knockout allele (Forward: 5′-GCCTGCGATGTCGGTTTCTGTGAGG, Reverse: 5′-CCAGCGCGGATCGGTCAAGACC).

**Euthanasia and Tissue Collection**
Each animal was given a lethal dose of ketamine/xylazine anesthetic cocktail via IP injection. Intracardiac perfusion with normal saline followed by ice-cold 4% buffered paraformaldehyde (PFA) solution containing 60mM KCl to arrest heart in diastole. All tissues were then removed and post-fixed at least 24 hours in PFA.

**Heart Histology**

Prior to embedding, the base and apex of each heart was dissected from the mid-ventricular portion. Tissues were moved to 70% ethanol for at least 24 hours, then embedded in paraffin. 4μm histological sections were taken and stained using Hematoxylin and Eosin (H&E), Mason’s trichrome and picrosirius acid red (PSR) reagents.

**Post-mortem Heart and Tibia recovery**

After mice were euthanized in a CO₂ chamber according to institutional and AAALAC guidelines, we removed the heart and tibia by gross dissection from the body. Weights were taken after the hearts were cleaned of pluck, chambers were emptied of blood, and tibia were cleaned of extraneous tissues.

**Electron microscopy**

Hearts collected immediately after euthanasia were fixed in 2.5% glutaraldehyde for a minimum of 24 hours and post fixed in 2% osmium tetroxide in 0.1M cacodylate buffer. Tissue was rapidly dehydrated in increasing concentrations of ethanol (30-100%), infiltrated with 100% acetone and embedded in low-viscosity polymerized epoxy resin (Spurr’s). 1200A sections were stained on grids with 1% uranyl acetate and lead
citrate. Electron micrographs were taken with an FEI Technai transmission electron microscope.

**Immunohistochemistry**

Brains and spinal cords were cut to 40μm-thick and stained as floating sections. Tissues were blocked in 10% donkey serum and 0.1-1% Triton-X 100 in TBS solution for 1 hour. Tissues were then incubated at 4°C for 24-48 hours in diluted primary antibodies in blocking solution. Primary antibodies used: Rabbit anti-GFP, 1:400 (Invitrogen); Mouse anti-TH, 1:500 (Sigma-Aldrich), Mouse anti-NeuN, 1:100 (Millipore), Goat-anti-ChAT, 1:200 (Chemicon); Mouse anti-neurofilament 160, 1:500 (Chemicon); Chicken anti-GFP, 1:400 (Abcam); Mouse anti-Dystrophin (Developmental Studies Hybridoma Bank, University of Iowa). After washing, tissues were incubated for 2 hours at room-temperature in FITC- or Cy3-conjugated secondary antibodies diluted 1:200 (Jackson ImmunoResearch), and mounted with PVA-DABCO.

**Data Analysis**

Data were analyzed using Prism statistical software (Graphpad). All results are expressed as mean ± SEM. Differences between groups were considered statistically significant at \( p < 0.05 \). A one-way ANOVA was used to compare echocardiography data between WT, SMA and scAAV9-treated groups. All echocardiographic parameters were assessed three times at different points of the cardiac cycle.
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References


Chapter 6: Muscle enhancement improves phenotype in a mouse model of mild spinal muscular atrophy.

This chapter is a collection of unpublished data that is part of an ongoing study. This study is the result of collaboration with the authors listed below. I designed and executed the experiments described herein.

Authors

Adam K. Bevan, Jennifer Pham, Min Liu, Leah Schmelzer, Dione Kobayashi, Karen Chen, H. Lee Sweeney, Brian K. Kaspar

Abstract

Proximal spinal muscular atrophy (SMA) is a devastating disease caused by motor neuron dysfunction, degeneration and subsequent muscular atrophy due to loss of neuronal stimulation. Recently, some groups have demonstrated pre-clinical success in treating the Δ7 mouse by increasing full-length SMN expression. While each of these studies represents a much-needed step forward for SMA therapy, it is unclear whether increasing SMN in patients late in the progression of their disease course will be of any benefit. Therefore, development of non-SMN-based therapies should also be pursued, especially in older patients with less-severe (Type 2 or 3) SMA. To this end, we tested whether muscle enhancement by follistatin gene therapy can improve the phenotype in the C/C mouse model of mild SMA. While similar methods of muscle enhancement have failed to alter disease course in Δ7 mice, we found that in the milder C/C mouse strength
could be increased despite neuromuscular transmission deficits. This is likely due to the increased proportion of intact motor units in C/C mice, which matches what occurs in Type 3 SMA patients. Therefore, we consider follistatin gene therapy to be an important new therapy option for older, stable, and less-severe SMA patients.

