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Neuropathology in respiratory-related motoneurons in young Pompe (*Gaa*^{-/-}) mice

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Abstract

Respiratory and/or lingual dysfunction are among the first motor symptoms in Pompe disease, a disorder resulting from absence or dysfunction of the lysosomal enzyme acid α -glucosidase (GAA). Here, we histologically evaluated the medulla, cervical and thoracic spinal cords in 6 weeks old asymptomatic Pompe (*Gaa*^{-/-}) mice to determine if neuropathology in respiratory motor regions has an early onset. Periodic acid-Schiff (PAS) staining indicated glycogen accumulation was exclusively occurring in *Gaa*^{-/-} hypoglossal, mid-cervical and upper thoracic motoneurons. Markers of DNA damage (Tunel) and ongoing apoptosis (Cleaved Caspase 3) did not co-localize with PAS staining, but were prominent in a medullary region which included the nucleus tractus solitarius, and also in the thoracic spinal dorsal horn. We conclude that respiratory-related motoneurons are particularly susceptible to GAA deficiency and that neuronal glycogen accumulation and neurodegeneration may occur independently in early stage disease. The data support early therapeutic intervention in Pompe disease.

Keywords

Phrenic; Hypoglossal; Intercostal; Neurodegeneration; Glycogen; Lysosomal storage disease

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1. Introduction

Pompe disease results from a mutation in the gene encoding the lysosomal enzyme acid α -glucosidase (GAA). Early-onset Pompe disease occurs when there is complete (or near complete) deficiency of functional GAA; late-onset patients have some residual GAA activity (Hirschhorn, 2001; Raben et al., 2002). Respiratory insufficiency is also common in both early- and late-onset Pompe disease (Burghaus et al., 2006; Mellies and Lofaso, 2009; Mellies et al., 2005; Pellegrini et al., 2005), and “respiratory difficulty” is often noted as the first symptom in Pompe infants (van den Hout et al., 2003). Neuropathology is prominent in Pompe disease (DeRuisseau et al., 2009; Mancall et al., 1965; Sidman et al., 2008), and this has important treatment implications. Intravenous delivery of recombinant GAA protein (*i.e.*, enzyme replacement therapy or ERT) targets skeletal and cardiac muscles but not neurons, and can provide a modest stabilizing impact on respiratory function with a possibility for slight improvements (reviewed in (Fuller et al., 2013)). There is also considerable variability in the success of ERT (Van den Hout et al., 2004) with ventilator-free survival observed in only one-third of ERT-treated early onset Pompe patients (Byrne et al., 2011). Collectively, the clinical and basic science literature indicates that treatments which can address neuropathology are likely to be necessary for optimal correction of Pompe neuromotor deficits (reviewed in (Fuller et al., 2013)). In this regard, a particularly important consideration is the potential need to therapeutically intervene early in the disease process. This is a primary concern in neurodegenerative diseases (Bellettato and Scarpa, 2010) because neuronal loss can lead to untreatable motor deficits.

Neuropathology is now established as a hallmark in Pompe disease, and profound glycogen accumulation has been described in respiratory neurons in Pompe mice (DeRuisseau et al., 2009; ElMallah et al., 2014; Lee et al., 2011), and in a Pompe infant autopsy sample following failed ERT (DeRuisseau et al., 2009). Our purpose was to determine if the systemic absence of GAA protein would result in respiratory motoneuron pathology very early in the disease progression (*i.e.*, before overt symptoms can be detected, and before neuropathology is detected in other regions). Prior work indicates that motor symptoms are not present in young *Gaa*^{-/-} mice (Sidman et al., 2008; Taksir et al., 2007; Ziegler et al., 2008); therefore, the medulla, mid-cervical and upper thoracic spinal cords were harvested in 6 weeks old, asymptomatic Pompe (*Gaa*^{-/-}) mice (Raben et al., 1998) and subjected to comprehensive histologic evaluation. Motor dysfunction in Pompe disease is often first detected in the lingual (Carlier et al., 2011) and respiratory motor systems (Fuller et al., 2013). Accordingly, our hypothesis was that the associated motoneurons would be the first to show signs of prominent neuropathology including glycogen accumulation, altered cell morphology, cell death and neuroinflammation.

