ANIMAL MODELS

α -Galactosidase A Knockout Mice

Progressive Organ Pathology Resembles the Type 2 Later-Onset Phenotype of Fabry Disease

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Fabry disease is an X-linked lysosomal storage disease caused by deficient activity of α -galactosidase A and the resultant systemic accumulation of globotriaosylceramide (GL-3) and related glycolipids. α -Galactosidase A gene knockout (*Gla* KO) mice have no α -galactosidase A activity and progressively accumulate GL-3 in tissues and fluids, similarly to FD patients. The nature and temporal effects of the progressive substrate accumulation on tissue histology in these mice have not previously been characterized. Here, we report the pathology of young to old (3 to 17 months old) *Gla* KO mice and compare these changes with those in strain-matched control animals. *Gla* KO mice accumulated GL-3 in various tissues and fluids with age. Lysosomal GL-3 inclusions increased with age in multiple cell types, including renal epithelial, intestinal, and vascular smooth muscle cells, and neurons in trigeminal and dorsal root ganglia, as detected by light and electron microscopy. However, unlike the case for male FD patients with the type 1 classic phenotype, GL-3 inclusions were not detected in vascular endothelial cells or cardiomyocytes. The histological changes in *Gla* KO mice better resemble the type 2 later-onset phenotype observed in patients with residual α -galactosidase A activity. GL-3 accumulation in the small intestine and sensory ganglia of *Gla* KO mice provides a model for study of enteropathy and neuropathy in Fabry disease. (*Am J Pathol* 2015, 185: 651–665; <http://dx.doi.org/10.1016/j.ajpath.2014.11.004>)

Fabry disease (FD) is an X-linked inborn disorder of glycosphingolipid catabolism caused by mutations in the *GLA* gene encoding the lysosomal hydrolase α -galactosidase A (α -Gal A).^{1,2} The deficiency or absence of α -Gal A activity results in progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids within lysosomes of multiple cell types, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), renal tubular cells, podocytes, mesangial cells, and cardiomyocytes.^{3–7} The two major clinical phenotypes are the type 1 classic phenotype and the milder, type 2 later-onset phenotype.^{1,8} Type 1 classically affected males have little or no α -Gal A activity and typically have onset of acroparesthesias, hypohidrosis, angiokeratomas, and a characteristic corneal dystrophy in childhood or adolescence. With advancing age, classically affected FD patients develop progressive

multisystem microvasculopathic disease culminating in renal failure, hypertrophic cardiomyopathy, and/or cerebrovascular disease.^{1,2} Males with type 2 later-onset FD have residual α -Gal A activity and lack the vascular endothelial GL-3 accumulations; thus, they do not present with the early clinical manifestations of the type 1 phenotype. In type 2 FD, patients typically present with predominantly cardiac or renal disease in the fourth to seventh decade, indicating delayed onset and slower progression.^{7–10} In patients with type 2 FD, the disease has been detected by screening

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hemodialysis, renal and cardiac transplant, and stroke clinics.^{9–11}

Knockout (KO) mouse models of FD, generated on the C57BL/6 or 129/Sv backgrounds by targeted disruption of the murine α -Gal A gene (*Gla*), have been used in various studies.^{12–14} *Gla* KO mice completely lack α -Gal A activity and exhibit the progressive GL-3 accumulation in blood and various organs. That is the metabolic hallmark of FD. These *Gla* KO mice have been used for preclinical efficacy and safety evaluation of enzyme replacement¹² and substrate reduction¹⁵ therapies and to probe the underlying basis of the disease pathophysiology.¹⁶

Although *Gla* KO mice recapitulate the metabolic defect in FD and accumulate GL-3 and lyso-GL-3 in tissues, they do not exhibit either the degree of tissue pathology or the clinical manifestations of the type 1 classic FD phenotype.^{12,15–17} No comprehensive analysis of the temporal pattern of substrate accumulation and its effect on tissue pathology of *Gla* KO mice has been reported. Furthermore, although age-dependent thermosensory deficits suggestive of a neuropathic disease have been reported in the 129/Sv-based *Gla* KO mouse,^{15,18} the histological basis for this phenomenon has not been well documented. Finally, the effect of aging on tissue pathology in these mice has not been characterized.

Here, we report our systematic characterization of the tissue pathology in young and old (3 to 17 months old) *Gla* KO mice on the 129/Sv background by light and electron microscopy. Comprehensive biochemical, histopathological, and immunofluorescence analyses of the major tissues were performed to examine the distribution of accumulated GL-3 and related glycolipids and to study the effect of aging on the progressive accumulation and resultant morphological changes in tissues. We observed progressively increasing GL-3 levels with age in plasma, heart, kidney, liver, and brain of *Gla* KO mice. Characteristic substrate inclusions were observed in multiple tissues, including neurons of the dorsal root ganglia (DRG), renal epithelial cells, vascular and intestinal smooth muscle cells, fibroblasts, and perineurial cells. These findings should facilitate a better interpretation of FD-related histology for research studies using the *Gla* KO mouse model.

Materials and Methods

Tissue Samples from FD Patients

Renal, skin, and cardiac biopsies from FD patients enrolled in a Phase 3 clinical trial of enzyme replacement therapy were processed as described previously.^{3–5} Pieces (approximately 1 mm³) of selected tissues were processed for epon embedding. In brief, tissues were fixed in 3% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer, pH 7.3 (Electron Microscopy Sciences, Hatfield, PA), postfixed in 1% osmium tetroxide in 0.2 mol/L sodium cacodylate buffer, and embedded in epon. Sections (1 μ m thick) of

epon-embedded tissues were stained with methylene blue and azure II (Richardson's stain) and were examined by high-resolution light microscopy (HRLM).