Introduction

Proximal spinal muscular atrophy (SMA) is a devastating neuromuscular disorder, affecting ~1:10,000 newborns worldwide [1]. It is caused by a homozygous deletion or loss of the survival motor neuron-1 (SMN1) gene [2]. Humans have a second survival motor neuron gene (SMN2) that is less efficient than SMN1 at generating full-length SMN product, but it is sufficient to rescue embryonic lethality [3, 4]. The decrease in full-length transcript from SMN2 is due to a single mutation in exon 7, leading to exon 7 exclusion in most mature mRNA transcripts (known as Δ7SMN) [5, 6]. SMA therefore presents in a spectrum of disease severity, depending on SMN2 copy number. The majority of SMA patients have 2 copies of SMN2 and a correspondingly severe phenotype, differentiated as Type 1 SMA. These patients present with severe weakness before 6 months of age, and most progress to almost complete paralysis by 2 years. However, some patients have more SMN2 copies or less-severe mutations leading to more FL-SMN production and a less-severe phenotype, denoted as Type 2, 3 or 4 SMA. Many mouse models of SMA have been generated since the link to SMN expression was discovered [2]. The most widely used model of SMA is known as the Δ7SMN mouse, where the mouse Smn gene is knocked out on a human SMN2 and the Δ7SMN double transgenic background [7]. These Δ7SMN mice mimic severe SMA very well, with
detectable onset within 3-5 days and an average survival of only 14-16 days. We and others have used this mouse model to test pre-clinical efficacy of SMA-specific treatment strategies, but due to the very rapid progression of Δ7SMN mice, we are unable to predict how well these therapies may work in older, less-severe patients [8-11]. Recently, however, a new model has been engineered to model patients with less-severe SMA, known as the C/C mouse [12]. This mouse has a normal lifespan but develops minor weakness over the first few weeks of life and has lower body mass than non-affected littermates. We decided this would be a more appropriate model to assess the efficacy of muscle-enhancement, which may be particularly valuable for less-severe SMA patients especially in older patients experiencing a much slower decline in weakness with more spared muscle fibers.

Previously we have shown that follistatin gene therapy can efficiently increase strength and improve phenotype in a model of muscular dystrophy [13]. Follistatin may increase muscle mass by multiple mechanisms, but the greatest effect is likely due to its ability to inhibit myostatin, a TGF-beta superfamily member that potently inhibits muscle growth, especially during periods of non-use. Others have shown by various methods that myostatin inhibition is unable to alter the course of disease in Δ7SMN mice [14-16]. It has become increasingly clear that myostatin inhibition alone is unable to compensate for loss of nervous stimulation to muscle, as occurs in affected motor units in SMA patients. However, as severity is decreased along the SMA spectrum, the number of affected muscle fibers is decreased. We hypothesize that enhancing the force generated by these
innervated muscle fibers will significantly improve quality of life in less-severe SMA patients.

In this study, we use the same transgene encoding the 344-kDa splicoform of follistatin (FS344) that was previously shown to be safe and efficacious [13, 17]. However, we have updated the delivery method to provide the most widespread efficacy while remaining clinically relevant. First, by using self-complementary vector, transgene expression is initiated more rapidly and infection is much more efficient. Second, the AAV serotype 9 (AAV9) has a very high natural tropism for striated muscle, and its vascular escape properties allow for efficient transduction of muscle throughout the body by a single systemic injection [18].

We demonstrate herein that follistatin gene therapy can sufficiently increase strength in the C/C mouse model of mild SMA. Also, we further characterize the C/C motor phenotype and describe an alternate platform by which the subtleties of this mouse model may be more appropriately tested.

**Results**

*FS344-treated C/C mice show early improvement*

Within 2 days of birth, 30 C/C mice (mixed background) were randomized into two groups, with 15 mice injected with $1 \times 10^{11}$ vector-genomes (vg) scAAV9-GFP (control vector) and 15 mice injected with $1 \times 10^{11}$ vg scAAV9 carrying the cDNA encoding the 344 kDa follistatin splice variant under control of the chick beta-actin promoter (scAAV9-FS344). Previous experience in our lab has shown that this dose of scAAV9 vector is sufficient to transduce fibers from all muscles throughout the entire mouse
(unpublished data). We chose this dose to most completely test the efficacy of follistatin treatment. We also included 15 healthy control littermates heterozygous for the C/C allele for comparison during the study.

Weight was recorded every-other day through the first two months of the study, then weekly thereafter. While the weight difference between C/C mice and healthy controls is small, C/C mice are significantly smaller by weaning at postnatal day 21 (PND 21). However, the weights of FS344-treated C/C mice segregate from control-treated C/C littermates by PND 23, and tend to be slightly larger than healthy controls by PND 33 (Figure 37 A). This approximate 21-day post-injection lag before detectible body mass increase matches what was previously observed in both mice and non-human primates, despite the young age at which these mice were injected [13, 17].
As demonstrated previously, C/C mice lag behind controls in righting ability from PND 8-12. We confirmed this in our untreated cohort using the same technique outlined in the initial descriptions of the model [12]. However, we also found that FS344-treated C/C mice were indistinguishable from healthy littermates in righting ability (Figure 37 B). This implies that high expression of FS344 can have early positive effects in muscle performance despite the lack of a detectible increase in body mass.