2. Methods

2.1. Experimental animals

All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Mice were housed in a standard room in an Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility with a 12:12 light/dark cycle and ad libitum access to rodent chow and water. The *Gaa*^{-/-} mouse (Taconic, Inc., Hudson, NY)

originally developed by Raben et al. (1998) was outbred to a 129SVE background (Falk et al., 2013). *Gaa*^{-/-} mice ($n = 7$) and wild-type mice ($n = 7$) from the syngeneic background strain were studied at 6 weeks of age.

In a separate cohort of *Gaa*^{-/-} animals ($n = 4$), intrapleural injection (20 μ l) of cholera toxin β subunit dissolved in sterile saline for a 2% solution (List Biological Laboratories, Campbell, CA) was used to retrogradely label phrenic motoneurons. This injection method was adapted for mice from Mantilla et al. (2009), as conducted previously by our group (Falk et al., 2013). Mice were maintained under isoflurane anesthesia (1.5%, oxygen balance) and injected using a 50 μ l Hamilton syringe with 23 gauge needle inserted 1–2 mm deep into the fifth intercostal space on the right side, 2–3 mm lateral from the sternum. Mice were monitored for 1–2 h post-injection for any signs of compromise. Mice returned to their home cages for 96-h prior to sacrifice (as described below).

All mice were sacrificed with an intraperitoneal injection of beuthansia (260 mg/kg; Patterson Veterinary Supply, Alachua, FL), and tissues were fixed either by 1) CNS removal and submersion into Gendres's fixative for 24–48 h, before transfer to 70% Ethanol ($n = 4$ per group) or by 2) perfusion with saline (100 ml) followed by perfusion with 4% Paraformaldehyde (100 ml; $n = 3$ per group). The Gendres's fixation method retained accumulated glycogen in neurons to allow for PAS staining (described below). Paraformaldehyde fixation was necessary to reduce non-specific and background staining for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and glial fibrillary acidic protein (GFAP; see below). All tissues were paraffin embedded and sectioned at 5 μ M. The medulla was sectioned in a rostral to caudal manner until the central canal and region of the caudal XII nucleus was visible. The cervical spinal cord was cut at the C3 ventral root, and serial tissue sections were obtained throughout the mid-cervical cord. The same approach was taken for the thoracic cord, beginning at the T2 ventral root, and obtained through the upper thoracic cord (T5). 3–4 tissue sections, separated by at least 30 μ M were evaluated in all animals. Tissue sections were slide mounted (Fisher, Waltham, MA) in series, deparaffinized and rehydrated through a graded series of ethanol exposures.

2.2. Histology protocols

2.2.1. Cholera toxin β subunit (CT β)—Retrograde labeled phrenic motoneurons were visualized with a goat anti-cholera toxin β subunit primary antibody (1:2000; List Biological Laboratories). In brief, slides were blocked with the Avidin/Biotin blocking kit (Vector Labs, Burlingame, CA). Following a 60 min 10% rabbit serum block, the primary antibody was applied for 48 h at 4 °C in a humidity chamber. Positive signal was identified with the ABC-Elite anti-rabbit detection kit using ImmPactDAB (Vector Labs). An isotype and concentration matched negative control section was included in the procedure. Slides were counterstained using Select Tech hematoxylin 560 (Leica, city state) and cover slipped in Vectamount (Vector Labs).

2.2.2. Periodic-acid schiff (PAS)—Glycogen accumulation was detected using the PAS reaction, as described previously (Falk et al., 2013), with minor modifications. Briefly, tissues were incubated in 0.5% periodic acid (Richard-Allan Scientific, Kalamazoo, MI) for

10-min at 60 °C, rinsed with tap water and stained with Schiff's reagent (Richard-Allan Scientific) for 5-min, and then rinsed again in tap water. Slides were counterstained using Select Tech hematoxylin 560 from Leica and cover slipped in Vectamount (Vector Labs).

2.2.3. Cleaved Caspase 3 (CC3)—Sections were stained with rabbit anti-Cleaved Caspase-3 (Cell Signaling Technologies, Danvers, MA). Slides were blocked for endogenous peroxidase and biotin activity then unmasked in citra buffer pH 6.0 using a microwave oven. Following a 30-min 2% serum blocking step, antibody was applied at 1:250 overnight, 4 °C. Positive signal was identified with the ABC-Elite anti-rabbit detection kit using ImmPactDAB (Vector Labs). An isotype and concentration matched negative control section was included in the procedure. Slides were counterstained with hematoxylin before being cover-slipped using Vectamount (Vector Labs).

