RETOGRADE GENE DELIVERY TO HYPOGLOSSAL MOTONEURONS: A POSSIBLE THERAPEUTIC APPROACH FOR POMPE DISEASE

By

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To my parents, with love:
Dr. Kamal O. ElMallah and Dr. Turkia A. Abdelal
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Pompe disease is a glycogen storage disorder caused by a deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA). Patients have macroglossia and tongue motor dysfunction with dysarthria, dysphagia and upper airway obstruction. A neural basis for tongue dysfunction is indicated by data from the Gaa<sup>−/−</sup> mouse Pompe model which shows profound glycogen accumulation in hypoglossal motoneurons (XII MNs) and impaired XII motor output. Retrograde viral transport (i.e., muscle to motoneuron) enables targeted gene delivery to specific motor pools. Recombinant adeno-associated virus serotype 9 (AAV9) robustly infects motoneurons, but the retrograde transport capabilities of AAV9 have not been systematically evaluated. Accordingly, we evaluated the retrograde transduction efficiency of AAV9 following direct tongue injection in 129SVE mice as well as a mouse model which displays neuromuscular pathology (Gaa<sup>−/−</sup>). Hypoglossal (XII) motoneurons were histologically evaluated 8 wks following tongue injection with AAV9 encoding green fluorescent protein (GFP) with expression driven by the chicken beta actin promoter (1x10<sup>11</sup> vector genomes (vg)). The retrograde transduction efficiency of AAV9 was similar between the 129SVE mice and those with Gaa<sup>−/−</sup>. Next, we tested the retrograde transduction of AAV
encoding human GAA. We hypothesized that tongue injection with adeno-associated virus serotype 9 (AAV9) encoding GAA would result in persistent GAA protein expression in XII motoneurons of Gaa\(^{-/-}\) mice. AAV9-GAA vector (titer: 1x10\(^{11}\)vg; promoter: chicken beta actin, CBA, or Desmin, DES) or lactated ringers solution (sham) was directly injected into the base of the tongue of 2 mo old mice. As expected, GAA could be immunohistochemically detected in XII motoneurons of 129SVE mice but not sham-injected Gaa\(^{-/-}\) mice. In contrast, robust GAA immunoreactivity could be detected in Gaa\(^{-/-}\) XII motoneurons up to 1 year following AAV9-CBA-GAA tongue injection. Similar results were seen 4 wks following AAV9-DES-GAA injection. Medullary sections (40 µm) showed that GAA-negative XII motoneurons had a swollen cell body and a granulated appearance. However, Gaa\(^{-/-}\) XII MNs that were immunopositive for GAA did not have swollen appearance of the soma. Our data suggest that AAV9 is an effective vector for retrograde gene delivery and gene replacement therapy targeting tongue motor units in Pompe disease.
CHAPTER 1
INTRODUCTION

Background and Significance

Pompe disease is an autosomal recessive disorder caused by a deficiency of acid alpha-glucosidase (GAA), an enzyme that hydrolyzes lysosomal glycogen. This condition results in extensive glycogen accumulation in skeletal muscle, including the muscles of respiration.\(^1-3\) Pompe patients often suffer from respiratory insufficiency which can lead to the need for mechanical ventilation\(^4\). Clinically, Pompe disease can be classified as early (infantile) and late-onset (juvenile/adult), depending on the extent of enzyme deficiency.\(^5\) Infantile Pompe disease is associated with complete or near complete GAA deficiency but late-onset patients maintain some residual GAA activity.\(^2\) Respiratory insufficiency is a common symptom in both infantile and late-onset Pompe disease. Pompe infants typically present at 4–6 months of age with respiratory distress and upper airway obstruction being noted as the first symptom in 25% of cases, with 19% of deaths occurring from respiratory failure.\(^6\) Late-onset patients usually show progressive respiratory weakness, upper airway obstruction, and eventual respiratory failure. Thus, approximately 75% of children and adolescents and 33% of adults with Pompe disease eventually require mechanical ventilation.\(^7\) Although the respiratory insufficiency has traditionally been attributed to respiratory muscle pathology, recent data suggest that there is also a CNS contribution to the respiratory-related morbidity and mortality of this disease.\(^1,8\) Glycogen accumulation is associated with apoptosis in neural cell cultures, and it appears that motoneurons in Pompe patients are particularly susceptible to excessive glycogen accumulation.\(^1,9,10\) Hypoglossal (XII) motoneurons which control the tongue muscles are affected by the glycogen accumulation. We used
a mouse model of Pompe disease (the \textit{Gaa}^{−/−} mouse) to determine if a “gene therapy” approach can simultaneously treat both tongue muscle and XII motoneuron pathology. Our overall hypothesis was that tongue injection of adeno-associated virus (AAV) encoding the GAA gene will cause transduction and therefore GAA gene expression in both tongue muscles and XII motoneurons.