*FS344-treatment increases strength in C/C mice*

Starting at PND 28 all mice were tested for forelimb grip strength and accelerating rotarod performance. Grip strength deficits in C/C mice were not detected compared to healthy controls. However, as expected, FS344-treated mice were significantly stronger.
than both C/C and healthy controls by the third trial (Figure 37 C). After 6 weeks
reliable strength testing became unreliable due to many treated and untreated C/C
subjects developing severe necrosis of the hindlimbs and perirectal area. As previously
demonstrated, rotarod performance is not negatively affected in C/C mice [12]. We also
found that C/C mice tended to have greater latency to fall than healthy controls, but this
was not significant. The increased strength in FS344-treated mice had no effect on
rotarod performance, either.

In order to further characterize the atrophy in various muscle groups throughout the body,
as well as to more closely observe the effects of FS344 treatment, a cohort of treated C/C,
control C/C and healthy control mice were aged to ~220 days before necropsy. By gross
observation we could see clearly that FS344 treatment had positive effects on muscles
throughout the body of C/C mice. After careful dissection, the quadriceps femoris
(quad.), gastrocnemius (gastroc.), tibialis anterior (TA), extensor digitorum longus (EDL)
and soleus (sol.) muscles were weighed. Muscle mass of C/C mice was significantly
decreased compared to control littermates in all muscles observed except for the TA in
the female cohort. Muscle mass of the FS344-treated C/C group was significantly
increased in all muscles when compared to C/C controls, again, with the only exception
being the TA of the female cohort. Moreover, the mass of quad, TA and soleus muscles
from male treated cohort and the quad, EDL and gastroc of the treated female cohort
were further increased above the corresponding muscles from healthy controls (Figure
38).
Figure 38 Muscle mass. Individual muscles were dissected from male (A) and female (B) mice. Healthy controls are in gray, C/C controls are in blue, and FS344-treated C/C mice are in red.

*Weakness in C/C mice is due to decreased NMJ transmission*

Due to the subtle deficits on motor behavior and potentially confounding non-motor aspects of the C/C phenotype, we decided to test muscular force generation using *ex-vivo* and *in-situ* measurements to more narrowly assess the neuromuscular deficit in C/C mice. The original description of the C/C model describes insufficient transmission in a very small number of NMJs analyzed [12]. Instead of assessing individual NMJ transmission, we collaborated with the Sweeney laboratory to quantify the maximal tetanic force generated from whole muscle preparations *ex vivo*. The ratio of maximal force generated via nerve vs. muscle stimulation is decreased in C/C mice, even when normalized by cross-sectional area (specific force) [19] The data generated by the Sweeney laboratory suggests that NMJ transmission deficits further contribute to the C/C weakness phenotype, independent of the already decreased muscle mass. Quantifying the difference in specific force generated by nervous vs. direct muscle stimulation is therefore a more
appropriate and sensitive assay of therapeutic benefit in C/C mice since by this method, one can remove many of the potentially confounding factors affecting muscle behavior measurements (i.e. hindquarter necrosis and decreased pain threshold).

*FS344-treatment increases muscle force despite NMJ transmission deficits*

After verifying that C/C mice indeed have an NMJ transmission deficiency, we sought to determine whether follistatin treatment would still increase the force generated by these muscles when tested *ex vivo* via direct stimulation of muscle and *in situ* via stimulation of the incoming nerve. In the male cohort, no difference in contraction force was detected among healthy controls, C/C controls or FS344-treated C/C mice by direct soleus m. stimulation. The C/C controls in the female cohort generated significantly less force than the healthy control and FS344-treated C/C groups (*Figure 39 A*). Stimulation of the nerve *in situ* evoked less forcible contractions in both muscle sets analyzed (TA and EDL) in the C/C mice compared to healthy controls. Remarkably, FS344 treatment restored force generation equal to that of healthy controls (*Figure 39 B and C*).

![Figure 39](image-url)

*Figure 39* Muscle force generation by direct stimulation (*ex vivo*) or indirect stimulation through the nerve (*in situ*). Healthy controls are in gray, C/C controls are in blue, and FS344-treated C/C mice are in red.
Discussion

Even in its milder form, SMA is a horrible disease. The recent advances in therapies designed to increase SMN is very encouraging, but for older patients, late in the course of their disease, it is unknown whether increasing SMN will be beneficial. The purpose of our study, then, is to provide another option for these patients that can be used to increase their quality of life. While this study is still ongoing, the data suggest that FS344 gene delivery to the muscle is therapeutic in less-severe SMA.