2.2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (Tunel)—Slides were blocked for endogenous peroxidase activity, then placed in 0.1 M citrate buffer pH 6.0 and permeabilized by exposure to 4-min of microwave irradiation (600 W). A known positive sample and 2 negative control slides were included. Staining was performed using a commercially available apoptosis kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Millipore, Billerica, MA) following the manufacturer's instructions. Tunel reaction mixture was incubated on the slides for 1-h at 37 °C, with negative control slides receiving labeling mixture devoid of TdT enzyme. Positive signal was detected using ImmPACT DAB (Vector Labs). Slides were counterstained with hematoxylin before being coverslipped using Vectamount (Vector Labs).

2.2.5. Glial fibrillary acidic protein (GFAP)—Slides were blocked in 3% horse serum for 1-h. Primary antibodies were then applied overnight at 4 °C. The antibody used was mouse anti-GFAP (1:500; Encor Biotechnology). Antigen retrieval with Trilogy reagent (Cell Marque, Rocklin, CA) at 95 °C for 15-min was required for optimal staining. Immunoreactivity was detected using 1:500 Alexa Fluor 594 anti-mouse raised in donkey. Sections were mounted in VectaShield with DAPI (Vector Labs) prior to imaging. Positive control tissues and concentration matched Ig controls were included with each immunoassay.

2.2.6. Ionized calcium binding adaptor molecule 1 (IBA-1)—The goal of these experiments was to provide a qualitative evaluation of the appearance and location of microglia. Slides were blocked in 3% horse serum for 1-h. Primary antibodies were then applied overnight at 4 °C. The antibodies used were rabbit anti-Iba-1 (1:300; Wako Chemicals, Cape Charles, VA), and mouse anti-NeuN (1:1000; Encor Biotechnology, Gainesville, FL). Antigen retrieval with Trilogy reagent (Cell Marque) at 95 °C for 25-min was required for optimal staining. Immunoreactivity was detected using 1:500 dilutions of species appropriate Alexa Fluor antibodies raised in donkey: anti-rabbit AlexaFluor 594 secondary and anti-mouse Alexa Fluor 488 secondary were used for IBA-1 and NeuN stains, respectively. Sections were mounted in VectaShield with DAPI (Vector Labs) prior to imaging. Positive control tissues and concentration matched Ig controls were included with each immunoassay.

2.3. Microscopy and evaluation of sections

Histological images were captured using an Olympus BX43 upright microscope, an Olympus DP80 camera and Olympus CellSens software. Using the software, images were white or black balanced for bright field or fluorescence. All images were qualitatively evaluated by multiple investigators. Glycogen accumulation was visualized using the PAS stain which imparts a deep pink color in neurons considered positive (Lee et al., 2011). For the CC3 immunochemical stain, a cell was considered positive if the soma was dark brown (Biosciences, 2012; Kressel and Groscurth, 1994). Cells were considered to be tunel-positive if the nucleus had a clearly discernable dark rim of staining, or if the entire soma was dark brown (Kressel and Groscurth, 1994). GFAP positive astrocytes were readily identifiable based on published criteria (Li et al., 2014). IBA-1 positive microglia were characterized by a hypertrophied and/or “bushy” appearance, or an amoeboid morphology (Karperien et al., 2013).

The *Gaa*^{-/-} and wild-type histological results were compared using a chi-square test of the null hypothesis that there were no differences in the qualitative appearance of tissues between the two strains. Thus, data were evaluated in a tabular format in which the numbers of animals showing a particular histological characteristic were listed (*e.g.*, see Table 1). For comparisons between the mutant (*Gaa*^{-/-}) and wild-type strains, statistical significance was set at $P < 0.05$.

3. Results

3.1. Neuronal glycogen accumulation as indicated by PAS staining

The results of the PAS staining in wild-type and *Gaa*^{-/-} tissues are summarized in Table 1. No neuronal PAS staining could be detected in the medulla of wild-type mice (Fig. 1A). In contrast, PAS staining revealed prototypical Pompe motoneuron histopathology (DeRuisseau et al., 2009; ElMallah et al., 2014; ElMallah et al., 2015; Lee et al., 2011) throughout the *Gaa*^{-/-} hypoglossal nucleus. Thus, hypoglossal motoneurons were robustly PAS positive in *Gaa*^{-/-} medullary sections, with a swollen soma including numerous vacuoles (Fig. 1B). Notably, the hypoglossal nucleus was the only region of the medulla in which these features could be identified in the *Gaa*^{-/-} tissues indicating that medullary glycogen accumulation was uniquely restricted to hypoglossal motoneurons.