XII motoneurons are important for upper airway patency as these cells regulate the shape, stiffness and position of the tongue.\textsuperscript{11-14} Contraction of the extrinsic tongue muscles, in particular the genioglossus, can dilate and/or stiffen the pharyngeal lumen, thereby minimizing airway narrowing and/or collapse in the face of normal negative inspiratory pressures.\textsuperscript{15,16} Infants with Pompe disease have problems maintaining upper airway patency and often present with macroglossia and tongue weakness.\textsuperscript{17,18} Figure 1-1 shows a mid-sagittal MRI from a 5 months Pompe infant with macroglossia. Tongue weakness leads to extremely limited tongue elevation and a decreased ability to achieve tongue cupping and lip seal for sucking. Therefore, weakness contributes to upper airway obstruction and oral stage dysphagia that results in a weak suck and feeding problems early in life. Additionally, these patients develop pharyngeal dysphagia leading to difficulty with saliva management causing pooling of secretions and drooling. These pooled secretions increase their risk for aspiration. In addition to dysphagia, obstructive sleep apnea is observed in Pompe disease and post-mortem histology has documented replacement of tongue musculature by fat and fibrous tissue.\textsuperscript{19}
Tongue and XII Motoneuron Pathology in a Murine Model of Pompe Disease

The murine model of Pompe disease (Gaa−/−) is an exon 6 GAA knockout model which lacks GAA enzyme activity. As in infantile Pompe disease, mice homozygous for disruption of the acid α-glucosidase gene lack enzyme activity and begin to accumulate glycogen in cardiac and skeletal muscle lysosomes by 3 weeks of age, with a progressive increase thereafter. By 8–9 months of age animals develop obvious muscle wasting and a weak, waddling gait. In addition, the Gaa−/− mice have respiratory difficulties which are evident by 6 months of age. Thus the Gaa−/− exon 6-knockout mouse provides an excellent opportunity to explore Pompe-related respiratory muscle deficits in a unique animal model.

In the Gaa−/− mouse, XII motoneurons show extensive glycogen accumulation (Figure 1-2), and XII motor output is impaired in Gaa−/− mice (Figure 1-3). Specifically, Figure 1-2 shows PAS staining of the XII motoneuron of a Gaa−/− mouse. An abundance of glycogen is seen in the XII motoneuron (A). A greater magnification (B) of an area in the motoneuron shows vacuole filled glycogen in the individual neurons. Figure 1-3 illustrates efferent XII nerve bursting and shows XII bursting in Gaa−/− mice is decreased (B) when compared to XII nerve activity in control (wild type) mice (A) in both integrated (C) and raw (D) XII signals. In addition, there is extensive pathology in the tongue muscle of Gaa−/− mice (Figure 1-4). Hematoxylin and eosin (H&E) staining of 18 mo old Gaa−/− mouse tongues were compared to those of 18-months-old wild type mice (Figure 1-4). Intrinsic muscles of Gaa−/− mouse tongue (A) show apparent atrophy and fibrosis of muscle cells when compared to wild type (B). In this project, we hope to correct deficits in XII output using AAV-based delivery of genes. The most effective method to
target a motoneuronal pool for infection is using retrograde transport following a direct muscle injection, however, effective retrograde transport has proven to be challenging. We proposed to test the effectiveness of retrograde transport using AAV serotype 9 (AAV9) which is more effective in permeating the CNS. To our knowledge, there have been no prior attempts to treat tongue pathology in Pompe disease (or relevant animal models) using AAV. This is of particular importance since the only FDA approved therapy for treatment of Pompe disease, intravenous (i.v.) enzyme replacement therapy, does not cross the blood brain barrier and therefore does not impact CNS pathology. On the other hand, AAV has the potential to reach the CNS by retrograde transduction. Another advantage of AAV is that it does not exhibit pathogenicity in humans and it confers significantly longer transgene expression.

**Retrograde AAV Transduction**

Muscle injection of recombinant adeno-associated virus (AAV) followed by retrograde axonal viral transport provides a means to target gene delivery to specific motoneuron pools. Following the initial report by Kaspar and colleagues several studies have confirmed motoneuron transduction following intramuscular AAV injection. The relative transduction efficacy, however, is typically quite low. For example, Hollis et al. found that self-complimentary (sc) AAV serotype 1 (scAAV1, $1.05 \times 10^9$ viral particles (vp)) transduced approximately 4% of the extensor carpi motoneuron pool after muscle injection, whereas scAAV6 ($1.05 \times 10^9$ vp) transduced <1% of the pool following a similar procedure. In contrast, Towne and colleagues found that gastrocnemius injection of AAV6 ($1.3 \times 10^{12}$ vp) in primates resulted in transduction of approximately
15% of the motoneuron pool.\textsuperscript{20} Thus, the efficacy of retrograde viral transport appears variable across AAV serotypes.

AAV9 is a recently identified serotype that effectively crosses the blood brain barrier\textsuperscript{27} and can robustly infect motoneurons.\textsuperscript{28} Intrathecal delivery of scAAV9-GFP (2.5 X10\textsuperscript{9} vector genomes (vg)) transduced approximately 700 motoneurons in adult mice, but this was not restricted to a given motor pool.\textsuperscript{29} Intravenously administered scAAV9-GFP (doses ranging from 4X10\textsuperscript{11} to 4 X 10\textsuperscript{12} vp) was reported to transduce motoneurons in neonatal but not adult mice.\textsuperscript{30} However, another study found that intravenous delivery of scAAV9-GFP caused transduction of approximately 15\% of motoneurons in both adult mouse (2 X 10\textsuperscript{12} vg) and cat (1.2 X 10\textsuperscript{12} vg) cervical spinal cords\textsuperscript{31}, with no efficient transduction achieved with single-stranded (ss) AAV9-GFP. Thus, AAV9 vectors are capable of gene delivery to motoneurons following intrathecal or intravenous delivery. To our knowledge, however, the present work represents the first systematic evaluation of retrograde transduction efficacy of AAV9 following intramuscular injection.