Animals

Procedures involving mice were reviewed and approved by the Genzyme Institutional Animal Care and Use Committee and were conducted in accord with guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care. Male and female *Gla* KO mice generated by targeted disruption of the *Gla* gene in 129/Sv mouse embryonic stem cells¹⁴ were contract bred at Charles River Laboratories International (Bedford, MA). Wild-type (WT) male and female 129S6/SvEvTac mice (here referred to as 129/Sv) were obtained from Taconic Biosciences (Germantown, NY). Mice were housed two to three per cage with unrestricted access to food and water. Male *Gla* KO mice and female homozygous *Gla* KO mice were used for these studies; for breeding, male KO mice were mated with homozygous KO female mice.

Measurement of Peripheral Sensory Function

Nociceptive response to heat stimulus was measured using a hot-plate test as described previously.¹⁵ In brief, mice were individually placed on a 55°C surface (hot-plate analgesia meter; Columbus Instruments, Columbus, OH), and the time taken to respond with a characteristic shake or flick of the hind paw was recorded as the latency period. If no response was evident by 60 seconds, the mice were removed from the hot plate, to prevent thermal injury.

Quantitation of Globotriaosylceramide and Related Glycolipids

Quantitative analysis of sphingolipids was performed by liquid chromatography–tandem mass spectrometry. In brief, tissue was homogenized in 10 times its volume of water; 10 μ L of the homogenate was then extracted with 1 mL of an organic solvent mixture. For extraction of GL-3 and lysosomal GL-3, the solvents consisted of acetonitrile–methanol–acetic acid (75:25:1 by volume) and 5 mmol/L ammonium acetate. For extraction of GL-1 and galactosylceramide, the solvents consisted of acetonitrile–methanol–acetic acid (90:10:1 by volume) and 5 mmol/L ammonium acetate. The samples were placed on a tube vortexer (VX-2500; VWR International, Radnor, PA) for 5 minutes and then were centrifuged. The supernatant was carefully transferred into high-performance liquid chromatography vials for analysis. For GL-3 and lysosomal GL-3 analyses, a hydrophilic interaction liquid chromatography column (2.1 $\times$ 100 mm, 1.7 μ m particle) (Acquity BEH; Waters Corporation, Milford, MA) was used to separate the sphingolipids, which were then analyzed by triple-quadrupole tandem mass spectrometry (API 4000; AB

SCIEX, Framingham, MA) in the MRM mode. Two Atlantis hydrophilic interaction liquid chromatography columns in tandem (2.1×150 mm, $3 \mu\text{m}$ particle) (Waters Corporation) were used to separate GL-1 and galactosylceramide before detection by tandem mass spectrometry (API 4000; AB SCIEX). Sphingolipid standards were obtained from Matreya (Pleasant Gap, PA).

Necropsy and Histology

At specified time points (3, 6, 8, 9, 12, and 17 months of age), *Gla* KO and WT mice ($n \geq 3$ per time point) were euthanized by asphyxiation via carbon dioxide inhalation; their tissues were harvested immediately and snap-frozen on dry ice for biochemical analyses or processed for histology as described below. Any gross lesions observed were recorded at the time of necropsy, and relevant tissue samples were collected for histopathology in addition to the predetermined tissue set.

Light Microscopy

For light microscopy, the following tissues from 3-, 6-, 9-, 12-, and 17-month-old *Gla* KO mice and age-matched WT 129/Sv mice were collected in 10% neutral buffered formalin: heart, aorta, kidney, liver, spleen, lung, brain, spine with spinal cord and DRG, trigeminal ganglia, peripheral nerves, eyes, small and large intestines, skin, and skeletal muscle. Formalin-fixed tissues were subjected to routine histology processing, embedded in paraffin, sectioned at $5 \mu\text{m}$, and stained with hematoxylin and eosin (H&E). For microscopic evaluation of DRG, whole spines fixed in 10% neutral buffered formalin were decalcified in buffered formic acid–trisodium citrate (Poly Scientific R&D, Bay Shore, NY), followed by routine histology processing and paraffin embedding. Cross sections of paraffin-embedded spines were obtained from the thoracic and lumbar regions and stained with H&E. Vacuolated and enlarged neurons were counted in two to four DRG per mouse, reported as a percentage of total DRG neurons. Representative cross sections of the spinal cord were also stained using Bielschowsky's silver stain to visualize axonal changes.

For frozen sections, tissue samples were first fixed with Zamboni's fixative (Newcomer Supply, Middleton, WI) for 48 hours, cryoprotected with 30% sucrose, and then embedded in cryomolds using OCT mounting medium (Tissue-Tek O.C.T.; Sakura Finetek USA, Torrance, CA). Sections were obtained at 5 to $10 \mu\text{m}$ using a cryotome and were stained with H&E or subjected to immunofluorescence assay (IFA) as described below.

HRLM and TEM

For HRLM, 1-mm^3 tissue pieces were fixed in 3% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer (Electron Microscopy Sciences), postfixed in 1% osmium tetroxide, subjected to epon embedding, sectioned at $1 \mu\text{m}$, and stained

with Richardson's stain, as described previously.⁵ Ultrathin sections from representative areas selected after HRLM evaluation were used for preparing grids stained with uranyl acetate and lead citrate, for transmission electron microscopy (TEM) ultrastructural evaluation (JEM-1400 transmission electron microscope; JEOL USA, Peabody, MA).

Immunofluorescence Assay

For IFA, unstained frozen sections were blocked using a serum-free protein block (Dako, Carpinteria, CA) for 10 minutes at room temperature. Serial sections were incubated with a 1:100 dilution of the primary antibody directed against CD77 (MCA579; AbD Serotec, Raleigh, NC) for 2 hours at room temperature. Sections were then incubated with a 1:500 dilution of the Alexa Fluor 488–conjugated goat anti-rat secondary antibody (Life Technologies, Carlsbad, CA) for 30 minutes at room temperature. Finally, sections were postfixed in 4% paraformaldehyde (in 0.1 mol/L phosphate buffer, pH 7.4) for 10 minutes, then coverslipped using Aqua-Poly/Mount medium (Polysciences, Warrington, PA). Primary and secondary antibodies were diluted in antibody diluent with background reducing components (Dako); all incubation steps were conducted in the dark. As an assay control, an appropriate isotype antibody (rat IgM) (Chemicon CBL607; EMD Millipore, Billerica, MA) was used in place of the primary antibody for each IFA experiment. Nuclei were detected using the nuclear dye DAPI. Slides were visualized and imaged using an Axiovert 200 mol/L fluorescence confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