Similar to the medulla, PAS staining was absent in the wild-type cervical spinal cord (Fig. 1C). In *Gaa*^{-/-} cervical spinal cords, PAS staining was prominent in the ventral gray matter (lamina 8 (Watson et al., 2009)) including the immediate vicinity of the phrenic motor nucleus (Fig. 1D) as indicated by our prior anatomical study of murine phrenic motoneurons (Qiu et al., 2010). In additional experiments, CT- β was administered intrapleurally to retrogradely label phrenic motoneurons in *Gaa*^{-/-} mice. CT- β positive phrenic motoneurons in lamina 8 of *Gaa*^{-/-} tissues showed evidence of vacuoles and swollen appearance of the soma (Fig. 1E). In the thoracic spinal cord, PAS staining was again absent in wild-type mice (not shown), but could be detected in *Gaa*^{-/-} tissue ventral gray matter (lamina 8 and ICo9), which is the location of intercostal motoneurons (Watson et al., 2009) (Fig. 1F).

3.2. Additional histological assessments

The medulla, as well as the cervical and thoracic spinal cords were further evaluated for evidence of cell death and/or neurodegeneration, and neuroinflammation. The overall results are summarized in Table 2. Our *a priori* hypothesis was that PAS-positive motoneuron soma (e.g., Fig. 1) would co-localize with histological evidence of “activated” microglia (Karperien et al., 2013) and degenerating neurons. Thus, additional staining procedures included IBA-1 to examine microglial morphology (Ito et al., 1998), GFAP to examine astrocyte morphology (Li et al., 2014), Tunel for evidence of DNA damage (Gavrieli et al., 1992; Kyrylkova et al., 2012; Yan et al., 2015; Zhang et al., 2014), and CC3 as a marker for ongoing apoptosis (Biosciences, 2012; Nicholson et al., 1995). Collectively, the data did not support the *a priori* hypothesis, but the results raise the possibility that neuronal glycogen accumulation (as indicated by PAS staining) and neurodegeneration may be occurring independently during early stages of the disease process.

Positive staining for CC3, Tunel, or GFAP was not observed in the wild-type rostral medulla (Fig. 2A–C, and Table 2). In the *Gaa*^{−/−} medulla, the robustly PAS positive hypoglossal nucleus (Fig. 1) showed no CC3 or Tunel positive cells, and no consistent alterations in microglia (IBA-1) or astrocyte (GFAP) appearance (Fig. 2, Table 2). However, three separate staining procedures indicated ongoing neurodegeneration in a relatively discrete region in the rostral medulla adjacent to the floor of the 4th ventricle (Fig. 2D). Thus, all *Gaa*^{−/−} tissues demonstrated robustly positive staining for CC3 in a region that included the medial NTS and cuneate nucleus (Ganchrow et al., 2014; Tokita et al., 2009; Wang and Bradley, 2010; Zaidi et al., 2008). The Tunel stain showed a similar pattern with positive staining in an area that extended from the area postrema through the NTS (Fig. 2E). Tunel and CC3 staining was contained dorsally, and was not present in the hypoglossal motor nucleus or the dorsal vagal motor nucleus (Ganchrow et al., 2014; Tokita et al., 2009; Wang and Bradley, 2010; Zaidi et al., 2008). The same region of the medulla also showed robust GFAP staining in all *Gaa*^{−/−} tissues (Fig. 2F).

Within the cervical cord, differences between wild-type and *Gaa*^{−/−} tissues were not as robust compared to the medulla, but some tendencies were observed (Fig. 3). For example, CC3 staining (Fig. 3C) was detected in the dorsal horn of 2 of 4 *Gaa*^{−/−} cervical spinal cords (lamina 3 (Watson et al., 2009)), in the intermediate gray in 2 of 4 (lamina 6 (Watson et al., 2009)), and in 1 of 4 ventral horns (lamina 8). In contrast, CC3 positive cells were never detected in the cervical cord of wild-type mice (0/4 for both dorsal and ventral horn) (Table 2, Fig. 3A). Cervical motoneurons in the ventral horn did not show evidence for Tunel staining in wild type or *Gaa*^{−/−} tissues, however, cervical neurons in the dorsal horn (lamina 1–3) were Tunel positive in 3 of 3 *Gaa*^{−/−} tissues (Table 2).