Study Aims

Aim 1

Our primary aim was to compare and contrast the relative efficiency of ssAAV9 retrograde motoneuron transduction. The ssAAV9 vector has approximately double the transgene capacity compared to scAAV9.\textsuperscript{24,32} This larger capacity offers distinct advantages for treating neuromuscular disorders. Accordingly, we compared the transduction efficiency of ssAAV9 with what could be achieved using a standard retrograde neuranoatomical tracer (cholera toxin beta subunit, CT-\(\beta\)). We and others have shown that CT-\(\beta\) will infect a majority of the associated motoneuron pool following
direct intramuscular injection. The efficiency of retrograde AAV9 transport was tested using the hypoglossal (XII) motor system.

**Aim 2**

Single-gene defect muscular dystrophies that are accompanied by motoneuron pathology would potentially benefit from parallel transduction of muscle and motoneurons following a single AAV injection. However, retrograde viral transport may be influenced by aspects of both muscle and neural pathology. Accordingly, our second aim was to determine if the retrograde transduction efficiency of motoneurons with AAV9 would be blunted in a mouse model with both muscular dystrophy and neuronal pathology. Accordingly, AAV9 retrograde transduction was evaluated in the $Gaa^{-/-}$ (Pompe) mouse where pronounced muscle wasting and motoneuron pathology has been reported.

**Aim 3**

In Pompe disease, tongue weakness contributes to upper airway obstruction, feeding difficulties, and aspiration pneumonia. In addition, obstructive sleep apnea is observed in Pompe disease and post-mortem histology has documented replacement of tongue musculature by fat and fibrous tissue. Thus, our final aim was to determine if tongue injection with AAV9 encoding the gene for GAA results in GAA expression in the tongue with retrograde transduction to the XII motoneurons. In addition, we assessed whether expression of GAA reverses tongue and XII neuropathology in Pompe ($Gaa^{-/-}$) mice.
Figure 1-1. A mid-sagittal MRI from a 5 months Pompe infant with macroglossia. Note the enlarged tongue obstructing the upper airway.
Figure 1-2. Plastic-embedded semi-thin medullary sections (2 µm) show neuronal glycogen accumulation in Gaa<sup>−/−</sup> mice. Sections were stained with PAS and toluidine blue. A) Low power view including both hypoglossal nuclei. B) Higher resolution image of the area indicated by the box in A. An abundance of glycogen is seen in the vacuoles of the motoneurons (purple), resulting in swollen motoneurons.

Figure 1-3. Blunted XII efferent nerve activity in a Gaa<sup>−/−</sup> mouse (B and D) compared to a wild type mouse (A and C). A) Integrated waveform of the raw signal in C. Note the increased amplitude and regular bursting in the wild type mouse compared to the Gaa<sup>−/−</sup> mouse.
Pathology of the intrinsic muscles of $\text{Gaa}^{-/-}$ mice. There is apparent atrophy and fibrosis of muscle cells, with disorganized nuclei. In contrast, the intrinsic tongue muscles of age matched wild type mice (B) show organized muscle fibers with peripheral nuclei and no evidence of fibrosis.
CHAPTER 2
MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. 129SVE (wild type) mice (N=20, age 4 weeks for the cholera toxin and AAV9GFP injections; 8 weeks ± 3 days for the LR injections (Aim 3) and Gaa⁻/⁻ mice (N=32, age 8 weeks ± 5 days) were used. The Gaa⁻/⁻ mice were created by disruption of exon 6 as previously described,¹³⁶ which results in completely blocking gene expression. These mice were obtained from Taconic, Inc.

**AAV Vectors**

Single stranded AAV vectors were used. They encoded either green fluorescent protein (GFP) or GAA. Expression for GFP was driven by the chicken beta actin (CBA) promoter (AAV9-CBA-GFP), whereas the expression for GAA promoters was driven by either CBA or Desmin (AAV-CBAd-GAA, or AAV9-Des-GAA). All vectors were given at a titer of 1 X 10¹¹ vg/mL, and were generated and titered at the University of Florida Powell Gene Therapy Center Vector Core Laboratory using previously published methods.³⁸ Vectors were purified by iodixanol gradient centrifugation and anion-exchange chromatography as described previously.³⁸ Final formulations of vectors were in lactated ringer solution.