Confocal Microscopy and Quantitative Morphometry

Immunofluorescence stained cryosections were imaged at $\times 20$ using a fluorescence-equipped Mirax slide scanner (Carl Zeiss Microscopy). A subset of slides were subjected to additional imaging using an LSM510 Meta laser scanning confocal microscope equipped with a $40\times$ Plan-Apochromatic water immersion objective (Carl Zeiss Microscopy). CD77 immunofluorescence was quantified using whole-slide scans. In brief, a region of interest encompassing the muscular wall in the cross section of the intestine was manually generated within the Mirax viewer. The fluorescence channel corresponding to CD77 staining within these regions of interest was then exported as monochrome TIFF files and imported into AxioVision image analysis software version 4.8.2.0 (Carl Zeiss Microscopy) for further analysis. Within the AxioVision software, a mask representing CD77-specific staining was generated by applying a top-hat transform–based algorithm and subsequent intensity threshold to eliminate background staining and autofluorescence. Parameters were established by reference to control samples stained with IgM isotype control in place of primary antibody and imaged identically to experimental samples. A separate mask representing total tissue area was generated first by applying a median filter to smooth the entire image and then applying an intensity

threshold to distinguish tissue from background. The area of both CD77-specific fluorescence and total tissue was then measured and exported to Microsoft (Redmond, WA) Excel software version 14.0.7.7015.100 and GraphPad Prism software version 5.01 (GraphPad Software, La Jolla, CA) for plotting and statistical analysis. All images were processed identically through execution of an automated script.

Statistical Analysis

Quantitative data were analyzed by one-way analysis of variance or parametric Student's *t*-test, depending on data distribution. Analyses were performed using GraphPad Prism statistical software (GraphPad Software). $P \leq 0.05$ was considered the indicator of a statistically significant difference.

Results

Pattern of Substrate Accumulation in Tissues from FD Patients

Analysis of representative biopsy samples from untreated male FD patients with the type 1 classic phenotype revealed numerous dark blue intracellular inclusions in various cell types in the kidney, heart, and skin (Figure 1). These inclusions consisted primarily of GL-3, which accumulates in the lysosomes of FD patients. Extensive lysosomal accumulation of GL-3 was observed within glomerular podocytes, capillary ECs, distal tubular epithelial cells, cardiomyocytes, vascular ECs, smooth muscle cells, and interstitial cells (macrophages and fibroblasts) of all tissue samples. In the skin samples, substrate inclusions were observed in dermal fibroblasts and in perineurial cells around the dermal nerves.

Morbidity and Mortality of *Gla* KO Mice

Morbidity and longevity were similar in *Gla* KO and control 129/Sv mice. Age- and strain-related pathology was observed in both *Gla* KO and control WT mice with similar incidence. Examples of age- and strain-related findings in these mice included chronic ulcerative dermatitis, balanoposthitis, hematoma, megaesophagus, hydronephrosis, and neoplasia (eg, testicular teratoma, osteosarcoma). These observations of age-related pathology associated with the 129/Sv mouse strain are consistent with previous report.¹⁹

Gla KO Mice Exhibit Progressive Accumulation of GL-3 in Tissues

A progressive, age-dependent increase in tissue GL-3 levels was observed in the 129/Sv-based *Gla* KO mice (Figure 2). The levels were significantly elevated, compared with tissues from age-matched 129/Sv healthy control animals, at all time points examined. Tissue GL-3 levels in normal 129/Sv mice were generally below the detection limit of the

Figure 1 Glycosphingolipid accumulation in different tissues from male patients with type 1 classic Fabry disease (FD). Representative photomicrographs of epon-embedded tissue sections, with methylene blue–azure II staining (Richardson's stain), show lysosomal GL-3 storage in the various cell types. **A:** Renal tubules. Dense granules indicative of substrate accumulation are evident in distal convoluted tubules (asterisks), capillary ECs (red arrow), and interstitial cells (black arrows). **B:** Glomerulus. Substrate inclusions are evident in capillary endothelium (red arrows), podocytes (green arrows), and mesangial cells (black arrow). **C:** Heart. Substrate accumulation is evident in cardiomyocytes (asterisks) and vascular ECs (red arrows). **D:** Skin (dermis). Substrate deposits are evident in vascular ECs (red arrows), smooth muscle cells (black arrows), and perineurial cells (blue arrow). Original magnification: $\times 600$ (A); $\times 1000$ (B–D).

sensitive mass spectrometry assay. *Gla* KO mice, however, exhibited elevated levels of GL-3 already at 3 months of age, the earliest time point in the present study; levels were highest in the spleen, followed by the liver, heart, and kidney in descending order. In these mice, GL-3 levels increased progressively with age in all of the tissues. At 12 months of age, tissue GL-3 levels in *Gla* KO mice were two- to threefold higher than at 3 months.

Pathology in Visceral Organs of *Gla* KO Mice

Previous reports on the pathology of *Gla* KO mice have focused on only a few tissues, and particularly on those of the cardiovascular, renal, and central nervous systems, because these are the more commonly and extensively affected tissues in FD patients.^{1,2,12,13,18,20} These studies typically examined *Gla* KO male mice and homozygous *Gla* KO female mice up to 6 or 10 months of age, with only a few older mice (>12 months old). Most studies describe the phenotype of *Gla* KO male mice. Given that FD is a slowly progressive, multisystemic disorder with lysosomal GL-3 accumulation as the major pathophysiologic driver of disease, we evaluated multiple tissues from *Gla* KO mice at ages 3, 8, 12, and 17 months. A comparison of various cell types affected by substrate accumulation in male FD patients (both type 1 and type 2) and in *Gla* KO mice is presented in Table 1. No meaningful differences in onset or severity of histological changes were observed between

Figure 2 Progressive substrate (GL-3) accumulation in visceral tissues of *Gla* KO mice. The GL-3 levels in various tissues of *Gla* KO (squares) and wild-type 129/Sv (triangles) mice were determined using mass spectrometry assay. Data are expressed as means $\pm$ SD. $n = 4$ mice per time point. KO, knockout.

age-matched male and female homozygous *Gla* KO mice. Pathology findings in the visceral organs are described below.