Collectively, the GFAP and IBA-1 results suggested that neuroinflammation was beginning in cervical *Gaa*^{−/−} tissues (Fig. 3E, F). Thus, GFAP staining was faint in gray matter of wild type cervical tissues, but robust in 2 of 3 *Gaa*^{−/−} cervical dorsal horns (lamina 1–4, (Watson et al., 2009)), and 1 of 3 ventral horns (lamina 8) (Fig. 3B, E and Table 2). Likewise, in 3 of 4 dorsal (lamina 1–4) and ventral (lamina 8) *Gaa*^{−/−} cervical cords showed evidence for activated microglia (IBA-1) vs. 1 of 4 wild-type (Fig. 3C, F and Table 2). IBA-1 positive

cells in *Gaa*^{-/-} tissues displayed a hypertrophied or “activated” morphology (Fig. 3F; (Karperien et al., 2013)).

No CC3 positive cells could be detected in the thoracic spinal cord of either strain (Table 2), but 3 of 3 *Gaa*^{-/-} thoracic dorsal horns contained Tunel positive neurons in lamina 1–3 vs. 0 of 3 wild type (Fig. 4A, 4D and Table 2). No tunel positive neurons were observed in the thoracic ventral horn (lamina 8) in either strain (Fig. 4A, D).

Evidence for neuroinflammation was more robust in the thoracic *Gaa*^{-/-} cord as compared to either the medullary or cervical regions. Thus, GFAP positive astrocytes in gray matter were readily identified in 3 of 3 *Gaa*^{-/-} thoracic cords but were not identified in wildtype tissues (Fig. 4B, E and Table 2). Gray matter GFAP staining was the most prominent in *Gaa*^{-/-} dorsal horns (lamina 1–3), however, all *Gaa*^{-/-} ventral horns also contained GFAP positive astrocytes in lamina 8 (Fig. 4B, E and Table 2). GFAP staining was also robust in white matter in the thoracic *Gaa*^{-/-} cord characterized by increased staining intensity, and increased astrocyte volume and process length (Fig. 4E). IBA-1 positive microglia had a hypertrophied appearance and neuronal co-localization in 4 of 4 *Gaa*^{-/-} spinal cords, but only 1 of 4 wildtype animals (Fig. 4C and Table 2). Microglial staining was more dense in the *Gaa*^{-/-} thoracic dorsal horn (Fig. 4F; lamina 1–4) and intermediate gray (lamina 5–7 (Watson et al., 2009)) compared to wildtype (Fig. 4C). Throughout the gray matter, *Gaa*^{-/-} tissues had examples of microglia with an amoeboid morphology, suggesting an “activated” state (Fig. 4F).

4. Discussion

There are two primary and novel observations from this histological evaluation of young (6 weeks old) Pompe (*Gaa*^{-/-}) mice. First, prototypical Pompe motoneuron histopathology was uniquely evident in respiratory- and lingual-related motoneurons. Thus, in the absence of GAA protein, these motoneuron pools appear to be particularly susceptible to glycogen accumulation. Second, several immunohistochemical markers indicated that neurodegeneration and/or neuroinflammation is ongoing in regions that do not show evidence of glycogen accumulation. For example, a region of the rostral medulla that included the NTS was robustly positive for CC3 and Tunel, but had no evidence for positive PAS staining and glycogen accumulation. These results raise the possibility that neurodegeneration and neural glycogen accumulation may be occurring independently in early stages of the disease process. Collectively, these data support the growing appreciation for neuropathology as an important component in Pompe disease (Fuller et al., 2013), and suggest that therapeutic intervention early in the disease process is likely to be critical, particularly for sustained correction of respiratory motor deficits.

4.1. Early-onset glycogen accumulation in respiratory and lingual motoneurons

Glycogen accumulation and overt histopathology in the spinal cord and brainstem has been reported in Pompe infants as young as 4–8 months ((Hansoul et al., 1999; Teng et al., 2004)). Several years ago, we published a report of an 18 month Pompe infant that succumbed to cardiorespiratory failure despite ongoing ERT treatments (DeRuisseau et al., 2009). Evaluation of autopsy samples revealed positive PAS staining, swollen soma, and