**In Vivo Delivery**

All animal studies were performed in accordance with the guidelines of the University of Florida IACUC. Mice were anesthetized with 3% isoflurane in oxygen administered via a nose cone. For Aims 1 and 2: Ten (5 females, 5 males) 4 week old 129SVE mice and four (1 female, 3 males) 8 week old Gaa⁻/⁻ mice were administered AAV9-CBA-GFP diluted in lactated ringers (20µL) directly to the left side of the base of
the tongue lateral to the lingual frenulum. Six 4-week old 129SVE mice were administered the same volume of lactated ringer solution. These sham injected mice were used as a control group for studies of ventilation and XII efferent nerve discharge (see below). Four (2 females, 2 males) 4 week old 129SVE mice received one injection of 5 µl CT-β (0.1%) diluted in 15 µl sterile water to the same area. CT-β is a monosynaptic tracer which we have previously used to label XII motoneurons in mice. For Aim 3: Eight Gaa<sup>−/−</sup> mice were administered AAV9-Des-GAA, 8 Gaa<sup>−/−</sup> mice were administered AAV9-CBAd-GAA, and 8 Gaa<sup>−/−</sup> mice were administered lactated ringers (sham injection). For all injections, the needle was held at a 45 degree angle and inserted to a depth of 2 mm. PE50 tubing was placed over the needle to insure that the depth of injection was uniform in each mouse.

**Vector Pharmacology**

For Aims 1 and 2: PCR was used to measure AAV genome copies in the tongue and medulla of 129SVE mice (N=4). At 8 weeks post AAV9-CBA-GFP lingual injection, tissues were harvested in a manner that prevented cross-contamination, snap frozen in liquid nitrogen and stored at −80°C until genomic DNA (gDNA) was extracted. The medulla was harvested en bloc whereas the entire tongue was harvested and divided into anterior, middle and posterior tongue. gDNA was isolated using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. gDNA concentrations were determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). AAV genome copies in the gDNA were quantified by real-time PCR using an ABI 7900 HT Sequence Detection System (SDS) (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions and results were analyzed using the SDS 2.3 software.
A region of the AAV vector as previously described. A standard curve was performed using plasmid DNA containing the same SV40 poly-A target sequence. PCR reactions contained a total volume of 100 µl and were run at the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. DNA samples were assayed in triplicate. In order to assess PCR inhibition, the third replicate was spiked with plasmid DNA at a ratio of 100 copies/µg gDNA. If this replicate was greater than 40 copies/µg gDNA then the results were considered acceptable. If a sample contained greater than or equal to 100 copies/µg gDNA it was considered positive for vector genomes. If a sample contained less than 100 copies/µg gDNA it was considered negative for vector genomes. If less than 1 µg of gDNA was analyzed the vector copy number reported was normalized per µg gDNA and the plasmid spike-in was reduced to maintain the ratio of 100 copies/µg gDNA. Data were reported as AAV genome copies per µg total genomic DNA ± SD.

**Biochemical Assessment**

For Aim 3: At four and twelve months post-injection, tissue homogenates were assayed for GAA enzyme activity. Animal tongues (divided into anterior and posterior), liver, brain, medulla, cervical cord, and thoracic cord were harvested and lysed in a lysis buffer. Alternatively, harvested muscle tissues were homogenized in water, then subjected to three freeze-thaw cycles. Lysates were centrifuged and clarified supernatants were assayed for GAA activity by measuring the cleavage of the synthetic substrate 4-methylumbelliferyl-α-D-glucoside (Sigma M9766, Sigma-Aldrich, St. Louis, MO) after incubation for 1 hour at 37°C. Successful cleavage yielded a fluorescent product that emits at 448 nm, as measured with a TKO100 fluorometer. Protein concentration was measured using a standard bicinchoninic acid method (Bio-Rad,
Hercules, CA), with bovine serum albumin as a standard. Data are represented as nanomoles of substrate cleaved in 1 hour per milligram of total protein in the lysate (nmol/hour/mg).

**Brainstem Histology**

Mice were anesthetized with isoflurane and urethane (1.5g/kg given intraperitoneal (i.p.)) and euthanized via systemic perfusion with 4% paraformaldehyde. Aims 1 and 2: Tissues were harvested at 72 hours post CT-β injection\(^{33}\) and 8 weeks post AAV9-GFP injection. Aim 3: Tissues were harvested 1, 4 and 12 months post AAV9-GAA. The post-injection time interval for CT-β was selected based on our prior reports.\(^{33,34}\) The brainstem and spinal cord tissues were extracted and post fixed by immersion in 2% paraformaldehyde (N=12) and stored at 4°C for sectioning. Transverse vibratome sections (40µm) were made through the brainstem and sections were placed in 2% paraformaldehyde in 96 well plates until ready for processing. CT-β labeled neurons were detected as previously described.\(^{33}\) The sections were incubated overnight in primary antibodies against CT-β (polyclonal goat antiserum to purified CT-β (choleragenoid isolated from *Vibrio cholerae* type Inaba 569B; List Biological Laboratories, Campbell, CA; product No. 703, lot No. 7032A5, 1:10,000). This antiserum labels a single immunoprecipitation band against 0.5 mg/ml of purified CT-β at a dilution of 1:16 on Western blot (manufacturer’s technical information). On the following day, tissue was washed in PBS, incubated in a biotinylated secondary antibody coupled with a Vectastain ABC Kit and DAB for bright field microscopy. GFP and GAA immunohistochemistry coupled with a Vectastain ABC secondary detection kit and 3,3’-Diaminobenzidine (DAB) was performed on the tissue from the AAV9-GFP and the AAV9-GAA injected mice. The tissue was incubated overnight in primary antibody
against either GFP, 1:20,000 (chicken anti-GFP, AVES Laboratories), or GAA, 1:2000. On the following day the tissue was washed in PBS, incubated in a biotinylated anti-chicken (for GFP) or anti-rabbit (for GAA) IgG secondary antibody, 1:200 (Vector laboratories) and coupled with a Vectastain ABC Kit and DAB for bright field microscopy. In four of the animals (2 sham and 2 vector injected mice) the medullae were post-fixed in 4% paraformaldehyde for 24 hours then transferred to 70% ethanol until processing. Paraffin serial sections (5 μm) were stained with hematoxylin and eosin (H&E) and by immunohistochemistry. Inflammatory cell immunophenotype was determined with monoclonal antibodies that recognize CD3. Periodic acid Schiff (PAS) staining to detect glycogen was also performed on the paraffin serial sections.