Kidney Pathology

Beginning as early as 3 months of age, *Gla* KO kidneys showed distinct inclusions indicative of substrate accumulation in epithelial cells of the collecting ducts, distal tubules, and proximal tubules, as well as in parietal epithelial cells of the glomerular Bowman's capsule (Figure 3). Ultrastructurally, these inclusions were multilamellar in arrangement and contained membrane-bound electron-dense materials, which resulted from substrate accumulation in the lysosomes, as described previously for the human disease.²⁴ A few aging *Gla* KO mice (15 to 17 months old) also had substrate deposits within the tubule lumina. Epithelial cells lining these tubules generally contained numerous GL-3 inclusions. In contrast to extensive podocyte involvement in types 1 and 2 FD,^{21,25} podocyte GL-3 storage in the *Gla* KO kidney was minimal, as evidenced by rare multilamellar membrane-bound inclusions and focal foot process effacement. In contrast to the corresponding sections from male FD patients with the type 1 classic phenotype, in *Gla* KO mice no substrate inclusions were observed in glomerular mesangial cells and ECs of the glomerular capillaries or other blood vessels, not even at an advanced age (>15 months old). These findings did, however, resemble those of males with the type 2 later-onset phenotype (with the exception of the extensive podocyte involvement).²¹ Substrate inclusions were generally absent in the kidneys of healthy control 129/Sv mice, except for the presence of rare inclusions in tubule epithelial cells of a few aging male mice. These inclusions in normal 129/Sv kidneys may represent testosterone-induced accumulation of GL-3, as has been reported in normal and *Gla* KO male mice.¹²

Cardiovascular Pathology

Substrate accumulation of GL-3 in cardiomyocytes is a prominent feature in male FD patients, both type 1 and type 2.^{1–3,7,21,25,26} In contrast to FD patients, in *Gla* KO mice the cardiac tissues showed no evidence of substrate accumulation in the cardiomyocyte lysosomes, as visualized by light or electron microscopy. Similarly, ECs of myocardial and coronary blood vessels in *Gla* KO mice were devoid of storage inclusions (which were observed only in VSMCs and interstitial fibroblasts of cardiac tissues). These inclusions were identifiable by HRLM, and the lysosomal location was confirmed by TEM. As expected, no substrate inclusions were observed in any cardiac cell type from WT mice (Figure 4). Overall, cardiac tissue of *Gla* KO mice showed less substrate accumulation than was observed in kidney, consistent with quantitative biochemical measurements.¹⁸ Aged male *Gla* KO mice (>12 months) exhibited an increased incidence of myocardial fibrosis, particularly in the left ventricular free wall and occasionally in the interventricular septum. However, the incidence and severity of this change was similar to that in older male WT mice. Thus, this histological change in the heart appears to be a strain-related rather than a disease-related phenomenon.

Neuropathology

Male FD patients with the type 1 classic phenotype have significant peripheral and autonomic neurological manifestations.^{1,2} In addition to GL-3 accumulation in the microvasculature throughout the body of FD patients, lysosomal storage inclusions have been reported in neurons, Schwann cells, perineurial cells, and DRG neurons, as well as in autonomic ganglia, leading to central and peripheral nervous system pathology.^{1,27} We therefore conducted detailed histological studies in nervous tissues of 3-, 6-, 9-, 12-, and 18-month-old *Gla* KO mice. In addition, the mice were assessed for thermosensory response using a hot-plate assay.

Thermosensory Deficits in *Gla* KO Mice

Although central nervous system (CNS) dysfunction is not observed in the 129/Sv-based *Gla* KO mice, thermosensory deficits and dorsal root ganglia neuronal vacuolation suggestive of sensory neuropathy have been reported.^{18,28} Glycosphingolipid accumulation and peripheral nerve dysfunction have also been described in the C57BL/6-based *Gla* KO mouse model.²⁸ To examine the effects of aging (and thereby increased pathology) on peripheral thermosensory activity, 3-month-old *Gla* KO mice and age-matched healthy control 129/Sv mice were subjected to the hot-plate assay at regular intervals (1 to 2 months) until 17 months of age. The time taken by the mouse to flick its paw when placed on a heated plate (55°C) was recorded as the latency period. Beginning as early as 3 months of age, *Gla* KO mice had clear deficits in thermosensation, as indicated by higher latency periods

Table 1 Tissue and Cell Types Affected by Substrate Accumulation, As Observed by Light and Electron Microscopy, in Male FD Patients and *Gla* KO Mice*

Tissue and cell type	Storage inclusions		
	FD type 1 ^{1,3-5}	FD type 2 ^{21,22}	<i>Gla</i> KO mice
Kidney (glomeruli)			
Capillary endothelial cells	+	—	—
Mesangial cells	+	—	—
Parietal epithelial cells	+	+/?	+ (rare)
Podocytes	+	+	+ (rare)
Kidney (tubules)			
Collecting ducts	+	—	+ [†]
Distal convoluted tubules	+	—	+
Proximal convoluted tubules	+	—	+
Interstitial cells	+	—	+ (rare)
Heart			
Endothelial cells	+	—	—
Cardiomyocytes	+	+	—
Valvular fibrocytes	+	—	—
Interstitial cells	+	—	+ (rare)
Aorta and blood vessels			
Endothelial cells	+	—	—
Vascular smooth muscle cells	+	NR	+
Perithelial cells	+	NR	—
Brain			
Neurons	+	NR	—
Glial cells	+	NR	+ (rare)
Vascular endothelial cells	+	—	+
Dorsal root ganglia			
Neurons	+	NR	+ [†]
Vascular smooth muscle cells	+	NR	+
Trigeminal ganglia			
Neurons	+	NR	+
Vascular smooth muscle cells	+	NR	+
Small and large intestine			
Myenteric plexus ganglion cells	+	NR	+ [†]
Smooth muscle cells	+	NR	+
Interstitial cells (fibroblasts)	+	NR	+
Peripheral nerve (including dermal nerve fibers)			
Axons	+	NR	+ (rare)
Schwann cells	+/?	NR	—
Perineurial cells	+	NR	+
Skin			
Epidermal cells	—	NR	—
Endothelial cells	+	—	—
Epithelial cells (eccrine gland)	+	NR	—
Fibroblasts	+	NR	+
Vascular smooth muscle cells	+	NR	+
Eye			
Epithelial cells (cornea, conjunctiva and lens)	+	—	—
Fibrocytes	+	NR	—
Liver			
Hepatocytes		NR	—
Portal bile duct epithelium	+ (rare)	NR	—

