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Correcting Neuromuscular Deficits With Gene Therapy in Pompe Disease

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Abstract

Objective—We have recently reported on the pathology of the neuromuscular junction (NMJ) in Pompe disease, reflecting disruption of neuronal and muscle homeostasis as a result of glycogen accumulation. The aim of this study was to examine how the alteration of NMJ physiology contributes to Pompe disease pathology; we performed molecular, physiological, and histochemical analyses of NMJ-related measures of the tibialis anterior muscles of young-, mid-, and late-stage alpha-glucosidase (GAA)-deficient mice.

Methods—We performed intramuscular injection of an adeno-associated virus (AAV)9 vector expressing GAA (AAV9-hGAA) into the tibialis anterior muscle of *Gaa*^{−/−} mice at early, mid, and severe pathological time points. We analyzed expression of NMJ-related genes, *in situ* muscle force production, and clearance of glycogen in conjunction with histological assessment of the NMJ.

Results—Our data demonstrate that AAV9-hGAA is able to replace GAA to the affected tissue and modify AChR mRNA expression, muscle force production, motor endplate area, and innervation status. Importantly, the degree of restoration for these outcomes is limited by severity of disease. Early restoration of GAA activity was most effective, whereas late correction of GAA expression was not effective in modifying parameters reflecting NMJ structure and function nor in force restoration despite resolution of glycogen storage in muscle.

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Potential Conflicts of Interest

B.J.B., D.J.F., D.D.F., Johns Hopkins University, and the University of Florida could be entitled to patent royalties for inventions described in this article. D.D.F., D.J.F., and B.J.B. are inventors of patents related to this research. B.J.B. is a founder of Applied Genetic Technologies Corporation (AGTC), although AGTC has no activity related to Pompe disease.

Author Contributions

A.G.T performed experimental procedures, analyzed and interpreted data and prepared the manuscript. J.A.M performed experimental procedures. R.W.G performed experimental procedures and assisted in interpretation of data and manuscript review. D.D.F interpreted data and assisted in study design and manuscript revision. B.J.B assisted in study design and manuscript revision. D.J.F designed the study, performed experimental procedures and revised the manuscript.

Interpretation—Our data provide new mechanistic insight into the pathology of Pompe disease and suggest that early systemic correction to both neural and muscle tissues may be essential for successful correction of neuromuscular function in Pompe disease.

Gene therapy strategies have the potential to advance treatment options for pediatric neuromuscular disorders. The capacity to restore or preserve integrity and functionality of the neuromuscular junction (NMJ) is presumably limited by multiple factors, including bioavailability of tissue composing the NMJ at the time of intervention and efficacy of transgene replacement to both pre- and postsynaptic components. For these reasons, evaluating adeno-associated virus (AAV) serotypes with favorable tissue transduction characteristics and establishing early markers of NMJ deterioration to define an optimal therapeutic window are essential to preclinical development of gene therapy strategies to treat neuromuscular disorders.

Maximum therapeutic benefit is presumably conferred by intervention at a prepathological stage; however, in the clinical setting, presymptomatic intervention is not always possible without newborn screening or clinical suspicion owing to an affected sibling. In the advanced stage of disease, the reversibility of pathology or plasticity of NMJ components may be limited. Examples from studies in spinal muscular atrophy have defined a narrow therapeutic window for scAAV9 therapy to impart functional benefit by restoration of survival motor neuron expression. Loss of therapeutic efficacy is related to rapid degeneration of the NMJ.¹ Therefore, understanding the influence of NMJ pathology on the optimal therapeutic window is critical in evaluating a therapeutic strategy for patients challenged with a variety of neuromuscular disorders.

Pompe disease is a neuromuscular disorder characterized by a deficiency in degradation of lysosomal glycogen resulting from reduced or absent acid alpha-glucosidase (GAA).² Severe glycogen accumulation causes muscle atrophy and weakness and leads to loss of muscle function and cardiorespiratory failure before 12 months of age in early-onset patients.^{2,3} We have previously characterized contractile dysfunction and weakness of the diaphragm in the murine model of Pompe disease^{4–6} that mirrors the progressive phenotype of human patients.⁷ More recently, we demonstrated NMJ dysfunction in both the diaphragm and tibialis anterior (TA) muscles in Gaa-deficient mice, confirming that both muscle and nerve pathophysiology are likely contributors to disease progression.^{8–10} These observations justify the development of AAV-based gene therapy as a viable therapeutic candidate for Pompe, based on the intrinsic properties of AAV9 to target transgene replacement in both muscle and motor neurons.^{4–6,9,11,12} Supporting this hypothesis, correction of the Pompe phenotype, as demonstrated by previous studies, is likely attributable to both muscle and neuronal targeting of AAV9 expressing GAA (AAV9-hGAA).^{1,4,5}

In this study, we used the Pompe mouse model to evaluate the therapeutic benefit of AAV9 vectors by direct intramuscular injection at early, mid, and advanced stages of NMJ pathology. We demonstrate that direct intramuscular administration of AAV9-hGAA is sufficient to promote glycogen clearance in gene-corrected muscle at all disease stages;

however, normalizing muscle glycogen is ineffective in restoring motor endplate gene expression or neuromuscular functional profiles in the setting of end-stage disease.

Overall, these data have important implications to guide the preclinical development of AAV9 vectors for treatment of Pompe disease and also provide potential markers of NMJ dysfunction that may be used to interpret therapeutic outcomes.

Materials and Methods

Experimental Animals

The *Gaa*^{-/-} and background strain control mice in this study have been described elsewhere.¹⁰ All animal studies were approved in accord with the guidelines set forth by the University of Florida Institutional Animal Care and Use Committee (#201207687). All animals were sacrificed at 1, 3, 6, 12, or 18 months for histological, biochemical, molecular, and *in situ* analyses.