displaced (eccentric) nuclei in motoneurons in the immediate region of the phrenic motor nucleus, and a biochemical assay indicated that the spinal cord had more glycogen accumulation than the brain. In studies of the *Gaa*^{-/-} mouse, glycogen accumulation and histopathology has been described in respiratory motoneurons as well as brainstem neurons involved in respiratory rhythm-generation (DeRuisseau et al., 2009; ElMallah et al., 2014; Lee et al., 2011). A qualitative histology study from Sidman and colleagues (Sidman et al., 2008) documented PAS positive labeling throughout the brain and spinal cord at early through advanced disease stages. In particular, tissues from 1 month old Pompe mice were described as robustly PAS positive in the sensory ganglia and “large neurons in the lower brainstem and spinal cord” (Sidman et al., 2008). However, the specific motoneuron pools were not identified. A recent study from our group documented that neuromuscular junction (NMJ) pathology is present in *Gaa*^{-/-} tissues by one month of age (Todd et al., 2015). Specifically, in tibialis anterior muscle, δ subunits of acetylcholine receptors are increased in *Gaa*^{-/-} tissues by four weeks, and by 12 weeks α -, δ -, and γ -subunits are all significantly elevated (Todd et al., 2015).

The current data confirm these earlier reports of early onset neuropathology in Pompe animal models, and is the first to clearly show that respiratory- and lingual-related motoneurons are uniquely susceptible to glycogen accumulation and the prototypical Pompe histopathology in young *Gaa*^{-/-} mice. With respect to the current data, the XII motor nucleus clearly (albeit qualitatively) had the densest PAS positive labeling, and there was no readily observable difference in appearance of PAS positive motoneurons when comparing the mid-cervical and upper thoracic spinal ventral horns. The reason that respiratory-related cells uniquely show glycogen accumulation so early in the disease process is not clear. However, it may relate to the relative amount of neurologic activity and associated metabolic demands in these motoneurons. For example, a portion of cells in the phrenic motor nucleus have depolarized and triggered diaphragm contraction approximately 80–200 times per minute since the birth of the animal. Similarly, the XII motor nucleus will be vigorously activated during suckling and feeding behaviors, and a portion of XII motoneurons are likely to be activated during inspiratory efforts to maintain upper airway stiffness/patency.

4.2. Neuroinflammation and neurodegeneration

Neuroinflammation is prominent in many lysosomal storage diseases including Gaucher (Vitner et al., 2012), Sandhoff (Kawashita et al., 2011) and Krabbe (Meisingset et al., 2013), and also in neurodegenerative diseases such as Alzheimer's, Creutzfeldt-Jakob (Stoeck et al., 2014) and amyotrophic lateral sclerosis (ALS; (Chiu et al., 2013)). This has important implications since neuroinflammation is associated with neuronal loss (Kawashita et al., 2011; Vitner et al., 2012), motoneuron degeneration (Chiu et al., 2013), and also a blunted capacity for neuroplasticity in respiratory pathways (Huxtable et al., 2013, 2011). In other lysosomal storage disorders, apoptosis and microglial activation occur in parallel during disease progression. For example, Gaucher's disease is characterized by severe neuronal loss and microglial proliferation which lead to ~30 fold increases in inflammatory cytokines, among other pathological outcomes (Vitner et al., 2012). Similarly, glial-neuronal metabolic disturbances correlate with the progression of astrogliosis, microglia activation, apoptosis and neurodegeneration in a murine model of Krabbe disease (Meisingset et al., 2013). Thus,

it is not surprising that we obtained evidence for ongoing neuroinflammation and neurodegeneration in *Gaa*^{-/-} mice. It is noteworthy, however, that apoptotic neurons and/or glial cells with “activated” morphology were not co-localized with glycogen-filled respiratory motoneurons. That is, the regions with early-onset prototypical Pompe neuropathology did not show histological evidence for marked neuroinflammation and neurodegeneration. In contrast, medullary and spinal regions associated with “sensory integration” both showed evidence for apoptosis and neuroinflammation. These data suggest that neuronal loss may contribute to Pompe disease progression and raise the intriguing possibility GAA deficiency triggers distinct mechanisms in sensory and motor neurons. Thus, future studies to elucidate the underlying molecular signaling pathways contributing to neuropathogenesis are warranted.