(table continues)

Table 1 (continued)

Tissue and cell type	Storage inclusions		
	FD type 1 ^{1,3-5}	FD type 2 ^{21,22}	<i>Gla</i> KO mice
Sinusoidal endothelial cells	+	(rare)	NR
Kupffer cells	+		NR
Spleen			
Reticular endothelial cells	+		NR
Skeletal muscle			
Skeletal myofibers ²³	+/?	(rare)	NR

*FD type 1, classic phenotype; FD type 2, later-onset phenotype.

[†]Cells were affected in *Gla* KO mice as early as 3 months of age.

FD, Fabry disease; KO, knockout; NR, not reported in the literature; +, present; —, absent; +/?, unverified or questionable report.

compared with control 129/Sv mice (Figure 5A). *Gla* KO mice exhibited a progressive, age-dependent prolongation of the latency period, which was significantly higher than those of age-matched control 129/Sv mice at all time points. By 12 months of age, the mean latency period in *Gla* KO mice was greater than 60 seconds, which was the predetermined humane threshold at which the mice were removed from the hot plate (Figure 5A).

Pathology in the Dorsal Root Ganglia

To delineate the pathological basis for the progressive thermosensory deficits in *Gla* KO mice, we examined multiple components of the thermosensory pathway in the brain, spinal cord, DRG, peripheral nerves, and footpads. Significant histological aberrations were observed only in the DRG. Light microscopic analysis of H&E-stained cross sections of the cervical, thoracic, and lumbar segments of the decalcified spinal column revealed many enlarged DRG neurons with vacuolated or foamy cytoplasm, scanty Nissl substance, and a compressed nucleus (Figure 5B). DRG in age-matched control 129/Sv mice were unremarkable, and DRG neurons had a uniform, granular basophilic cytoplasm. Under HRLM, numerous discrete to coalescing dark blue inclusions (Figure 5B) were observed in the cytoplasm of enlarged DRG cells from *Gla* KO mice. TEM revealed that these cells contained numerous membrane-bound inclusions, with many exhibiting the multilamellar zebra-like morphology (Figure 5, D and E) characteristic of tissues from patients with FD and various other sphingolipidoses.^{24,27} Substrate inclusions were also observed in perineurial cells in the nerves, but Schwann cells and axons were unaffected.

We directed our efforts to quantifying the vacuolated DRG neurons in the decalcified H&E-stained cross sections from the lumbar segment of the spine. Vacuolation was first in the DRG neurons of *Gla* KO mice already at 3 months of age, which was coincident with the onset of thermosensory deficits. Approximately $30.5 \pm 3.9\%$ of DRG neurons were vacuolated

Figure 3 Substrate storage in renal tissue of 12-month-old *Gla* KO mice. **A:** Photomicrographs of epon-embedded kidney show substrate deposits in epithelial cells of collecting ducts (**black arrows**) and glomerular parietal epithelial cells (**red arrows**) in a *Gla* KO mouse. By contrast, the specimen from a WT 129/Sv mouse is unremarkable. **B** and **C:** Representative TEM images show many lysosomal storage inclusions (**red arrows**) in a distal tubule (**B**) and glomerular parietal epithelial cells (**C**) in a *Gla* KO mouse. A lysosome with abundant electron-dense storage material is shown in the **inset** (**B**). Original magnification, $\times 600$ (**A**). Scale bars: 2 μm (**B** and **C**); 0.5 μm (**B**, **inset**). En, endothelial cell; N, nucleus; TEM, transmission electron microscopy.

by 3 months, and this proportion increased progressively with age, reaching $65.8 \pm 6.3\%$ at 17 months (**Figure 5C**). In general, the progressively higher number of vacuolated DRG neurons with age corresponded with the trend toward an increased latency period in the hot-plate assay. Both female and male *Gla* KO mice exhibited similar onset, incidence, and severity of DRG neuronal vacuolation. *Gla* KO mice had similar numbers of vacuolated DRG neurons in the thoracic and cervical segments as in the lumbar segment. WT 129/Sv mice did not show any evidence of vacuolated DRG neurons.

Substrate Storage in Trigeminal Ganglia

Because glycosphingolipid inclusions have been reported in the trigeminal ganglia and autonomic ganglia of type 1 classically affected male FD patients,^{1,27} we examined these tissues from *Gla* KO mice and age-matched 129/Sv mice.

Interestingly, the trigeminal ganglia of *Gla* KO mice showed many enlarged neurons with vacuolated or foamy cytoplasm, similar to those in the DRG (**Figure 6**). HRLM and TEM revealed that lysosomal storage inclusions similar to those observed in DRG neurons were present in the trigeminal ganglia neurons of *Gla* KO mice; by contrast, the trigeminal ganglia of healthy control 129/Sv mice were histologically unremarkable (data not shown).

