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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.

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

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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 μl) 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 μl 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 Gendre'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 Gendre'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 μM. 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 μM 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 (CTβ)

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 coverslipped 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