Nucleic Acid Isolation and Quantitative Real-Time Polymerase Chain Reaction

Whole TA muscles were homogenized in Trizol (Life Technologies, Carlsbad, CA) using MB lysing matrix B (MP Biomedicals, Santa Ana, CA). After ethanol precipitation, nucleic acid isolation was performed using the Zymo-spin IC column (Zymo Research, Irvine, CA) with DNase treatment, following the manufacturer's protocol. Yield and purity were confirmed by nanodrop spectrophotometry before complementary DNA (cDNA) synthesis using SuperScript VILO (Life Technologies) and 1.6µg purified RNA. Resulting cDNA libraries were diluted for quantitative polymerase chain reaction (qPCR) performed using iTaq Universal SYBR Green (Bio-Rad, Hercules, CA) on a CFX-96 real-time detection platform (Bio-Rad) using 20ng cDNA as template per reaction. The following primer sequences were used for their specified targets; mouse: GAPDH 5'-TGTGTCCGTCGTGGATCTGA, 3' - CCTGCTTCACCACCTTCTTGA; CHRNA 5'-ACAATCTCACGGTGGTCCTC, 3'- CTGTTCTCCTGCTGCTAGGC; CHRND 5'-ATCTTGGTGGTTGGTGCTGT, 3'- AGGAGGAAAACAACCGCTCT; CHRNE 5'-CGAGGGGATGTAGCATGAGT, 3'-ACTTCTCTGAGCGTGCCACT; CHRNG 5'-GCTCAGCTG-CAAGTTGATCTC; 3'- CCTCCTGCTCCATCTCTGTGTC. Changes in gene expression were quantified using the $\Delta\Delta C_t$ method and statistical analysis quantified using 2-way analysis of variance (ANOVA) with either Sidak's multiple comparisons test (1 month time point) or Tukey's multiple comparison test (all other time points). All values are reported as mean and standard error of the mean (SEM).

In Situ Isometric Force Analysis

Isometric force analysis was performed on the TA muscle with modifications to procedures described elsewhere.¹³⁻¹⁶ Under anesthesia (2.0–2.5% isoflurane, 97.5–98.0% O₂, flow rate 1 liter per minute); skin and fascia surrounding the distal hindlimb were surgically removed exposing the TA. 4-0 braided silk surgical suture (Teleflex Medical, Manfield, MA) was tied just distal to the myotendinous junction, and then the tendon was cut distal to the suture knot. In addition, to prevent movement of the paw during stimulation, the extensor digitorum longus and peroneus longus tendons near the ankle were cut. Mice were

positioned in dorsal recumbency on a heated platform to maintain body temperature at 37°C. A clamp was used to secure the hindlimb at 90° at the knee and the paw secured to the physiology table using transpore surgical tape (3M). The TA tendon was secured to a 300C-LR-FP muscle lever (Aurora Scientific, Aurora, Ontario, Canada). Cathode and anode electrodes were inserted distal to the fibular head to stimulate the peroneal nerve. Under control of the Dynamic Muscle Control and Analysis Software suite (Aurora Scientific), optimal electrode placement was determined by repositioning the electrodes and stimulating the nerve at 1Hz until maximum twitch amplitude was recorded indicating bracketing of the peroneal nerve. Optimal length tension was determined by performing isometric twitch stimulation at an increasing range of amplitude and varying tensions until maximum twitch amplitude was observed. Three successive tetanic stimulations (200Hz, 100 pulses per train, 60 seconds of rest between) were performed and the muscle was allowed to rest for 5 minutes. Single stimulations at 15, 30, 60, 100, 120, 160, and 200Hz were then performed with 30 seconds between each stimulation. Data were processed with the DMA software, and a force-frequency curve was derived. Significance was determined using 2-way ANOVA with Sidak's multiple comparison between individual groups and frequencies. All values are reported as mean and SEM.

Analysis of NMJ Endplate Area

Tissues were processed for NMJ staining as described previously.¹⁰ Endplate area was measured using active contour detection in Icy¹⁷ and verified manually for accurate detection of single endplates. Measurements of individual or partial endplates were excluded from analysis. Measurements were exported to Excel and Prism6 for statistical analysis. Significance was determined by Kruskal-Wallis and Dunn's multiple comparison test. All values are reported as mean and SEM.

Periodic Acid–Schiff Staining

Assessment of glycogen clearance was performed by preparing segments of treated and untreated tibialis anterior fixed overnight in 2% glutaraldehyde/0.2M sodium cacodylate buffer (pH 7.3), embedded in epon, sectioned into 0.5-µm sections and stained with periodic acid–Schiff (PAS), as described previously.⁴

GAA Activity and Vector Genome PCR

GAA activity assays and vector genome qPCR were performed as described previously.⁴

Glycogen Content Analysis

Quantitative measurement of glycogen content within TA muscles was performed using the Glycogen Assay Kit (ab65620; Abcam, Cambridge, MA), following the manufacturer's instructions. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test (GraphPad Prism6; GraphPad Software Inc., La Jolla, CA). Semiquantitative analysis was performed by visual analysis of PAS-stained cross-sectional area tissue sections and determining the number of glycogen positive fibers within each section from 3 independent animals. Statistical significance was determined by 2-way ANOVA with Tukey's multiple comparison test (GraphPad Prism6; GraphPad Software).

Innervation Scoring

Innervation status of the NMJ was determined by colocalization between nerve terminals (stained with neurofilament heavy and synaptotagmin antibodies) and nicotinic acetylcholine receptors (AChRs; labeled with α -bungarotoxin). Photomicrographs of NMJs were processed using Adobe Photoshop CC with brightness and contrast editing to minimize background noise before analysis. Innervated junctions were defined as overlap (either full or minimal) between nerve terminal staining and AChRs. Denervated junctions were defined as the presence of AChR staining with no visible presence of nerve terminals. A minimum of 100 junctions from 3 independent animals were analyzed by blinded evaluators. Statistical significance was performed using 2-way ANOVA with Sidak's multiple comparison test (GraphPad Prism6; GraphPad Software).