Histopathology of Spinal Cord, Brain, and Peripheral Nerves

Examination of the spinal cord of *Gla* KO mice did not reveal significant histological changes, compared with control 129/Sv mice. No evidence of storage inclusions was noted in neurons or glial cells in the spinal cord of *Gla* KO mice under HRLM or EM. In 6-month-old *Gla* KO mice, several eosinophilic spheroid-like structures were observed bilaterally in the dorsal horn gray matter, corresponding to lamina I in the lumbar and thoracic segments of the spinal cord (**Figure 6**). With Bielschowsky's silver staining, these spheroids revealed argyrophilic aggregates, which is suggestive of axonal origin. Axonal spheroids in the spinal cord were not detected in the younger *Gla* KO mice (3 months old). Older *Gla* KO mice exhibited an age-dependent progression in the incidence and severity of axonal spheroids. Approximately 70% of *Gla* KO

Figure 4 High-resolution light microscopy and TEM imaging of heart. **A:** Photomicrographs of epon-embedded heart sections from a 10-month-old *Gla* KO mouse reveal no substrate storage in cardiomyocytes. A representative photomicrograph of cardiac tissue from an age-matched 129/Sv wild-type mouse is histologically unremarkable. **B:** In a *Gla* KO mouse heart, TEM reveals electron-dense membranous inclusions (**red arrows**) in smooth muscle cells around a blood vessel and relatively unremarkable cardiomyocytes (**asterisks**). **C:** In the same animal, TEM reveals dark structures near the nucleus in a cardiomyocyte; these are deposits of lipofuscin, a wear-and-tear pigment occasionally observed in cardiomyocytes of aging animals. Cardiomyocytes are indicated by **asterisks**. Original magnification, $\times 400$ (**A**). Scale bar = 2 μm (**B** and **C**). bv, blood vessel; N, nucleus.

mice exhibited axonal spheroids at 6 months, and 100% of mice did so at 9 months of age. The anatomical location of axonal spheroids in the spinal cord and their bilateral distribution, in conjunction with the age-dependent progression in incidence and severity of onset, suggest that these structures develop

secondary to substrate accumulation in the DRG neurons. No such spheroids were observed in spinal cord sections from control 129/Sv mice at any age.

Under HRLM, no significant histological changes were observed in brain and peripheral nerves of *Gla* KO mice, compared with age-matched 129/Sv mice. Older *Gla* KO and 129/Sv mice exhibited a few degenerative changes (eg, mineralized foci in the thalamus and spheroids in the gracile and cuneatus tracts), which were likely age-related incidental changes in this mouse strain. In *Gla* KO mice, HRLM and TEM revealed glycosphingolipid inclusions in a few vascular smooth muscle cells and occasionally in glial cells (Figure 7, A and B). No storage inclusions were observed in neurons or axons.

Unmyelinated axons in a dermal (footpad) nerve of *Gla* KO mice showed degenerative changes, including a few electron-dense membranous inclusions (Figure 7, C and D). Rarely, perineurial cells contained a few electron-dense membranous inclusions.

Enteric Pathology

Male and some heterozygous female FD patients with the type 1 classic phenotype commonly experience intestinal dysfunction, especially postprandial cramps and diarrhea,^{1,29} and extensive lysosomal GL-3 inclusions have been reported in colon and small intestine of FD patients.^{22,30,31} Although overt clinical signs of gastrointestinal dysfunction have not been reported in *Gla* KO mice, in cross sections of the ileum and colon HRLM and TEM revealed lysosomal storage inclusions in a few smooth muscle cells in the intestinal wall. Interestingly, several neurons in the myenteric and submucosal plexuses of both the ileum and colon were markedly enlarged and vacuolated, as observed under HRLM (Figure 8A). Both HRLM and TEM revealed membrane-bound lysosomal inclusions within enlarged myenteric plexus ganglion cells and smooth muscle cells of *Gla* KO mice. Storage inclusions were abundant in neurons, but were relatively sparse in intestinal smooth muscle cells. Ultrastructurally, these storage inclusions consisted of osmiophilic whorl-like inclusions

Figure 5 Thermosensory deficits and dorsal root ganglial pathology in *Gla* KO mice. **A:** Mice were placed on a 55°C hot plate, and the time for them to respond (latency) was recorded. Compared with 129/Sv wild-type mice, *Gla* KO mice exhibited progressive loss of thermosensation, as indicated by the higher latency period in older mice. **B:** Sections stained with H&E show vacuolated and enlarged DRG cells (black arrows) in a *Gla* KO mouse, but normal DRG cells in an age-matched 129/Sv mouse. Corresponding epon-embedded sections show dark blue inclusions in many DRG cells (asterisks) of a *Gla* KO mouse. **C:** The proportion of vacuolated neurons in DRG from the lumbar spinal segment. **D:** TEM reveals storage inclusions (red arrows) in DRG cells of a *Gla* KO mouse. **E:** A higher magnification view of **D** reveals numerous inclusions in a DRG cell. At still higher magnification (inset), the zebra body-like morphology of DRG inclusions is evident. Data are expressed as means $\pm$ SD. $n \geq 10$ mice per time point (**A**); $n \geq 4$ mice per time point (**C**), with ≥ 2 DRG evaluated per mouse. Original magnification: $\times 200$ (top row, **B**); $\times 600$ (bottom row, **B**). Scale bars: 5 μ m (**D**); 2 μ m (**E**); 0.5 μ m (**E**, inset). DRG, dorsal root ganglia; H&E, hematoxylin and eosin; N, nucleus.

Figure 6 Substrate storage and histopathology of trigeminal ganglia and spinal cord of *Gla* KO mice. H&E-stained sections show enlarged and vacuolated trigeminal ganglion cells (arrows) in an approximately 15-month-old *Gla* KO mouse and spheroid-like structures (asterisks) in the dorsolateral fasciculus of the spinal cord in an approximately 12-month-old *Gla* KO mouse. Corresponding tissues from age-matched 129/Sv mice are unremarkable. **Inset:** Silver staining in a serial section reveals argyrophilic spheroids (arrows) in the spinal cord. Original magnification: $\times 400$ (top); $\times 600$ (bottom).

(Figure 8, B and C), similar to what was seen in DRG neurons. The myenteric and submucosal plexus ganglia cell inclusions were detected as early as 3 months of age; this timing is similar to the early presentation in DRG neurons. Intestinal ganglion cells and smooth muscle cells of age-matched control 129/Sv mice were unremarkable.