The C/C mouse provides us a model of Type 3 SMA, in that it develops weakness early, but reaches a “plateau phase” after the first few weeks of life where progression is greatly slowed. We have also confirmed that the weakness in these mice is indeed due to SMN deficiency since scAAV9-SMN treatment rescues this weakness (unpublished data). However, C/C mice develop necrotic tails and hindquarters, and they have increased sensitivity to painful stimuli [12]. This non-motor phenotype potentially confounds behavior data, and makes working with the model very difficult.

Therefore, while the resulting behavior data generated by our lab were very encouraging, we had to find a way to test the success of FS344 treatment outside the context of these potentially confounding factors. Due to the work done at the Sweeney lab at the University of Pennsylvania, we have found that the neuromuscular phenotype can be more specifically assayed by using ex vivo or in situ protocols. Indeed, the finding that FS344 treatment can rescue strength generated even in the context of decreased neural input leaves us hopeful that this option will be beneficial for Type 2 or Type 3 SMA patients. The fact that FS344 gene therapy is currently being tested in the clinic for
treatment of inclusion body myositis and Becker muscular dystrophy makes this approach even more attractive.

Methods

**Viral vector production**

The scAAV9.CB.GFP and scAAV9.CB.FS344 vectors were produced using transient transfection of 293 cells with plasmids containing double-stranded AAV2-ITR–based CB-GFP or –FS344 vector, Rep2Cap9 and an adenoviral helper plasmid pHHelper (Stratagene) as previously described [20].

C/C mouse breeding and use

All mouse use was approved by the IACUC at the Research Institute at Nationwide Children’s Hospital (TRINCH).

C/C mice were obtained from Psychogenics and housed and bred at TRINCH. On the day of birth, gDNA was extracted from tails of the pups for genotyping. The presence of the “C” allele was determined by PCR using the following primers:

Forward: 5’-TACCCAGATGCAGTGCTCTTGTAG-3’
Reverse: 5’-CCTTATGGCATAGACACCAACTTCT-3’

**AAV9 injection of neonatal mice**

Mice were injected as previously described with $1 \times 10^{11}$ particles of scAAV9-FS344 or scAAV9-GFP [20].

**Righting assay**

Between P6 and P12 pups were briefly removed from their cage to a smooth stainless steel surface. Pups were turned upside-down and given 3 seconds to right themselves.
This was repeated a total of 10 times each day, and the number of successful righting attempts was recorded.

References


Chapter 7: An AAV-based approach to model the full spectrum of spinal muscular atrophy in mice

This chapter is currently unpublished and is the result of collaboration with the authors listed below. I helped design and execute all experiments.

Authors

Abstract
Objective: Spinal muscular atrophy (SMA) presents with an array of severity that results in early lethality in the most severe form. A number of mouse models recapitulate severe SMA, but attempts to model the less severe type 2 and 3 SMA patients in mice have been complicated by genetically titrating levels of SMN, the gene implicated in SMA, to create a later onset, less severe disease model. The aim of this study was to use vector-based strategies to alter the expression of SMN to more closely model type 2 and 3 SMA patients.

Methods: To model mild SMA, we utilized adeno-associated virus type 9 to postnatally deliver RNAi against murine Smn on a background of human SMN2 to create a spectrum of phenotypes ranging from mild to severe by varying the vector dose administered.
Results: High dose animals lived ~14 days post injection and had symptoms similar to established models of severe SMA. More importantly, low dose animals reproducibly developed a later onset weakness and neurogenic atrophy with no necrosis or apparent decrement in lifespan. Muscle weakness was not associated with motor neuron loss and remained constant throughout the study (~250 days). Compound muscle action potentials were similar between treated and age matched controls, but motor unit number estimation showed a reduced number of motor units with larger potentials than control littermates indicative of a denervation and subsequent re-innervation. Importantly animals injected with an off-target RNAi construct developed no phenotype indicating that the symptoms are specific to Smn knockdown.

Interpretation: We have created a robust mouse model of type 3 SMA suitable for study of the disease process and therapy development. Further testing of whether SMN replacement can rescue the phenotype is still required to fully determine the cause of the SMA-like phenotype.

Introduction

Type 1 spinal muscular atrophy (SMA) is a leading genetic cause of death among infants [1]. There has been much recent progress in the understanding of type 1 disease cause and progression as well as therapy development largely due to the robust animal models available [2, 3]. However later onset, less severe forms of SMA, such as type II and III, are not as well understood. Attempts to model type II and III SMA in mice have proven difficult due to the exacting levels of survival motor neuron (SMN), the protein involved in SMA, required to produce disease. The phenotypes of the resulting mice are subtle
and would require large numbers for assessing differences, or manifest with a distal necrosis that may be related in some way to SMA, but is not generally seen in less severe patients [4].