Results

Animal Groups

Our initial goal was to determine whether efficacy of AAV9-hGAA to impart therapeutic benefit is modified by disease severity in Pompe disease. Animals were assigned to 3 study groups based on the age at which AAV9-hGAA was administered by intramuscular injection into the TA muscle and the age at which analyses were performed. The early group consisted of wild-type (WT), *Gaa*^{-/-}-untreated, and *Gaa*^{-/-}-AAV9-hGAA-treated animals injected at 1 month of age. These animals were analyzed for gene expression at 3 months and additional measures at group termination (6 months of age). The mid-stage diseased group consisted of WT, *Gaa*^{-/-}-untreated, and *Gaa*^{-/-}-AAV9-hGAA-treated animals injected at 9 months of age and analyzed at 12 months of age. The late-stage disease group consisted of WT, *Gaa*^{-/-}-untreated, and *Gaa*^{-/-}-AAV9-hGAA-treated animals injected at 15 months of age and analyzed at 18 months of age. All *Gaa*^{-/-}-AAV9-hGAA-treated animals received a single intramuscular injection of 1×10^{11} vg AAV9-CMV-hGAA delivered to the TA muscle.

Early and Persistent Elevation of AChR Subunit Transcript Expression in *Gaa*^{-/-} Mice

We have previously reported on the pathology of the NMJ in TA and diaphragm muscles of 9-month-old *Gaa*^{-/-} mice relative to age-matched WT controls.¹⁰ Altered gene expression of AChRs has been reported to accompany pathophysiology in a number of neuromuscular disorders reflecting abnormal homeostasis or compromised integrity of the NMJ.^{18–20} It is not presently known whether changes in expression result from alterations in receptor turnover dynamics, or from additional expression from nonsynaptic nuclei within the myofiber as a result of loss of nerve imprinting. To determine whether altered AChR messenger RNA expression occurs in Pompe disease, and evaluate the effectiveness of AAV9-hGAA administration to prevent abnormal gene expression, we examined AChR gene expression in the TA muscle at a number of time points ranging from 1 to 18 months of age (Fig 1).

We analyzed gene expression at 1 month of age and observed significant elevation of AChR δ -subunit (Fig 1A; mean fold change: 2.15 ± 0.37 ; $p = 0.01$). We did not observe any difference in expression of the α - or γ -subunits at 1 month of age (1.51 ± 0.18 and $1.59 \pm$

0.34 [$p = 0.05$], respectively). We therefore designated 1 month of age as the “early NMJ pathophysiology” time point. By 3 months of age, α -, δ -, and γ -subunits were all significantly elevated in *Gaa*^{-/-} TA relative to age-matched WT control muscles (Fig 1B; mean fold change: 2.23 ± 0.42 [$p = 0.01$]; 6.31 ± 1.88 [$p = 0.0001$], and 2.26 ± 0.20 [$p = 0.01$], respectively). Administration of AAV9-hGAA at 1 month of age modified the otherwise elevated expression of α - and δ -subunits to WT levels (mean fold change: 1.31 ± 0.34 and 1.30 ± 0.40 [$p = 0.05$], respectively), although did not modify the expression of the γ -subunit to WT levels in 3-month-old (Fig 1B; 2.57 ± 0.67 ; $p = 0.01$) treated *Gaa*^{-/-} mice.

The elevated expression of α -, δ -, and γ -subunits in untreated *Gaa*^{-/-} mice persisted to 6 months of age (Fig 1C; mean fold change: 2.50 ± 0.63 [$p = 0.001$], 7.53 ± 2.08 [$p = 0.0001$], and 2.49 ± 0.77 [$p = 0.001$], respectively), with treatment at 1 month of age modifying expression of α -, δ -, and γ -subunits in 6-month-old treated *Gaa*^{-/-} mice to a range similar to WT mice (Fig 1C, mean fold change: 1.51 ± 0.17 , 1.39 ± 0.08 , and 1.15 ± 0.13 , respectively; $p = 0.05$). We also observed reduced expression of the ε -subunit in AAV9-hGAA-treated *Gaa*^{-/-} mice, relative to WT animals (Fig 1C, mean fold change: 0.52 ± 0.05 ; $p = 0.05$).

These initial data suggested that AAV9-hGAA can impart long-term modifications to gene expression of AChR if administered before onset of severe neuromuscular junction pathology. We next determined how AAV9-hGAA modifies gene expression in later stages of the disease.

At 12 months of age, there was significant elevation of the AChR δ -subunit (Fig 1D; mean fold change: 7.16 ± 2.25 ; $p = 0.05$), with no significant elevation observed in either the AChR α - or γ -subunits in untreated *Gaa*^{-/-} mice, relative to age-matched WT control muscles (Fig 1D; mean fold change: 1.91 ± 0.63 and 1.05 ± 0.4 , respectively; $p = 0.05$). AAV9-hGAA administration at 9 months of age was sufficient to modify the otherwise elevated expression of the AChR δ -subunit to WT levels 3 months post-treatment in 12-month-old *Gaa*^{-/-} mice (Fig 1D; mean fold change: 1.89 ± 1.39 ; $p = 0.05$) and did not further modify expression of AChR α - or γ -subunit to levels significantly different from WT mice (Fig 1D; mean fold change: 1.55 ± 1.19 and 2.26 ± 1.55 , respectively; $p = 0.05$).