5. Conclusions

A number of case reports have confirmed motoneuron pathology in Pompe patients (Gambetti et al., 1971; Hogan et al., 1969; Mancall et al., 1965; Martin et al., 1973; Teng et al., 2004), and this pathology is not responsive to ERT (DeRuisseau et al., 2009). Preclinical data indicate that neuropathology contributes to respiratory dysfunction in Pompe disease (DeRuisseau et al., 2009; Mah et al., 2007, 2010), and this is supported by a few recent studies in patients. First, the amplitude of the diaphragm compound muscle action potential (CMAP) evoked by phrenic nerve stimulation is blunted in adult Pompe patients (Smith et al., 2016a). Further, those patients requiring the most extensive mechanical ventilator support have the greatest relative reduction in the evoked diaphragm CMAP. Second, direct electrical stimulation of the diaphragm (*i.e.*, “diaphragm pacing”, (Onders et al., 2014)) can effectively maintain ventilation for extended periods in both adults and children with Pompe disease (Smith et al., 2016b). If diaphragm myofiber pathology was the sole contributing factor to respiratory insufficiency in Pompe disease, then direct muscle stimulation would be unable to produce sustainable ventilation.

The current data indicate that pathology in respiratory-related motoneurons occurs very early in the Pompe disease progression, and support the notion that early therapeutic intervention will be necessary for optimal outcomes (Falk et al., 2015; Todd et al., 2015). We suggest that effective Pompe respiratory therapies will need to target both myofibers and motoneurons (*i.e.*, the entire respiratory motor unit needs to be treated). Gene therapy approaches show high promise in this regard (Smith et al., 2013) since the entire motor unit can be transduced with the therapeutic vector with apparent correction of both muscle and motoneuron pathology (ElMallah et al., 2012, 2014). A recent study shows that delivering adeno-associated virus (serotype 9) encoding human GAA (AAV9-hGAA) to the *Gaa*^{-/-} mouse tibialis anterior can restore muscle force production, “innervation status”, and motor endplate area, but treatment is much more effective when initiated in young mice (Todd et al., 2015). In fact, when AAV9-hGAA was given to older *Gaa*^{-/-} mice, muscle glycogen accumulation was ameliorated, but neuromuscular junction pathology persisted (Todd et al., 2015).

The current data also raise the possibility that neurodegeneration and neuronal glycogen accumulation may be occurring independently in early stages of the disease process. To our

knowledge, this possibility has not previously been considered in Pompe disease, and evidence from the rostral medulla (*e.g.*, Figs. 1–2) indicates that this should be explored in future experiments.

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Fig. 1.

PAS staining indicates glycogen accumulation in respiratory-related motoneurons in *Gaa*^{-/-} but not WT tissues. Neuronal PAS staining is absent in the wild-type medulla (A), but is prominent throughout the *Gaa*^{-/-} hypoglossal nucleus (B). The hypoglossal nucleus was the only medullary region that was positive for PAS in *Gaa*^{-/-} tissues. PAS staining is absent in the wild-type cervical spinal cord (C). Retrogradely labeled *Gaa*^{-/-} phrenic motoneurons showed evidence of vacuoles and swollen soma (D), and robust PAS staining is present in *Gaa*^{-/-} ventral cervical gray matter (lamina 8) (E). Similarly, *Gaa*^{-/-} thoracic spinal cords showed positive neuronal labeling in the ventral horn (F). In panel D, the top right image was obtained from another histological section of the same mid-cervical spinal cord. Calibration bars indicate 200 μM (lower power images) and 20 μM (higher power images).

Fig. 2.

CC3, TUNEL and GFAP staining indicate neurodegeneration in the *Gaa*^{-/-} medulla. Positive staining for CC3 (A), TUNEL (B) or GFAP (C) was not detected in the wild-type medulla. In contrast, all three immunochemical stains suggested that a region of the *Gaa*^{-/-} medulla near the base of the 4th ventricle had ongoing neurodegeneration. Thus, the *Gaa*^{-/-} medulla had CC3 positive cells in the region of the cuneate nucleus and NTS (D). In addition, TUNEL positive cells (E) and robust GFAP staining (F) were observed to extend approximately from the Area Postrema to the NTS and cuneate nucleus (E). Calibration bars indicate 100 μ M (lower power images) and 20 μ M (higher power images). In Panels A–F, *i* indicates NTS and cuneate region, and *ii* indicates the XII motor nucleus. GFAP staining is shown in white.