Because SMA severity is largely determined by SMN levels, we asked if postnatal reduction in murine Smn levels could be used to model less severe SMA [5, 6]. To do this, we utilized adeno-associated virus type 9 (AAV9) to deliver a short hairpin RNA (shRNA) against Smn with a single intravenous injection in one-day-old mice. Importantly for future therapy development, the mice carried a genomic SMN2 transgene that was resistant to shRNA mediated knockdown. In the following work, we present on the creation of a spectrum of mouse models for SMA, and further characterize a model that closely resembles SMA type III. The phenotypes created were dependent on the dose of AAV9 administered at postnatal day one. High dose animals had phenotypes similar to commercially available mouse models of severe SMA and died at two weeks of age. However low dose animals first presented with motor deficits at weaning, and the symptoms were maintained for the duration of the study (~250 days). Electromyography and histological analyses indicated a neurogenic atrophy without accompanying motor neuron loss. Importantly, the low dose animals had no apparent decrease in life span or necrosis. Together, these data describe a mouse model of type III SMA that will be useful for the study of disease course and therapy development, and further demonstrate the utility of systemic gene delivery for the rapid modeling of disease.
Materials and Methods

**AAV9 Production and Characterization**

The shRNA cassette together with the H1 promoter were cloned into a self-complementary AAV-GFP vector, with the promoter inserted back-to-back with the chick beta-actin promoter. The resulting plasmid was used to make virus by being co-transfected into HEK-293 cells with AAV Rep2Cap9 and adeno pHelper plasmids to create virus as described previously [7].

**Animal Care and Use**

All procedures performed were in accordance to either the Research Institute at Nationwide Children’s or The Ohio State University Institutional Animal Care and Use Committees.

**Neonatal intravascular injection**

All mice were genotyped and injected within 48 hours after birth. Δ7 carrier mice, heterozygous for Smn (SMN2+/+, Δ7SMN+/+, Smn+) were cryoanesthetized and injected with either scAAV9 or PBS via the temporal vein as described previously. The low- mid- and high- doses of scAAV9-shRNA corresponded roughly with $2 \times 10^{14}$ vg/kg, $4 \times 10^{14}$ vg/kg and $1 \times 10^{15}$ vg/kg respectively, each in a total volume of 50 µl. Off-target scAAV9-shRNA was delivered in an identical manor at high-dose ($1 \times 10^{15}$ vg/kg).

**Motor Behavior Testing**
Mice were monitored daily for weight. After weaning, mice were tested for grip strength (Columbus Instruments), accelerating rotarod and open field testing as described previously.

**Electrophysiology**

A cohort of low-dose and PBS control mice underwent EMG analysis at either 30 or 200 days of age after sedated with a mix of 100 mg/kg Ketamine and 10 mg/kg Xylazine.

**Euthanasia and tissue collection**

Each animal was given a lethal dose of ketamine/xylazine anesthetic cocktail via IP injection.

For muscle collection, individual muscles were immediately dissected and snap frozen in liquid nitrogen-cooled isopentane, and stored at < -70°C until sectioned. Sectioning was done on a cryostat to produce 10-12um sections, which were also stored at < -70° until stained.

Prior to isolating internal organs, including brain and spinal cord, mice were perfused with normal saline followed by ice-cold 4% buffered paraformaldehyde (PFA) solution. All tissues were then removed and post-fixed at least 24 hours in PFA.

**Heart, and liver histology**

Tissues were moved to 70% ethanol for at least 24 hours, then embedded in paraffin. 4μm histological sections were taken and stained using standard Hematoxylin and Eosin (H&E) methods.

**Spinal cord immunofluorescence**
Fixed spinal cords were sectioned on a vibratome and stained for immunofluorescent detection as was done previously [8].

Results

*Design and in vitro validation of a mouse-specific shRNA construct*

The Δ7-SMN mouse has greatly contributed to the SMA field as a model for studying SMA pathogenesis as well as therapy development [3]. Colonies of Δ7 mice are propagated by crossing mice homozygous for the Human *SMN2* and SMNΔ7 transgenes and heterozygous for mouse *Smn* (*SMN2+/+, SMNΔ7+/+, Smn+/−*), and these heterozygous mice show no phenotype. We chose to use these heterozygous mice for developing an AAV9-shRNA-based mouse model of less-severe spinal muscular atrophy, since we could design a mouse-specific shRNA that would leave the human *SMN2* transcript intact. This is very important for two reasons. First, *SMN2* would be able to produce at least a small amount of full-length SMN (FL-SMN) in every cell despite the extent of shRNA-mediated knockdown of Smn. A complete depletion of SMN, which is shown to be detrimental to every cell type examined, would not reflect the true pathogenesis of SMA [9, 10]. Second, *SMN2* is the target of many therapeutic strategies such as HDAC-inhibitors, antisense oligonucleotides and stop-codon read-through compounds [11-15]. By leaving SMN2 intact these therapies could be tested in the resulting model.

The sequence of SMN is very well conserved between humans and mice. However, exon 4 has sufficient variability to create an shRNA specific to mouse Smn mRNA (Figure 40A). We also transfected human embryonic kidney 293 (HEK293) cells to determine
whether there was any cross-reactivity with human SMN mRNA. Following transfection, mRNA was extracted and subjected to quantitative RT-PCR, which revealed no change in SMN expression when compared to control-transfected HEK293 cells. (Figure 40 B).