In 18-month-old *Gaa*^{-/-} mice at an advanced disease stage, elevated gene expression of α -, δ -, and γ -AChR subunits were observed (Fig 1E; mean fold change: 3.91 ± 0.25 ; [$p = 0.01$], 9.93 ± 1.01 [$p = 0.0001$], and 7.07 ± 1.01 [$p = 0.0001$], respectively). The ability of AAV9-hGAA administration at 15 months of age (during advanced disease stage) to correct expression of AChR subunit quantities was severely limited and, in some instances, led to increased AChR subunit gene expression, relative to age-matched WT animals. In 18-month-old *Gaa*^{-/-}-treated animals (3 months post-treatment), the α -subunit remained elevated (Fig 1E; mean fold change: 5.88 ± 1.48 ; $p = 0.0001$). Similarly, AAV9-hGAA did not modify expression of the δ -subunit, which remained elevated in 18-month-old treated *Gaa*^{-/-} mice, relative to age-matched WT controls (Fig 1E; mean fold change: 10.87 ± 2.28 ; $p = 0.0001$). We observed a striking upregulation in gene expression of the γ -subunit in 18-month-old AAV9-hGAA-treated *Gaa*^{-/-} mice, relative to WT controls (Fig 1E; mean fold change: 29.79 ± 9.64 ; $p = 0.0001$). AAV9-hGAA did appear to modify gene expression of ε -

subunit to levels not different from WT in 18-month-old treated animals (Fig 1E; mean fold change: 2.05 ± 1.55 ; $p = 0.05$). Throughout the course of 18 months in *Gaa*^{-/-} TA muscles, we did not observe any significant increase in expression of the ϵ -subunit (Fig 1; mean fold change 1 month: 0.87 ± 0.05 ; 3 months: 1.26 ± 1.11 ; 6 months: 1.05 ± 0.4 ; 12 months: 0.54 ± 0.18 ; 18 months: 1.56 ± 0.12 ; $p = 0.05$).

Our data suggest that elevation of gene expression of AChR subunits is correlated with onset, progression, and associated severity of Pompe disease, with significant changes in transcript level detectable at 1 month of age that progress with disease onset and impact the ability of AAV9 to normalize myofiber gene expression of key AChR subunits to WT levels.

Intramuscular Injection of AAV9-hGAA Attenuates Physiological Deterioration Postintervention During Early- and Mid-, but Not Advanced-Stage, Pompe Disease

Physiological correction of muscle function is essential for treatment of neuromuscular disorders. Our aim was to determine whether improvement in muscle function accompanies the molecular correction of Pompe NMJ pathophysiology. Post-tetanic isometric force analysis was determined *in situ* from TA muscles in WT, *Gaa*^{-/-} and *Gaa*^{-/-}-AAV9-treated mice to assay muscle function (Fig 2; Supplementary Table 1).

At 6 months of age, the force generated by the muscles normalized to animal body mass of *Gaa*^{-/-} mice was impaired, compared to age-matched WT mice (Fig 2A; WT: peak normalized force 15.5 ± 0.94 mN/g; *Gaa*^{-/-}: 10.9 ± 0.8 mN/g; $p = 0.001$) at stimulation frequencies of 60Hz or greater (fused tetanic contractions). Intramuscular injection of AAV9-hGAA at 1 month of age significantly increased peak normalized force production above those of untreated *Gaa*^{-/-} mice to levels comparable to WT (Fig 2A, *Gaa*^{-/-}+AAV9 peak normalized force: 17.2 ± 1.1 mN/g).

Force production in animals injected at 9 months of age and measured at 12 months of age was significantly higher in AAV9-treated *Gaa*^{-/-}, compared to untreated *Gaa*^{-/-}, mice at stimulation frequencies ranging from 100 to 160Hz (Fig 2B; $p = 0.05$); however, no significant difference was observed at 200Hz, which corresponded to peak stimulation frequency of *Gaa*^{-/-} mice, but not the *Gaa*^{-/-}-AAV9-treated group (Fig 2B; $p = 0.05$). Despite the improvement in physiological function in AAV9-treated *Gaa*^{-/-}, compared to untreated *Gaa*^{-/-}, mice, peak force production was significantly impaired, relative to WT mice (160Hz *Gaa*^{-/-}-AAV9 12.49 ± 0.62 ; WT 15.18 ± 0.88 mN/g; $p = 0.05$).

No significant differences in physiological function at 18 months were observed between *Gaa*^{-/-} and *Gaa*^{-/-}-AAV9 animals treated at 15 months of age across all stimulation frequencies (Fig 2C; $p = 0.05$). Force production of both groups was significantly impaired, compared to WT mice, at stimulation frequencies between 60 and 160Hz (Fig 2C; $p = 0.05$).

Taken together, these data suggest that the physiological benefit of AAV9 administration is limited by pathological state, with early intervention before indication of neuromuscular junction deterioration sufficient to correct physiological function to normal levels.

Vector Genomes and GAA Activity

We chose to evaluate whether disease-specific pathology influenced vector genome biodistribution and relationship to GAA activity. qPCR for vector genome number (vg) and GAA activity assays from TA muscle harvested from 6-, 12-, and 18-month-old groups was performed (Fig 3).

Vector genomes were readily detectable in the injected TAs at all time points (Fig 3A; 6-month-old *Gaa*^{-/-}+AAV9: $5.2 \times 10^4 \pm 1.9 \times 10^4$ vg/ μ g DNA; 12-month-old *Gaa*^{-/-}+AAV9: $6.6 \times 10^4 \pm 1.3 \times 10^4$ vg/ μ g DNA; 18-month-old *Gaa*^{-/-}+AAV9 $1.6 \times 10^5 \pm 2.7 \times 10^4$ vg/ μ g DNA).