Fig. 3. CC3, TUNEL and GFAP staining in the *Gaa*^{-/-} cervical spinal cord. Positive staining for CC3 (A), GFAP (B) or “activated” microglia (C) was largely absent from wildtype cervical spinal cords. CC3 positive neurons were observed in cervical *Gaa*^{-/-} tissues (D), and GFAP positive astrocytes were more prominent (E). Hypertrophied or “bushy” microglia with amoeboid phenotypes were observed in gray matter (F), particularly in the dorsal horn (lamina 1–3). Calibration bars indicate 100 μ M (lower power images) and 20 μ M (higher power images). In Panels A–F, *i* indicates the dorsal horn region. In A, B, D, and E, *ii* indicates the intermediate gray, while in C and F, *ii* shows the ventral horn. In B and E, *iii* indicates the ventral horn and *iv* represents the ventral white matter; GFAP positive staining is shown in white. In C and F, IBA-1 staining is shown in red, NeuN is shown in green, and DAPI is in blue.

Fig. 4.

Tunel, GFAP and IBA-1 staining indicate on-going neurodegeneration and neuroinflammation in the *Gaa*^{-/-} thoracic spinal cord. Positive staining was absent in wildtype thoracic cords for Tunel (A), GFAP (B) or “activated” microglia (C). All *Gaa*^{-/-} thoracic tissues had Tunel positive neurons in the dorsal horn (lamina 1–3; D), intense GFAP staining in the white matter, and GFAP positive astrocytes throughout the gray matter—especially in lamina 1–3, and 6 (E). IBA-1 positive microglia were “bushy” and co-localized with neurons in lamina 1–3. Microglial cells had multiple examples of the amoeboid morphology in lamina 8 (F). Calibration bars indicate 100 μ M (lower power images) and 20 μ M (higher power images). In Panels A–F, *i* indicates the dorsal horn region. In A, B, C, and F, *ii* indicates the ventral horn. In B and E, *ii* shows the lateral white matter, *iii* indicates the ventral horn and *iv* represents the ventral white matter; GFAP positive staining is shown in white. In C and F, IBA-1 staining is shown in red, NeuN is shown in green, and DAPI is in blue.

Table 1

Summary of neuronal PAS staining in the medulla, and the cervical and thoracic spinal cord. The PAS stain imparts a pink color in neurons considered positive (*e.g.*, Fig. 1). The Table depicts the proportion of *Gaa*^{-/-} and wildtype (WT) mice showing positive PAS staining in the region examined (*e.g.*, 3/3 indicates that 3 of 3 mice examined showed PAS positive neurons).

CNS region	<i>Gaa</i> ^{-/-}	WT
XII motor nucleus	3/3 *	0/3
Cervical ventral horn	3/3 *	0/3
Thoracic ventral horn	3/3 *	0/3

The proportions were examined using a chi-square test;

* indicates $P < 0.05$.

Table 2

Summary of caspase 3 (CC3), Tunel, GFAP and IBA-1 staining in the medulla and the cervical and thoracic spinal cord. The Table depicts the proportion of $Gaa^{-/-}$ and wildtype (WT) mice showing positive staining in the region examined (*e.g.*, 0/4 indicates that 0 of 4 mice examined showed positive staining). The proportion of animals showing positive staining in $Gaa^{-/-}$ vs. WT mice was examined using a chi-square test. There were no observable differences between $Gaa^{-/-}$ and wild-type (WT) tissues in the XII motor nucleus. However, the dorsal part of the medulla showed consistent CC3, Tunel and GFAP staining in $Gaa^{-/-}$ but not WT tissues. Robust cervical spinal IBA-1 staining was observed in a more $Gaa^{-/-}$ than WT tissues, although this did not reach significance ($P=0.157$). Differences in Tunel, GFAP and IBA-1 staining were more pronounced in the thoracic spinal cord.

Stain	Caspase 3		Tunel		GFAP		IBA-1	
	$Gaa^{-/-}$	WT	$Gaa^{-/-}$	WT	$Gaa^{-/-}$	WT	$Gaa^{-/-}$	WT
Medulla	XII nucleus	0/4	0/4	0/3	0/3	0/3	1/4	1/4
	Dorsal medulla	4/4 *	0/4	3/3 *	3/3 *	0/3	2/4	1/4
Cervical cord	Dorsal horn	2/4	0/4	3/3	1/3	2/3	0/3	3/4
	Ventral horn	1/4	0/4	0/3	0/3	1/3	0/3	3/4
Thoracic cord	Dorsal horn	0/4	0/4	3/3 *	0/3	3/3 *	0/3	4/4 *
	Ventral horn	0/4	0/4	0/3	0/3	3/3 *	0/3	4/4 *

* , indicates $P < 0.05$ (chi square).