Figure 40 Characterization of mouse-specific anti-Smn shRNA. Binding of shRNA is specific for mouse Smn, and has multiple mismatches compared to human SMN2 (A). shRNA construct does not decrease SMN expression in HEK-293 cells (human cell line) (B). Western blot of whole spinal cord (C) and muscle (D) lysates demonstrating Smn knockdown.
Validation of the shRNA construct in vivo

We proceeded to clone the shRNA construct into a self-complementary AAV, serotype 9 (scAAV9) vector, which also expresses GFP under control of the ubiquitous chick beta-actin promoter. We have previously shown that AAV9 is uniquely able to efficiently transduce motor neurons and other neurons throughout the CNS and periphery after a single intravenous dose in neonatal mice [7]. Additionally, self-complementary vectors are able to start expressing detectable levels of gene products within 24 hours after injection [16, 17]. We therefore injected mice with scAAV9-shRNA at doses previously shown to be sufficient for near-complete motor neuron transduction (2E+14 vg/kg) and extracted tissues at postnatal day 8 (P8) for protein extraction (Figure 40 C and D). In the spinal cord, AAV9 primarily transduces motor neurons and sensory fibers and very few interneurons and glia. In contrast, AAV9 at this dose almost completely transduces muscle fibers throughout the body. Therefore, we confirmed the occurrence of Smn knockdown in the spinal cord (Figure 40 C). However, the extent of knockdown in each cell is better indicated by protein derived from muscle, since the spinal cord contains many cell types not well transduced, such as interneurons and glia. This was indeed confirmed by western blot analysis by probing for both total SMN and human-specific SMN protein in muscle, where total SMN expression was decreased by ~33% in scAAV9-injected animals, while human SMN2-derived protein was unaffected (Figure 40 D).
Postnatal knockdown of Smn is sufficient to cause an SMA-like phenotype in mice

To determine whether postnatal knockdown of Smn was sufficient to cause SMA in mice, we injected neonatal mice with either low-, medium- or high-dose scAAV9-shRNA (corresponding roughly to 2E+14 vg/kg, 4E+14 vg/kg and 1E+15 vg/kg, respectively). Indeed, we found that all shRNA-treated animals developed SMA-like phenotypes, with high-dose mice showing a phenotype very similar to the Δ7-SMN mouse (median survival 12 days), the medium-dose mice showing slightly less-severe disease (median survival 20 days) and the low-dose mice surviving long-term with obvious weakness and atrophy evident within the first 2-3 weeks of life (Figure 41 A). The weakness in the low-dose mice was accompanied by a typical hind-limb clasp upon lifting the mouse by the tail (Figure 41 B).
Figure 41 Range of SMA-like phenotype of high-, medium-, and low-dose mice. Varying the dose of AAV9-shRNA can reliably recreate the wide spectrum of SMA severity (A). Low-dose shRNA mice develop weakness during the first 2 weeks post injection and clasp upon raising by the tail (B). The numbers of cells transduced between low- and high-dose mice is similar (C).

We next verified that the shRNA was not causing disease in cell-types other than motor neurons. AAV9 is known to efficiently target skeletal muscle, cardiac muscle and liver [8, 18]. These tissues are also susceptible to death upon total depletion of SMN [9, 10]. Histological evaluation revealed that there was no pathology in these tissues at either low or high doses, despite high levels of mouse-specific Smn knockdown (Figure 42).
The potential for shRNA toxicity of certain sequences, especially within the CNS, has been well documented [19-21]. However, we saw no signs of shRNA-mediated toxicity, including gliosis and microglial activation, in either our high- or low-dose mice (Figure 43).

![Image of tissue samples showing control and shRNA expression in quad, liver, and heart tissue.](image)

Figure 42 No toxicity is associated with high shRNA expression in quad, liver and heart tissue.
Figure 43 Immunofluorescent staining of microglia in lumbar spinal cords of control and shRNA-treated mice. Microglia were detected by anti-Iba1 antibody (blue) and motor neurons are stained with anti-ChAT antibody (red). No activation of microglia was seen in any tissues analyzed.

All doses of scAAV9-shRNA transduced similar percentages of motor neurons

The relatively mild phenotype observed in the low-dose mice could be due to either fewer numbers of motor neurons being transduced or to fewer infections events in each motor neuron transduced. Due to the shRNA construct also expressing GFP under a very strong, ubiquitous promoter we could easily determine whether the proportion of motor neurons differed between high- and low-dose groups. While we observed that in low-dose spinal
cords GFP fluorescence was dimmer in unstained sections compared to high-dose cords, upon immunofluorescent staining, we found that similar numbers of motor neurons were transduced in both groups (Figure 41 C and D). This suggests that the major difference among the three dose cohorts is how many particles infect each cell. Indeed, it has been shown that a single cell can be simultaneously infected by multiple AAV particles before saturating infection processes, thereby making it possible to modulate the level of Smn knockdown within targeted motor neurons in a dose-dependent fashion.