GAA activity in the injected TA reached supraphysiologic levels (Fig 3B; 6-month-old: *Gaa*^{-/-} with AAV9-hGAA had activity of 165.1 ± 30.7 nmol/mg protein/hr; 12-month-old: *Gaa*^{-/-} with AAV9-hGAA had activity of 678.7 ± 150.5 nmol/mg protein/hr, 18-month-old: *Gaa*^{-/-} with AAV9-hGAA had activity of 644.8 ± 198.5 nmol/mg protein/hr), compared to WT (Fig 3B; 6-month-old WT: 36.1 ± 1.9 nmol/mg protein/hr; 12-month-old WT: 36.00 ± 1.80 nmol/mg protein/hr; 18-month-old WT: 47.41 ± 2.12 nmol/mg protein/hr). GAA activity in untreated *Gaa*^{-/-} animals remained negligible (Fig 3B; 6-month-old *Gaa*^{-/-}: 1.6 ± 0.6 nmol/mg protein/hr; 12-month-old *Gaa*^{-/-}: 4.1 ± 1.3 nmol/mg protein/hr; 18-month-old *Gaa*^{-/-}: 2.2 ± 0.6 nmol/mg protein/hr). These data support the observation that AAV9-hGAA confers gene expression and restores enzyme activity at the site of administration.

AAV9-hGAA Promotes Glycogen Clearance From Myofibers at All Stages of Disease Severity

AAV9 is an attractive therapeutic candidate for systemic diseases because of its broad tissue tropism. To assess whether efficiency of myofiber transduction is modified by disease severity and could partly explain the inability to provide physiological correction, we analyzed sections of AAV9-hGAA-treated TAs to determine the relative effectiveness of glycogen clearance. Levels of glycogen deposition in the AAV9-hGAA-injected TA of all time points (Fig 4C, F, I) were comparable to WT muscle (Fig 4A, D, G), whereas profound accumulation of glycogen remained in untreated *Gaa*^{-/-} muscles (Fig 4B, E, H). Despite direct administration of AAV9-hGAA to the TA, a small population of myofibers still appear to be untreated and show glycogen deposition (Fig 4C, F, I).

Given that physiological function is not improved after AAV9-hGAA injection at an advanced-stage disease, we performed biochemical analysis to determine relative glycogen content of treated TA muscles from mid- and advanced-stage animals (Fig 4J). AAV9-hGAA treatment at 9 and 15 months of age significantly reduced total glycogen content in both 12-month-old (WT: 7.02 ± 1.60 μ g/mg tissue; *Gaa*^{-/-} 32.36 ± 11.19 mg/mg tissue; AAV9-hGAA-treated: 3.51 ± 3.09 μ g/mg tissue; $p = 0.05$) and 18-month-old mice (WT: 6.47 ± 0.53 μ g/mg tissue; *Gaa*^{-/-}: 227.3 ± 10.49 μ g/mg tissue; AAV9-treated: 0.25 ± 2.98 μ g/mg tissue; $p = 0.0001$). No significant difference in glycogen content was observed between WT and AAV9-treated mice in either group ($p = 0.05$). To determine whether myofiber transduction efficiency was impaired, semiquantitative counts on percentage of glycogen-positive and -negative fibers in each treatment group was performed (Fig 4K). No

significant difference in the percentage of glycogen-positive fibers was observed (Fig 4K; 12-month-old + AAV9: $34.22 \pm 7.95\%$; 18 month old + AAV9: $37.30 \pm 9.88\%$; $p = 0.83$). Together, these data suggest that the ability of AAV9-hGAA to transduce myofibers and clear glycogen is not modified by disease severity.

AAV9-hGAA Has Limited Ability to Reduce Motor Endplate Area and Innervation Status After Intramuscular Injection

We have previously reported significant increases in the area occupied by AChRs on the muscle surface (motor endplate area) in 9-month-old WT and *Gaa*^{-/-} mice, indicative of a neuromuscular phenotype.¹⁰ Innervation and stabilization of the neuromuscular junction results in reduction of end-plate area by clustering AChR to the specific synaptic site on the myofiber in addition to selectively limiting gene expression to those subsynaptic nuclei. Because the effectiveness of AAV9-hGAA therapy to modify AChR gene was variable, depending on the time of treatment, we examined the ability of AAV9-hGAA to modify motor endplate area (Fig 5).

In 6-month-old animals, a significant increase in motor endplate area was observed for *Gaa*^{-/-} (mean area: $853.8 \mu\text{m}^2 \pm 23.72$), compared to WT (mean area: $580.2 \mu\text{m}^2 \pm 17.1$) mice (Fig 5A–D). Motor endplate area in 6-month-old *Gaa*^{-/-}+AAV9 (treated at 1 month of age) occupied a significantly reduced area (*Gaa*^{-/-}AAV9: $704.3 \pm 26.36 \mu\text{m}^2$; $p = 0.01$), relative to untreated *Gaa*^{-/-} mice (Fig 5D); however, the area occupied by AChR receptors in the *Gaa*^{-/-}+AAV9 mice remained significantly larger than those of WT controls ($p = 0.001$; Fig 5D). This effect appeared to be independent of neuromuscular junction innervation status, given that no difference in the number of innervated or denervated neuromuscular junctions was observed between 6-month-old groups (Fig 6A–D; denervated WT: $4.1 \pm 1.9\%$; *Gaa*^{-/-}: $5.2 \pm 3.4\%$; *Gaa*^{-/-}AAV9: $4.6 \pm 0.8\%$; $p = 0.05$).

AAV9-hGAA administration at mid-stage disease did not modify motor endplate area. Thus, the mean motor endplate area of both 12-month-old *Gaa*^{-/-}AAV9-treated mice ($852.5 \pm 33.62 \mu\text{m}^2$) did not differ from *Gaa*^{-/-} mice ($856.3 \pm 28.31 \mu\text{m}^2$), with both significantly larger than WT mice ($647.6 \pm 20.86 \mu\text{m}^2$; $p = 0.001$; Fig 5E–H). However, consistent with gene expression data (Fig 1), the otherwise elevated frequency of denervation observed in 12-month-old *Gaa*^{-/-} mice (Fig 6F, H; $41.3 \pm 7.2\%$) was significantly reduced in AAV9-treated *Gaa*^{-/-} mice (Fig 6G, H; $8.6 \pm 5.9\%$; $p = 0.05$) back to WT levels (Fig 6H; $15.8 \pm 6.2\%$) accompanying the rescue of gene expression (Fig 1B).