**Tongue Histology**

After perfusion, the tongue was removed, post-fixed in 4% paraformaldehyde for 24 hours, then transferred to 70% ethanol until processing. Paraffin serial sections (5 μm) were stained with hematoxylin and eosin (H&E). Inflammatory cell immunophenotype was also assessed using monoclonal antibodies that recognize CD3. CD3 is a protein complex important in transducing the signal that initiates a T cell activation and differentiation pathway. The presence of CD3 indicates activation of a T-cell inflammatory response. In addition, PAS staining was performed on paraffin embedded sections of the tongue. In a subset of mice the tongue was processed differently to enable visualization of GFP immunofluorescence. In those animals, the tongue was frozen in optimal cutting temperature (OCT) compound and liquid nitrogen. Using a cryostat, the tongue was then sectioned at 8 μm and directly examined using fluorescent microscopy.
**Microscopy and Quantitative Analyses**

Brightfield and standard fluorescence photographs were taken with a Zeiss AxioPhot microscope and an AxioCam HRc digital camera linked to a PC. By using brightfield microscopy, CT-β and GFP-positive XII motoneurons with visible nuclei were counted at X10 magnification in consecutive transverse sections of the medulla from each animal. Only cells with a visible nucleus were counted, and an Abercrombie correction was applied to raw counts (total motoneurons counted*[T/(T + h)], where T = section thickness and h = diameter of the nuclei). 33,35,43

**Ventilation**

Ventilation was quantified using whole-body plethysmography in unrestrained, unanesthetized mice as previously described.1 Mice were placed inside a 3.5” × 5.75” Plexiglas chamber which was calibrated with known airflow and pressure signals before data collection. Data were collected in 10 second intervals and the Drorbaugh and Fenn equation 44 was used to calculate respiratory volumes including tidal volume and minute ventilation. During both a 30 minutes acclimation period and subsequent 30–60 minutes baseline period, mice were exposed to normoxic air (21% O₂, 79% N₂). At the conclusion of the baseline period, the mice were exposed to a brief respiratory challenge which consisted of a 10 minute hypercapnic exposure (7% CO₂, balance O₂). Experiments were conducted using 129SVE mice and Gaa knockout mice that had received tongue injection of AAV9-GFP, and Gaa knockout mice that received AAV9-GAA, or sham (N = 8). Data were collected 8 weeks post-injection for the AAV9-GFP group and 2, 4, 6 and twelve months after injection for the AAV9-GAA groups.
Statistical Analysis

Statistical comparisons of the number of labeled neurons and body weight between experimental groups were made using an unpaired $t$-test. Comparison of ventilation parameters was made using a two-way repeated measures analysis of variance. Data were considered to be statistically different when $p < 0.05$. 


CHAPTER 3
RESULTS

CT-β Labeling

Initial experiments verified the extent of XII motoneuron labeling following tongue injection with the standard retrograde tracer CT-β.\(^{35,45,46}\) The XII nucleus was observed caudal and lateral to the medullary central canal as previously reported in mice\(^{34,47}\) (Figure 3-1). On average (n=4), 818±88 XII motoneurons per animal were immunopositive for CT-β. Labeling was restricted to the immediate region of the hypoglossal nucleus which is consistent with prior reports that CT-β does not cross neuronal synapses.\(^{39}\). It has been estimated that the XII nucleus in the adult mouse contains 974 motoneurons.\(^{48}\) Accordingly, CT-β was able to infect approximately 84% of XII motoneurons following a single injection to the base of the tongue.

Retrograde Transport of AAV9 in 129SVE and Gaa\(^{-/-}\) Mice

Evaluation of AAV9-GFP Transduction

As expected, fluorescence microscopy confirmed robust GFP expression in lingual myofibers at eight weeks post tongue injection. GFP expression was immunohistochemically examined in the brainstem of the same animals (Figure 3-1). On average, 234±43 GFP-positive XII motoneurons could be detected per animal. This was significantly less than the total number of XII motoneurons which were labeled following CT-β injections (p<0.01). Thus, retrograde transport of AAV9 to XII motoneurons appears to be approximately 30% as effective as CT-β. There was no evidence for transynaptic transport of AAV9 as GFP expression was limited to the immediate region of the XII motor nucleus and could not be detected in neighboring glial
cells or interneurons. Similarly, GFP expression could not be detected with immunohistochemistry in rostral brainstem regions or the spinal cord (not shown).