GL-3 levels in representative samples of ileum from 10-month-old *Gla* KO and control 129/Sv mice were determined by mass spectrometric analysis. Significantly elevated GL-3 levels were noted in the small intestine of *Gla* KO mice ($8000 \pm 1790 \mu\text{g/g}$ of tissue), compared with the small intestine of control mice (Figure 9A). Immunofluorescence staining using an anti-CD77 antibody confirmed the molecular specificity of the storage inclusions (see below). The histological and biochemical findings of elevated GL-3 levels in the small intestine of *Gla* KO mice were corroborated by quantitative morphometric measurements. The total GL-3 fraction estimated by morphometry in representative cross sections of the small intestine of *Gla* KO mice was approximately sixfold higher than that in the age-matched 129/Sv control mice (Figure 9B).

Substrate Accumulation in Other Tissues

Glycosphingolipid accumulation has been reported in many other cell types of FD patients with the classic phenotype, including corneal epithelium, skeletal myofibers,³² and bronchial epithelial cells.⁶ However, in the 129/Sv-based

Gla KO mice, there was no evidence of GL-3 inclusions in hepatocytes, corneal epithelium, retinal ganglion cells, lung epithelium, or skeletal myofibers by either HRLM or TEM (Table 1). Swollen Kupffer cells with foamy cytoplasm were observed in 17-month-old *Gla* KO mice, but not in age-matched 129/Sv mice (data not shown). Hepatocytes in both *Gla* KO and 129/Sv mice were unremarkable, similar to those in type 1 and type 2 FD patients.

Immunofluorescence Localization of GL-3 Accumulation in *Gla* KO Mice

Tissue cryosections were used for immunofluorescence assay with a rat monoclonal antibody specific for GL-3 (CD77), as has been used on tissues from FD patients³³ and mice.³⁴ The most prominent CD77⁺ cells in *Gla* KO mouse tissues were epithelial cells (of the renal tubules and collecting ducts), Kupffer cells, VSMCs, intestinal smooth muscle cells, ganglion cells (in the DRG, trigeminal ganglia and intestinal myenteric plexus), and perineurial cells (in the sciatic nerves) (Figure 10). Importantly, ECs, cardiomyocytes, hepatocytes, renal mesangial cells, CNS neurons, and skeletal muscle fibers were CD77⁻. As expected, tissues from 129/Sv mice showed no or minimal CD77⁺ immunofluorescence. Serial tissue

Figure 7 Ultrastructural pathology of brain and peripheral nerve in *Gla* KO mice. **A:** TEM reveals substrate inclusions (arrows) in smooth muscle cells of a blood vessel in the cerebellum of an approximately 17-month-old *Gla* KO mouse. **B:** A higher magnification view of the inclusions from **A** reveals dense substrate deposits (asterisks). **C:** TEM image of a nerve in the footpad of a *Gla* KO mouse. Dense GL-3 inclusions (arrows) are observed in a few unmyelinated axons and Schwann cells. **D:** Higher magnification of **C** shows an unmyelinated axon with dense GL-3 inclusions (asterisks). Scale bars: 2 μm (A and C); 0.5 μm (B). Original magnification: $\times 25,000$ (D). end, endothelial cell; m, myelin sheath.

Figure 8 Substrate storage pathology in *Gla* KO mice. **A:** H&E sections (top and middle rows) show enlarged myenteric plexus ganglia (outlined in blue) in colon and ileum of an approximately 15-month-old *Gla* KO mouse; the age-matched 129/Sv mouse is unaffected. In epon-embedded thin sections of ileum (bottom row) with Richardson's stain, dark blue inclusions are evident within ganglion cells (black arrows) and smooth muscle cells (red arrows). **B:** TEM reveals storage inclusions in myenteric plexus ganglia (black arrows) and smooth muscle cells (red arrows) in the muscular layer of the ileum of a 15-month-old *Gla* KO mouse. **C:** A higher magnification view of the same sample reveals storage inclusions (asterisks) within a ganglion cell. A concentric membranous pattern (myelin figure) is evident within these inclusions. Original magnification, $\times 600$ (A). Scale bars: 2 μ m (B); 0.2 μ m (C). N, nucleus; smc, smooth muscle cell.

sections subjected to IFA with an isotype control antibody showed no immunofluorescence (data not shown).

Discussion

Knockout mice lacking lysosomal α -Gal A activity have been used as a model for testing therapeutic concepts for FD for nearly two decades.^{13–15,18,35} These models have proven

useful for preclinical pharmacokinetic and pharmacodynamics studies of enzyme replacement, substrate reduction, and gene therapy strategies for FD.^{12,15,17,18} These *Gla* KO mice also provided important data on the biodistribution of the first intravenously delivered enzyme via mannose 6-phosphate receptors.¹² Nonetheless, despite absence of α -Gal A activity and despite progressive GL-3 accumulation in various tissues and plasma, these mice do not exhibit the same extent of tissue pathology or clinical disease as do FD patients with the severe early-onset type 1 classic phenotype. In the present study, we conducted a detailed histopathological characterization of young and aged *Gla* KO mice (3 to 17 months old) to determine whether the GL-3 storage pathology increases with age and whether older mice have GL-3 storage in additional cells or tissues.

Progressive accumulation of GL-3 and related glycolipids in tissues and blood due to absent α -Gal A activity has been reported in both 129/Sv-based and C57BL/6-based *Gla* KO

Figure 9 Quantitation of GL-3 accumulation in the small intestine of 12-month-old mice. **A:** GL-3 levels in the mouse small intestine were assessed by mass spectrometry. GL-3 levels in the 129/Sv mice were below the detection limit of the assay. **B:** Histomorphometric quantitation of GL-3 in mouse small intestine. Frozen cross sections of mouse intestine were subjected to immunofluorescence assay with anti-CD77 antibody followed by a fluorescently labeled secondary antibody. The CD77 immunopositive area was quantitated and expressed as a fraction of the total tissue area. Data are expressed as means $\pm$ SD (A) or as means $\pm$ SEM (B). $n = 9$ (A, *Gla* KO); $n = 10$ (A, 129/Sv WT) $n = 3$ per group (B).