Motor endplate area or innervation was not modified by AAV9-hGAA administration in the advanced-stage group. Eighteen-month-old *Gaa*^{-/-} ($682.9 \pm 18.69 \mu\text{m}^2$) and *Gaa*^{-/-} + AAV9-treated mice ($717.2 \pm 21.62 \mu\text{m}^2$) had significantly lower endplate areas than age-matched WT mice ($861.4 \pm 28.28 \mu\text{m}^2$; Fig 5 I–L). No difference was observed between the number of denervated junctions between WT and *Gaa*^{-/-} mice (Fig 6L; WT: $8.5 \pm 4.2\%$; *Gaa*^{-/-}: $18.6 \pm 4.7\%$; *Gaa*^{-/-}+AAV9: $29.7 \pm 5.6\%$), which is consistent with lack of modification to the molecular (Fig 1B) and physiological (Fig 2C) profiles after late treatment.

Collectively, these data suggest that innervation status of the NMJ partially correlates with the molecular and physiological profiles, and the ability of AAV9 to rescue innervation at the NMJ is influenced by the stage of neurological disease progression.

Discussion

Our data confirm that NMJ pathology is an early part of the pathophysiology in Pompe disease and plays a significant role in reduction in strength and mobility described previously.⁷ In keeping with other disorders affecting the NMJ, elevated transcript levels of AChR subunits within the muscle are more pronounced with age or disease progression.^{18,19} These findings support defects in nerve-muscle signaling as a primary factor contributing to Pompe pathology that manifests early in disease onset and persists throughout the course of the disease.²¹

Unequal change in the transcription of AChR subunits at any given stage of Pompe disease remains unclear, however, this finding could be attributed to specific signaling cascades induced by motor neuron connectivity. We speculate that defects in neuronal imprinting and synaptogenesis or plasticity of the NMJ accounts for the increased transcription of AChR subunits throughout the course of Pompe disease.

Additionally, we have evaluated the potential of AAV9-hGAA to impart both molecular and functional correction after direct intramuscular injection at early, mid, and advanced stages of Pompe disease. Given that synaptogenesis involves precise coordination and signaling between a variety of specialized cell types, it is critical that expression of GAA is properly expressed in the motor system (pre- and postsynaptic cells) to achieve a therapeutic effect.

A key finding of this work is that early intramuscular delivery of AAV9-hGAA led to correction of gene expression and force generation in the TA muscle. The therapeutic effect was attenuated in animals treated during the mid-stage of Pompe disease pathology, where improved force measurements remained lower than those of WT animals, even with correction of gene expression profiles and innervation status. Quantification of NMJ innervation status demonstrated a greater number of denervated motor endplates in 12-month-old untreated *Gaa*^{-/-} mice than 18-month-old untreated *Gaa*^{-/-} mice. Without analyzing all junctions within the muscle, we speculate that, at mid-stage disease, there are a higher percentage of denervated endplates relative to the total number. This could be reflective of a time point where the endplate and nerve are no longer able to maintain a proper connection reflecting actively occurring neuronal dieback during the transition from mid to advanced stage of the disease. In the advanced stage, it is possible that there is selective preservation of specific motor endplates that result in a higher number of innervated NMJs relative to the total number.

Interestingly, no improvements in either gene expression, force production, endplate area, or innervation status were observed in advanced-stage Pompe animals after AAV9-hGAA treatment, despite high levels of AAV9-hGAA tissue transduction and clearance of glycogen from myofibers. High copy numbers of vector genomes were detected within the injected TA at all time points studied, with no evidence to suggest either impairment in ability of

AAV9-hGAA to transduce injected tissues or activity of the expressed enzyme to deplete glycogen with disease progression. It should be noted that quantified vector genome copies from 18-month-old treated animals were elevated, compared to 12-month-old animals. This observation suggests that transduction efficiencies may vary with disease status in the Pompe mice. Although we have no definitive explanation, there are several possibilities to elucidate the observed results. In brief, the dependence of the AAV life cycle on cellular stress has been exploited to generate higher transduction efficiency of AAV-mediated gene delivery. Several approaches, including proteasome, DNA synthesis, and topoisomerase inhibitors, have generated varied success in improving AAV transduction efficiency.¹⁻⁷ More recently, alternative cellular stressors have been used. Arsenic trioxide has been shown to enhance transduction significantly *in vitro* and *in vivo*.⁸ Whether these compounds have a similar action, compared to the cellular stress observed in Pompe disease, is unclear. An alternative explanation to the observed increase in transduction and GAA activity levels in advanced-stage cells may be the result of vector dosage. In this study, all animals received a fixed dose of 1×10^{11} vg. At this stage of disease, animals demonstrate muscle atrophy and weight loss, and therefore the amount of vg administered may have been higher on a vg/mg of tissue basis. Nonetheless, we believe the lack of AAV-GAA to impact neuromuscular function in aged animals supports the urgency of treating before advanced stages of Pompe disease.

No functional detriment resulting from the observed levels of GAA expression was detected in this study. Our observations are in keeping with preclinical toxicology studies for intramuscular rAAV1-CMV-hGAA, where a 3-fold higher dose (3×10^{11} vg) did not find evidence of histopathology.²² These findings have been corroborated by results from a phase I/II clinical trial in human patients who received 5×10^{12} vg of rAAV1-hGAA directly injected into the diaphragm, where no adverse events have been detected⁴⁹.