In additional experiments, PCR was used to quantify AAV vector genome copies in samples from the tongue and brainstem of 129SVE mice following AAV9-CBA-GFP injection. A predictably high number of copies (60,533±28,784 vg copies per µg DNA) were detected in the base of the tongue (i.e., the site of injection). The middle portion of the tongue also had a high number of copies (25,460±8160vg copies per µg DNA). In contrast, the anterior third of the tongue had much less copies (820±273vg copies per µg DNA). A reduced number of vector genome copies were detected in the en bloc medulla (673±306 vg copies per µg DNA). Since the entire medulla was processed for PCR studies, it is likely that that relative number of AAV9 vector copies (i.e., copies per µg tissue) in the XII nucleus was substantially higher. Nevertheless, these data are consistent with the histological results (Figure 3-1) and with retrograde AAV9 transport.

Additional AAV9-GFP tongue injections were performed in Gaa⁻/⁻ mice. The intent was to compare the retrograde transport of AAV9 in healthy animals to those with a known neuromuscular pathology.¹,³⁶ In Gaa⁻/⁻ mice, an average of 333±102 GFP-positive XII motoneurons could be detected following tongue injection (Figure 3-1). This value was similar to the number of labeled cells in the 129SVE mice (p=0.336).

**Evaluation of AAV-GAA Transduction**

Glycogen accumulation in the muscle myofibers of the tongue results in muscle architecture disruption. Periodic acid Schiff (PAS) staining to detect glycogen revealed clearing of glycogen in the AAV9-GAA injected animals. Figure 3-2A illustrates PAS staining in the tongue of a wild type mouse. As expected, there is minimal PAS
staining. In contrast Figure 3-4B illustrates PAS staining of a Gaa<sup>-/-</sup> untreated tongue. Figure 3-2C shows PAS staining of a treated Gaa<sup>-/-</sup> tongue which was treated one year prior with AAV9-GAA. As a result of treatment, Figure 3-2C illustrates glycogen clearance and resultant improved muscle morphology which persisted one year after AAV9-GAA injection.

GAA expression was immunohistochemically examined in the brainstems of the same animals. Retrograde transduction and GAA expression in the XII nucleus was evident 1, 4 and 12 months (Figure 3-4) after intralingual injections with AAV9-GAA (n=4 in each group). Figure 3-3 illustrates GAA immunohistochemistry with DAB staining in 129SVE (wild type) mice and Gaa<sup>-/-</sup> (Pompe) mice. As expected, there is positive GAA staining in the wild type mouse, but none in the Gaa<sup>-/-</sup> mouse. Figure 3-4 illustrates that there was persistent GAA expression in the XII motor nucleus one year after AAV1-GAA intralingual injections. Note in Panel C the histological appearance of the treated (GAA positive) compared to the untreated motoneuron.

In additional experiments GAA activity assay was performed in the AAV9-GAA injected Gaa<sup>-/-</sup> mice and compared to the LR injected group and the wild type mice. GAA activity levels in the base of the tongue were high in the AAV9-GAA treated animals compared to the wild type animals (Figure 3-5). The base of the tongue was the sight of injection. The GAA activity in the en bloc medulla did not show higher GAA activity in the AAV9-GAA injected mice. This finding is explained by the low relative proportion of GAA activity in the XII motoneurons compared to the whole medulla. These results are similar to the vector PCR data from the AAV9-GFP injected animals.
Immune Response and Impact on Breathing

Histological evaluation of tongue and brainstem tissues indicated no persistent immune response following AAV9-GFP or AAV9-GAA injection (N=3 in each group). Specifically, H&E stained tissues showed no evidence for inflammatory cells, and CD3 immunostaining did not detect an increase in T-lymphocytes (Figure 3-6). In addition, tongue muscle fibers showed no signs of fibrosis or necrosis. Myonuclei were of normal diameter, number and position with no evidence of disorganization of nuclei.

Weight and ventilation Aims 1 and 2: In the wild type mouse weight gain following AAV9-CBA-GFP injection was not different compared to sham injection (p=0.66). Whole body plethysmography (unanesthetized mice) was used to confirm that viral infection and GFP protein expression in tongue myofibers and XII motoneurons had minimal impact on the respiratory control system. In the AAV9-GFP injected group, 8 weeks post-injection, respiratory frequency (breaths*min\(^{-1}\), p=0.93), tidal volume (mls*breath\(^{-1}\), p=0.28) and minute ventilation (mls*min\(^{-1}\), p=0.37) were similar between AAV9-CBA-GFP and sham injected mice.