Low-dose AAV9-shRNA causes weakness and atrophy typical of type III SMA

While low-dose mice were in no apparent distress, able to easily access food and water, their body weights were all about 10-15% lower than sex- and age-matched controls (Figure 44 A). Since the low-dose mice survive past weaning age, we were able to perform weekly tests of motor function, including grip strength, rotarod and open field ambulation. We found that these mice were consistently weak and performed worse on all these measures (Figure 44 B-E).
To determine the cause of the decreased strength and mass of these animals, we first assessed muscle fiber size in both proximal (quadriceps femoris m.) and a distal muscle (gastrocnemius m.) groups. We utilized a succinate dehydrogenase (SDH) staining protocol to distinguish among fiber types 1, 2a and 2b (Figure 45). It became clear that low-dose scAAV9-shRNA mice showed atrophy in both muscle groups analyzed, but that the extent of atrophy was greater in the quadriceps. In quadriceps of shRNA-treated mice, fiber types 1, 2a and 2b were all significantly atrophied compared to controls by 15.18%,
16.55%, and 20.30%, respectively (Figure 45 A-E). Gastrocnemius muscle fibers were also significantly atrophied, but to a lesser degree (9.64%, 17.62% and 6.28%) (Figure 45 F-I). The angular fibers with an absence of any dystrophic fibers or necrosis are consistent with neurogenic atrophy, such as is typical of SMA patients. We did not, however, observe any hypertrophy of type 1 fibers as may also be seen in SMA, though not as commonly in less-severe forms.
Figure 45 Comparison of muscle atrophy by fiber type in quadriceps and gastrocnemius muscles. Staining for succinate dehydrogenase can differentiate type 1 (dark purple) from type 2a (intermediate purple) and type 2b (unstained) fibers types.
Neuromuscular electrophysiology confirms loss of functional motor units in low-dose mice

To further explore the cause of atrophy in the low-dose mice, we performed a battery of electrophysiological tests in a cohort of young (P30-40) and old (P215-220) mice. The compound muscle action potential (CMAP) in these mice was similar to controls, although there was a trend to decreased amplitude in the older cohort (Figure 46 A). Even in the context of motor neuron disease, it is possible for CMAP values to remain almost normal due to rapid sprouting and collateralization of surviving motor neurons. This would be indicated by an increased single motor unit potential (SMUP), whereby stimulating a single motor unit would evoke a larger motor response. This is indeed what we saw in our mice (Figure 46 B). When we calculated the motor unit number estimation (MUNE), we found that these low-dose mice have significantly decreased numbers of functional motor units (Figure 46 C).
Loss of functional motor units could be due to pathology in the motor neuron soma, axon or NMJ. In Δ7 mice, motor neuron loss is evident early on in disease progression, but only ~20% of total motor neurons are lost by end stage [3]. In our mice, we also counted motor neurons in the spinal cord and found that there is no loss of MN soma in neither young nor old mice (Figure 46 D). Additionally, we found no pathology of the neuromuscular junctions of P30 low-dose mice (Figure 46 E).

Discussion

In this study, we have successfully used AAV9 technology to deliver a mouse-specific shRNA and thus created much-needed models of spinal muscular atrophy. Of particular importance, we have created a mild form of SMA-like disease in mice that has no signs of necrosis or other vascular abnormalities, problems that have hindered the creation of
mild SMA models thus far. The unique abilities of AAV9 have been used before to treat models of neurological disease and to overexpress mutant protein to create a model of amyotrophic lateral sclerosis (ALS), this is the first, time to our knowledge, that a full-spectrum of a neurological disease has been modeled using this vector and RNAi technology [22]. By using this approach, we have learned that, at least when using very high titers with optimized shRNA constructs, one can very precisely modulate the level of infection, and therefore knockdown, of target genes within motor neurons. However, since this is a new modeling approach, it is necessary to further validate that the model phenotype is indeed due to loss of SMN by preventing the phenotype by by replenishment of human SMN prior to disease onset.

Variability among different viral preparations is a potential limitation to the expansion of this approach to other disease models, but this can be overcome by pooling of multiple preparations until enough virus is produced to be used for a given study. In the current study, the use of the same viral prep allowed for very similar phenotypes among animals of the same dose cohort.

We have very little information about the pathogenesis of milder forms of SMA, but we see that our model of mild SMA has many signs that match what is seen in these patients [23]. In type 3 SMA, patients often develop normally until 18-24 months, followed by a sub-acute loss of strength. Importantly, many of the patients stabilize and do not see much subsequent loss of strength for years. We have captured that phenotype in our low-dose model, as weight, grip strength and rotarod performance don’t decline after the initial phase of disease onset (Figure 44). This model mirrors the hallmark pathological
features of neurogenic atrophy with signs of reinnervation in the absence of other muscle pathology. Despite the seemingly even distribution of AAV9 transfection of all pools of lower motor neurons, we see that proximal muscle groups are more affected compared to distal ones, typical of other mouse models and SMA patients.