This study provides the first evidence of *in situ* force deficits at different stages of disease progression in the mouse model of Pompe disease. A critical difference between these and previous *ex vivo* skeletal muscle contractile measurements^{5,6,8} is the evaluation of muscle function after *in situ* nerve stimulation and is the only method for direct assessment of an intact neuromuscular system. Importantly, these findings are consistent with the alterations in activation of the TA after volitional or stimulated neurophysiological responses in a cohort of Pompe subjects.²¹

Consistent with other myopathies,¹⁸ we have previously demonstrated increased motor endplate area and loss of innervation as a phenomenon accompanying the progression of NMJ deterioration in Pompe disease.¹⁰ Innervation of the NMJ regulates both gene expression and motor endplate area and presumably reflects the potential for neuromuscular signaling and adequate physiological response. This variable appeared the least responsive to AAV9-hGAA treatment and warrants further investigation. Motor endplate expansion could be indicative of either innervation problems (in keeping with other diseases^{18,19,25}) or a physiological response in AAV9-treated mice to increase signaling at the NMJ.²⁶ Understanding the mechanisms leading to these adaptations will provide greater insight into maintenance of the NMJ, influenced by both muscle and nerve function in Pompe disease.

Our findings contribute to mounting evidence of neuropathology associated with glycogen accumulation in Pompe disease and demonstrate the complexity of developing therapies to attenuate multi-tissue pathology. Neuromuscular function is dependent on both pre- and postsynaptic inputs. Pathological changes in the muscle and or neural input may act together or independently leading to weakness. Traditional neurophysiological testing in Pompe patients has identified some abnormalities of motor control^{21,27–31}; however, further mechanistic studies are needed, especially to evaluate the response to specific therapies targeting the NMJ.²¹ Our data support the finding that early intervention is important in treatment and management of Pompe disease,^{28,32} given that some changes in the lower motor neuron may be irreversible or lead to cell loss. Similarly, we do not know the proportional contribution of muscle and nerve pathology to NMJ pathology in Pompe disease. In our previous studies on the phrenic motor system, restricting restoration to lower motor neuron function lead to rescue of ventilation in the mouse model,³³ suggesting that the dominant aspect is the neural deficit. The current US Food and Drug Administration–approved treatment for Pompe disease is enzyme replacement therapy (ERT) involving biweekly infusions of recombinant GAA enzyme. A critical drawback to this treatment option is the inability of GAA to cross the blood–brain barrier, therefore providing little therapeutic benefit to the central and peripheral nervous system. The notion of early partial correction (ie, muscle only), such as would be elicited by ERT, could explain some early impact on NMJ development, especially if the rate of glycogen accumulation in muscle and nerve differ. Correction of the muscle may be sufficient to stymie NMJ deterioration in an ERT-treated population, but not fully prevent inevitable dysfunction resulting from progressive nerve pathology. We speculate that neuromuscular junction pathology limits ERT-mediated improvements in respiratory and ambulatory function in Pompe disease given that treatment of both nerve and muscle pathology is essential to advance long-term patient outcomes.

Our data provide new insight into the pathogenesis of neuromuscular dysfunction in Pompe disease. We speculate that restoring the defective gene to both the motor neuron and muscle before onset of significant abnormalities is sufficient to restore neuromuscular function in *Gaa*^{−/−} mice, leading to *in situ* force production, which is indistinguishable from WT animals. Further work will be required to support the independent role of the NMJ in this process and investigate both the mode of neuromuscular failure and how neural and muscle restoration of GAA contribute to preserving neuromuscular function. The potential to rescue neuromuscular performance with delivery of AAV9-hGAA is impaired with disease progression, and the degree of neuromuscular plasticity in patients with partial GAA deficiency will be a key determinant of the success of such therapeutic efforts. Early-onset patients would be expected to have more rapid loss of motor neuron function; therefore, early intervention in this group is of paramount importance. Efficient clearance of glycogen in ERT is hampered by both the potency of the hGAA to efficiently bind to the cell surface, as well as in trafficking to the lysosomal compartment.^{34–37} Consistent with other related studies,^{1,4,38,39} our data support the development of AAV9-hGAA to treat disorders associated with pathology of NMJ although highlight the importance of establishing markers of NMJ deterioration that could limit the functional benefit after gene therapy administration.

This study highlights the importance of establishing physiological biomarkers that aid in the diagnosis of early functional decline and post-treatment outcomes. Magnetic resonance imaging (MRI) and electrical impedance myography (EIM) are two candidates capable of detecting alterations in muscle composition.^{40,41} MRI in late-onset Pompe disease patients has demonstrated infiltration of fat into affected tissues,⁴² whereas recent evidence from retrospective analysis of human Pompe patients demonstrates the importance of monitoring specific muscle groups that accurately reflect the development and progression of neuromuscular involvement in Pompe disease.⁴³ The refinement of EIM to quantify changes in diseased muscle with subcutaneous fat lends its use to determine changes throughout the clinical spectrum in Pompe disease patients in addition to its current use in patients with related neuromuscular disorders.^{41,44–48}