Weight and ventilation Aim 3: As expected, with corrected tongue pathology, weight gain in the Gaa\(^{-/-}\) mice injected with AAV9-GAA was greater than those injected with LR. However, the difference in weight was not statistically significant (p=0.16). Whole body plethysmography was used to assess the effects of intralingual gene therapy on ventilation (N=8 in each group). Although, not statistically significant, the AAV9-GAA injected Gaa\(^{-/-}\) mice had a lower inspiratory time when compared to the non-injected mice (p=0.22) (Figure 3-7A). Otherwise, there were no significant changes in ventilation between the Gaa\(^{-/-}\) mice injected with AAV9-GAA and those injected with LR.
Figure 37B illustrates the similarities in minute ventilation between the AAV9-GAA and the LR injected Gaa<sup>-/-</sup> animals (p=0.48).
Figure 3-1. Medullary sections showing retrograde transduction of XII motoneurons. Transverse sections through the rostral medulla immunolabeled for the presence of GFP (A, B, C, D, G and H) or CT-β (E, F) and counterstained with cresyl violet. A and B: Sham tongue injections show no GFP labeled XII motoneurons. C and D: AAV9-CBA-GFP tongue injections result in robust retrograde transduction of XII motoneurons. Panel B shows a higher magnification (40X) of the inset in A. E and F: Retrograde XII labeling using CT-β tongue injections was performed as a comparison to the AAV9 experiments. Note that CT-β is distributed throughout the cytoplasm, but not the nucleus. Panels G and H: AAV9-CBA-GFP injected into the tongue of a Gaa⁻/⁻ mouse also resulted in robust retrograde transduction. Note the difference in neuronal morphology in this tissue. This difference is due to the extensive accumulation of glycogen in the lysosomes. Scale bars: A,C,E, G: 200µm; B,D,F, H: 50µm.
Figure 3-2. Periodic Acid Schiff staining for glycogen in the genioglossus (GG) muscle of the tongue in a wild-type mouse (A), LR injected \( Gaa^{-/-} \) mouse (B), and AAV9-GAA tongue injected \( Gaa^{-/-} \) mouse (C). The presence of glycogen is indicated when PAS staining results in a dark pink/magenta color. Note the wild-type tongue muscle fibers are a pale pink which indicates an absence of glycogen accumulation. In contrast, B shows dark pink staining of tongue myofibers of a \( Gaa^{-/-} \) mouse indicative of abundant glycogen. Note also the empty vacuoles in the myofibers (black arrow) where glycogen has been washed out from the lysosomes. This finding is frequently seen in Pompe muscle tissue. C is a section of GG muscle of a tongue of a \( Gaa^{-/-} \) injected with AAV9-GAA a year earlier. Note that overall they myofibers have less pathologic empty vacuoles and that their appearance is healthier. In addition there is much less glycogen than in the LR injected animal. Scale bars: 50µm.
Figure 3-3. GAA immunostaining in XII motoneurons of a wild-type mouse (A) and a Gaa<sup>−/−</sup> mouse (C). The dark brown color indicates the presence of GAA. Transverse sections through the rostral medulla immunolabeled for the presence of GAA and counterstained with cresyl violet. A and B: As can be expected, wild type 129SVE mouse XII motoneurons express GAA. C and D: Gaa<sup>−/−</sup> XII motoneurons express no GAA as evidenced by the lack of positive staining (purple indicates cresyl violet counterstain). B and D show a higher magnification (40X) of the inset in A and C, respectively. Note the difference in neuronal morphology in D when compared to B. The Gaa<sup>−/−</sup> XII motoneurons are swollen and full of vacuoles from glycogen accumulation, with the nucleus pushed to the periphery. Scale bars: A,C: 200µm; B,D: 50µm.
Figure 3-4. Medullary sections showing GAA expression after retrograde transduction of XII motoneurons one year after a single intralingual injection of AAV9-GAA. Transverse sections through the rostral medulla immunolabeled for the presence of GAA and counterstained with cresyl violet. B and D are higher magnifications (20X) of A and C, respectively. Note the difference in neuronal morphology in the GAA positive motoneurons (black arrow) compared to the adjacent motoneurons not expressing GAA (white arrow). Scale bars: A,C: 200µm; B,D: 100µm.
Figure 3-5. GAA activity assay performed one year after AAV9-GAA (AAV9) injection. GAA activity in the base (TB) and anterior (TA) portions of the tongue are higher in the AAV9-GAA injected \(Gaa^{-/-}\) mice compared to the LR injected \(Gaa^{-/-}\) mice and the wild type mice. However, there is no difference in GAA activity in the medulla (Med) between the treated and untreated \(Gaa^{-/-}\) mice.
Figure 3-6. CD3 and H&E staining to evaluate inflammatory responses to AAV9-CBA-GFP. A and B: Tongue CD3 staining (T cell activation) showed no difference between the sham (A) and AAV9-CBA-GFP (B) groups. C and D: Tongue H&E staining did not show increased inflammatory cells in AAV9 (D) vs. sham mice (C). E and F: CD3 staining of the XII region did not show inflammatory activity. Scale bars: A-F: 100µm.
Inspiratory time and inspired minute ventilation (ml/min) measured 4 months following AAV9-GAA tongue injected unanesthetized mice using whole-body plethysmography. A) Inspiratory time in $\text{Gaa}^{-/-}$ mice injected with AAV9-GAA (n=8) is similar to wild type mice (n=8). However, the inspiratory time in the treated animals is less than that of the $\text{Gaa}^{-/-}$ mice injected with sham (LR) (n=8). This result can be attributed to a treated tongue which is smaller and less weak. This results in decreased upper airway resistance thereby decreasing inspiratory time. B) Minute ventilation during quiet breathing (baseline conditions of 21% O2 with balance of N2) and a hypercapnic respiratory challenge (7% inspired CO2 in air) – there is no statistically significant difference between the AAV9-GAA and the LR injected $\text{Gaa}^{-/-}$ treated mice (0.48) and untreated animals, but the wild type group had a greater minute ventilation, statistically significant during the hypercapnea (p=0.04).
Our most significant finding is that an AAV9 vector can effectively transduce a significant portion of the XII motor nucleus following direct intramuscular tongue injection. In this regard, the retrograde transport capability of AAV9 is significantly higher than previously characterized AAV serotypes. In addition, AAV9 was equally effective at motoneuron transduction following tongue injection in a mouse model of Pompe disease. Pompe mice show both motoneuron and muscular pathology but this did not impair the retrograde transport ability of AAV9. The vector therefore a good candidate for targeted gene delivery to motoneurons in animal models associated with neuromuscular pathology and/or degeneration such as ALS, spinal cord injury, and Pompe disease.