Type 3 SMA pathogenesis is very poorly understood. The few published autopsy have conflicting reports as to quantification of spinal motor neurons, with some patients even having normal numbers of MNs well into their teenage years [24]. Electrophysiological studies such as CMAP and MUNE only confirm that SMA patients lose effective NMJ connectivity. These findings, together with our study in mice, support the possibility that mild disease results more from distal defects in NMJ connectivity. It is therefore of utmost importance to determine at what stage of disease progression are MNs able to be rescued. This is perhaps the most pressing question for existing SMA patients today. This new model enables us to systematically determine the window of opportunity for SMN-mediated rescue of the SMA phenotype in the full-spectrum of SMA severity.

Additionally, with a robust model such as this, translational investigators can better evaluate preclinical efficacy of potential treatments aimed at treating SMA type 2 and 3, possibly increasing the speed at which existing therapies can be utilized for Phase 2 clinical trials.

References


Chapter 8: Future directions and conclusion

Not 4 years have passed since the discovery of AAV9’s ability to transcend the blood-brain barrier, but this finding has already impacted neurological research in remarkable ways [1]. As the studies herein have outlined, spinal muscular atrophy (SMA) research and therapy development has primarily benefited from AAV9-based gene delivery, but others are also finding this technology to be very beneficial when applied to other CNS disorders.

AAV9 can be a useful tool for studying SMA pathogenesis

The translation of AAV9-based SMA gene therapy to the clinic is of utmost importance, and our studies represent a major step toward establishing safety, efficacy and the best conditions that will ultimately lead to the first-in-human clinical trial. However, many questions remain about SMA disease pathogenesis that AAV9 technology can help to answer. The most clinically-relevant issue that begs for resolution is whether SMN replacement will be clinically useful after disease onset. This applies to the entire spectrum of SMA disease severity, but especially so in older children and adults with Type 2 or Type 3 SMA. This question has been well studied in the Δ7SMN mouse model [2-4], implying that SMN replacement is required prior to the precipitous loss of function seen early in Type 1 patients. Working with the less severe vector-based model we have created may provide better data to link the “point of no return” to specific markers of
disease progression, such as electrophysiological measures or changes in strength or muscle mass.

AAV9-based strategies can also aid in the basic understanding of SMN function, disease modifying genes and their individual or combined roll in SMA pathogenesis. Since we have established that AAV9 can transduce sufficient motor neurons to affect disease course, we can now create a kind of rapid transgenic animal by which we can assess the functionality of well-crafted SMN mutants or screen putative modifying genes without having to invest the time and resources required for traditional mouse transgenics.

The mechanisms of AAV9 neuronal transduction still need to be elucidated

It is now clear that wild-type AAV9 is highly useful for neurobiological research due to its ability to surpass the blood-brain barrier, but it is still unclear how AAV9 infects target cells. Additionally, the amount of vector needed for high-level transduction of the CNS is almost prohibitive to therapies targeting older patients. By understanding how AAV9 targets cells, it may be possible to orchestrate a more efficient scenario leading to lower titers of virus needed for CNS transduction. Two potential receptors have been identified, but whether these receptors play any role in infection of CNS tissue remains untested [5, 6]. Better understanding the specific moieties involved in attachment and uptake of AAV9 capsid may be helpful in designing more-efficient vectors or strategies to increase target cell receptor expression to lower viral requirements.

Similarly, knowing the cellular location of AAV9 uptake will be necessary to better anticipate the barriers to neuronal transduction. For example, in the original report of systemic AAV9 delivery, our lab demonstrated the importance of astrocyte polarity, since
AAV9 could more efficiently transduce glia through the bloodstream than when injected intraparenchymally [1]. If primary infection of motor neurons occurs in a specific region, it may be possible to develop strategies to enhance or widen this permissible region to increase likelihood of AAV9 infection.

Conclusions

Now is an exciting time to work on the leading edge of gene therapy research, and especially to see the translation of our findings to the clinic. Along with the discovery of AAV9’s ability to transcend the blood-brain-barrier, SMN gene therapy has rapidly gained traction and, due largely to the lessons learned from the studies presented herein, is pushing the limits of gene therapy farther than any single study since the first AAV human trial in 1996 [7]. Specifically, we have learned that systemic AAV9-SMN therapy is efficacious and well-tolerated. We have also demonstrated the potential for concomitant heart disease in SMA patients, which has already guided how we are to run the planned clinical trial.

We are just now starting to understand the breadth of AAV’s utility. The future holds so much promise for gene therapy, and I am excited to continue using these tools to combat human neurological disease.

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