The results of this current study raise additional questions related to NMJ pathogenesis and the capacity of therapies to specifically preserve or restore NMJ integrity. Currently, the extent of NMJ alterations and the effect on muscle activation in the patient population is unknown. The current study to evaluate AAV1-hGAA-mediated correction of GAA deficiency in Pompe disease has provided some early evidence that restoration of GAA activity in the diaphragm and phrenic motor pool can lead to improvements in some parameters of respiratory function.⁴⁹ Certainly, defining a potential therapeutic window and required effective dose will be an essential next step in developing AAV9-hGAA as an effective treatment for global neuromuscular dysfunction in Pompe disease. As with related neuromuscular disorders, further optimization of administration routes, promoter, and viral serotypes to maximize systemic targeting and replacement of defective gene products is crucial to facilitating maximum therapeutic benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Elevated gene expression of acetylcholine receptor (AChR) subunits accompanies onset and progression of Pompe disease. (A) Significant (** $p < 0.01$) elevation of AChR δ -subunit gene expression in 1-month-old *Gaa*^{-/-} mice. (B, C) Significant (** $p < 0.01$) increase in expression of AChR subunit α - and δ -subunits at (B) 3 months of age and (C) 6 months of age that is corrected after intramuscular TA injection of AAV9-hGAA at 1 month of age. (D) Significantly elevated expression of the AChR δ -subunit in 12-month-old animals that is reduced by intramuscular TA injection at 9 months of age. (E) Significant elevation in gene expression of α -, δ -, and γ - AChR subunits in 18-month-old *Gaa*^{-/-} mice that is not modified by administration of AAV9-hGAA at 15 months of age. Data presented as mean $\pm$ standard error of the mean ($n = 3$ animals per group per time point). Dotted line indicates wild-type (WT) value. AChR = acetylcholine receptor; *Gaa* = acid alpha-glucosidase; AAV = adeno-associated virus; AAV9-hGAA = AAV9 vector expressing GAA; TA = tibialis anterior; n.s. = non significant.

FIGURE 2.

Ability of AAV9-CMV-hGAA to impart neuromuscular correction to contractile function is limited by disease severity in *Gaa*^{-/-} mice. (A) AAV9 significantly improves normalized force (force production relative to body mass) in treated animals relative to *Gaa*^{-/-} ($p = 0.001$) restoring values to wild-type (WT) levels ($p = 0.05$). (B) Intramuscular injection at 9 months of age significantly improves peak force production in treated mice relative to untreated *Gaa*^{-/-} mice 3 months post-treatment ($^{\#}p = 0.05$). Peak force production in WT mice was significantly higher than *Gaa*^{-/-} mice ($****p = 0.001$). (C) Intramuscular AAV9 injection at 15 months of age does not result in improved peak force 3 months post-treatment relative to *Gaa*^{-/-} mice ($p = 0.05$). WT peak force is significantly larger than both AAV9-treated and untreated *Gaa*^{-/-} mice ($****p = 0.001$). Data presented as mean $\pm$ standard error of the mean (6 months $n = 8$ wild-type $n = 6$ *Gaa*^{-/-} and $n = 5$ *Gaa*^{-/-}+AAV9; 12 months $n = 15$ WT, $n = 8$ *Gaa*^{-/-} and $n = 8$ *Gaa*^{-/-}+AAV9; 18 months $n = 5$ WT, $n = 3$ *Gaa*^{-/-} and $n = 6$ *Gaa*^{-/-}+AAV9). Gaa = acid alpha-glucosidase; AAV = adeno-associated virus; n.s. = not significant.

FIGURE 3.

Myofiber transduction efficiency and GAA activity is not modified by disease severity. (A) Quantification of viral vector genomes in TA tissue harvested either 5 months (early-stage disease) or 3 months postinjection of mid- or late-stage disease *Gaa*^{-/-} mice. In all treated mice, vector genome (vg) copies are readily detectable at elevated levels. (B) Elevated levels of GAA enzyme activity in 6-, 12-, and 18-month-old treated muscle. Data presented as mean ± standard error of the mean (n = 3–6 animals per group per time point). GAA = acid alpha-glucosidase; TA = tibialis anterior; WT, wild type.

FIGURE 4.

AAV9-CMV-hGAA promotes glycogen clearance in early-, mid-, and late-stage diseased muscle. (A–I) Representative cross-sectional micrographs of glycogen staining within the TA muscle from 6-month-old (A–C), 12-month-old (D–F), and 18-month-old (G–I) wild-type (WT), *Gaa*^{-/-}, and *Gaa*^{-/-}+AAV9 mice, respectively. Quantitative analysis of TA glycogen content of mid- and late-stage diseased groups (J). Semiquantitative glycogen content counts in 12-month-old and 18-month-old treated animals (K). Scale bar represents 40μm. Gaa = acid alpha-glucosidase; AAV = adeno-associated virus; TA = tibialis anterior; n.s. = not significant.

FIGURE 5.

Pompe disease progression modifies the efficacy of AAV9 to attenuate motor endplate area expansion. (A–C) Representative micrographs of motor endplates and quantified area (D) in early-stage treated mice. Motor endplate area (D) is significantly reduced in early-stage treated AAV9-treated mice, when compared to *Gaa*^{-/-} mice ($p < 0.01$); however, it remains significantly larger than wild-type (WT) controls ($p < 0.01$). (E–L) Motor endplate area is not modified by AAV9 administered in mid- (E–H) or late-stage (I–L) diseased animals, relative to age matched *Gaa*^{-/-} controls, but remains significantly altered relative to WT controls ($p < 0.001$). Data presented as mean $\pm$ standard error of the mean ($n = 3$ animals per group per time point). Scale bar represents 40 µm. *Gaa* = acid alpha-glucosidase; AAV = adeno-associated virus.

FIGURE 6.

Pompe disease severity limits the effectiveness of AAV9 to preserve innervation of the NMJ. (A–C) Representative micrographs of innervated motor endplates and (D) quantified numbers of innervated junctions in 6-month-old wild-type (WT), *Gaa*^{-/-} and AAV9-treated mice. (D) No difference in innervation status of the NMJ between 6-month-old WT, *Gaa*^{-/-}, and AAV9-treated mice. (E–L) NMJ innervation is modified by intramuscular AAV9 administration at mid-stage (H), but not late-stage (L), stage disease animals (**p* < 0.05). Data presented as mean ± standard error of the mean (n = 3 animals per group per time point). Scale bar represents 40μm. Gaa = acid alpha-glucosidase; AAV = adeno-associated virus; NMJ = neuromuscular junction; n.s. = not significant.