Commentary on AAV and Retrograde Transport

AAV2 was the first AAV-serotype proposed for extensive use in gene therapy. Since then, a variety of different AAV serotypes have been described and evaluated. Tissue tropism varies across the serotypes, and this can be exploited for gene therapy applications.

Isolated originally by James Wilson and colleagues, AAV9 can cross the blood brain barrier and scAAV9 efficiently transduces neuronal and glial cells following intravenous or intrathecal delivery. However, ssAAV9 does not have a high level of transduction efficiency with intravenous delivery. Additionally, a relatively high dose of AAV9 is required for motoneuron transduction following systemic intravenous delivery in mice (~ 1×10\(^{13}\) vg/kg), and this could provide an obstacle for translation to clinical use. Overall, an emerging body of evidence supports the use of
the AAV9 serotype as perhaps the most ideal vector for gene delivery to the CNS.\textsuperscript{30,66}

The current data indicate that ssAAV9, which has a larger transgene capacity compared to scAAV9, is also an appropriate vector for retrogradely targeting gene delivery to a specific motor pool. Compared to a standard retrograde tracer (CT-β) capable of robust motoneurons infection\textsuperscript{33,35}, we observed that retrograde transport of AAV9 was approximately 1/3 as efficient. Thus, our AAV9 approach was able to transduce approximately 25% of the XII motor nucleus. This degree of retrograde motoneuron transduction exceeds the relative transduction previously reported for other AAV serotypes that have ranged from approximately 1-15%.\textsuperscript{20,23-25}

\textbf{Pompe Disease and AAV9}

Pompe disease is an autosomal recessive metabolic myopathy caused by the deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA). This deficiency results in cellular lysosomal and cytoplasmic glycogen accumulation.\textsuperscript{6} Myopathy is a hallmark of this disease, but a growing data base indicates that motor failure in Pompe disease also involves CNS pathology.\textsuperscript{1,3,8,17,40,67-69} However, the current clinical strategy of i.v. infusion of recombinant GAA.\textsuperscript{5,70} Current treatment by enzyme replacement therapy has been responsible for improving the overall survival in early onset Pompe disease.\textsuperscript{6} For example, the 18 months survival rate of Pompe infants receiving systemic enzyme replacement therapy (ERT) is dramatically increased compared to historical control patients. However, variability in the success of ERT has been noted, and the long-term survival rates and motor function capabilities of patients on ERT have not been carefully evaluated. In this regard, it is interesting that Muller and colleagues found that Pompe children on ERT are at high risk for developing speech disorders.\textsuperscript{71} These authors speculated that this could reflect lower motoneuron involvement which will not
be effectively targeted by ERT because recombinant GAA enzyme does not effectively cross the bloodbrain-barrier. In addition, Rohrbach and colleagues reported that ERT effectively delayed the muscular progression of a Pompe infant over a 44 months period, but neurological symptoms including impaired language development remained. Thus, much evidence supports the view that intravenous GAA delivery may target cardiac and skeletal muscle, but will not effectively mitigate CNS glycogen accumulation. Based on the current data and both recent and historical reports, we hypothesize that therapy targeting both skeletal muscle and the CNS may be required to fully correct respiratory related deficits. It also is important to emphasize that in the face of generalized weakness, the associated problems of diminished pharyngeal tone, limited secretion clearance, and ineffective cough all culminate in respiratory failure. New therapeutic options in Pompe disease could target a reduction in CNS glycogen storage or could provide a means of pharmacologically activating endogenous gene products. Here, we show a promising method to restore GAA activity to both muscle and neuronal tissue: the use of AAV for gene transfer to muscle coupled with retrograde gene delivery to motor neurons. Additional AAV serotypes which are potentially capable of more robust retrograde transduction (i.e., muscle-to-motoneuron) may be candidates for future studies. Clinical application of gene transfer strategies, alone or in combination with ERT, may provide an important advancement in treatment of Pompe disease. A Phase I/II study administering rAAV2/1-CMV-hGAA to the diaphragm in children with Pompe disease is ongoing (ClinicalTrials.gov Identifier: NCT00976352).
Conclusion

Based on the retrograde transport described here, we suggest that the AAV9 serotype may be a prime candidate for use in future clinical studies to increase retrograde transduction of motor neurons. Tongue injection could be considered in the patient population since there are clinical reports of impaired upper airway function including swallow dysfunction\(^7^4\), speech problems\(^7^1\) feeding difficulties\(^5\) and obstructive sleep apnea.\(^1^9\) In Pompe disease, direct intralingual injections can target both the tongue and XII motoneuron pathology. Ideally, intralingual retrograde gene therapy will be given in conjunction with diaphragm injections to target both the upper airway pathology and the diaphragm pathology. These therapies have the potential to reverse respiratory pathology and prolong ventilator free survival.


BIOGRAPHICAL SKETCH

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