Figure 10 Localization of GL-3 accumulation in tissues of *Gla* KO mouse (approximately 10 months old) by immunofluorescence. In immunofluorescence assay, bright green staining with anti-CD77 denotes GL-3 accumulation. Note the intense specific immunofluorescence in various cell types of *Gla* KO mouse tissues, but only background staining in the corresponding tissues from age-matched 129/Sv wild-type mice. Original magnification: $\times 200$ [kidney (tubule) and sciatic nerve]; $\times 400$ [liver, ileum, kidney (glomerulus), heart, brain (choroid plexus), and DRG].

mice.^{12,15,18,20} Biochemically, the progressive increase in tissue and plasma GL-3 levels of *Gla* KO mice is consistent with the progressive glycolipid storage in FD patients. However, most previous reports have not provided histological documentation of widespread storage inclusions or pathology in mouse tissues, a situation that likely reflects the greater sensitivity of the biochemical assay compared with histological methods. Moreover, the mass spectrometric assay measures total GL-3 (cell membrane, cytosolic or lysosomal, and extracellular) levels in the tissue homogenate; in the present study, our use of light and electron

microscopy, and of the anti-CD77–based IFA, was focused principally on determining the cellular and subcellular sites where GL-3 is concentrated.

Gla KO Mice Lack Vascular Endothelial GL-3 Accumulation

Tissue pathology and organ dysfunction in type 1 classic FD patients result principally from lysosomal GL-3 storage in vascular, cardiac, and renal tissues.^{1–3,36} The vasculopathy in type 1 classically affected FD patients is attributed to

substrate storage primarily in vascular ECs and to a lesser extent in the VSMCs.³⁶ Similar to *Gla* KO mice, male FD patients with the type 2 later-onset phenotype have residual α -Gal A activity and do not exhibit vascular endothelial GL-3 accumulation.³⁷ Endothelial dysfunction secondary to GL-3 storage in vascular endothelial cells and VSMCs has been implicated in the multisystemic vasculopathy seen in FD patients with the type 1 phenotype.^{1,2,38} Widespread substrate storage in cardiomyocytes, renal mesangial cells, podocytes, and capillary ECs results in the tissue pathology, leading to multiorgan failure, nephropathy, cardiomegaly, and/or stroke.^{3,5,6}

In the present study, HRLM showed that GL-3 accumulation in these cell types was generally absent in *Gla* KO mice at earlier time points (3 to 6 months of age); however, minimal to mild renal tubular degeneration was evident in older *Gla* KO mice (>10 months old). Under HRLM and TEM, dense inclusions indicative of lysosomal GL-3 storage were observed in the epithelial cells of renal tubules, collecting ducts, and Bowman's capsule, and rarely in glomerular podocytes. Lysosomal storage inclusions were not detected in mesangial cells and ECs of the glomerular capillary or extraglomerular vasculature of *Gla* KO mice at any age. Similarly, no lysosomal GL-3 storage was detected in cardiomyocytes, although a few inclusions were detected in the interstitial fibroblasts and VSMCs of coronary blood vessels. In the liver, no substrate inclusions were detected in hepatocytes, and Kupffer cells and VSMCs of hepatic blood vessels showed few storage inclusions. To complement our HRLM and TEM observations, we used an immunohistochemistry assay with a monoclonal rat anti-CD77 antibody known to specifically detect GL-3 in murine tissues.³⁴ CD77⁺ immunofluorescence was observed in multiple cell types, including those that showed inclusions under HRLM and/or TEM (Table 1). In accord with previous reports describing the pathology of the C57BL/6-based *Gla* KO mouse,^{12–14} we could not detect evidence of substrate storage in the vascular ECs, cardiomyocytes, or CNS neurons of 129/Sv-based *Gla* KO mice.

Species-Specific Differences in Physiology May Explain Differing GL-3 Storage Pathology in *Gla* KO Mice and FD Patients

The documentation of minimal tissue pathology with age and no overt clinical disease in *Gla* KO mice can be attributed to the lack of lysosomal GL-3 storage in the cell types commonly affected in FD patients.^{12,20} A major pathological difference between FD patients and *Gla* KO mice is that the prominent vascular endothelial GL-3 accumulation in patients with type 1 classic FD does not occur in *Gla* KO mice, nor in males with type 2 later-onset FD, presumably because the type 2 FD patients have residual α -Gal A activity.^{1,6} Species-specific differences in lipoprotein transport and metabolism may explain the vascular endothelial differences. In humans, the majority of the glycosphingolipids, including GL-3, are synthesized in

hepatocytes and are transported out of the liver into the circulation with the very low density and low density lipoproteins (VLDL and LDL, respectively).^{39,40} A portion of the circulating LDL-bound GL-3 is taken up via LDL receptors into the vascular endothelial and smooth muscle cells. Deficient α -Gal A activity in FD patients with the classic type 1 phenotype results in markedly elevated GL-3 levels and progressive lysosomal accumulation in vascular ECs, resulting in clinical disease.¹ The LDL/HDL profile of mice, with low LDL and relatively high HDL fractions, is the inverse of the human profile.⁴¹ As a result, murine ECs have reduced LDL receptor-mediated uptake of plasma GL-3, which accounts in part for the absence of vascular endothelial storage pathology in *Gla* KO mice, despite the complete absence of α -Gal A activity.

Reduced GL-3 synthesis in mice, relative to that in humans, may also contribute to the attenuated GL-3 accumulation in *Gla* KO mice, compared with type 1 FD patients. Turnover of senescent erythrocyte membranes is thought to be a significant contributor to circulating GL-3 levels in humans.¹ By contrast, erythrocytes in mice have very low levels of GL-3 and of its globoside, the catabolic precursor molecule. Ohshima et al²⁰ proposed that the barely detectable levels of GL-3 and globoside in erythrocytes from 10-week-old C57BL/6 mice, in contrast to normal human erythrocytes, was likely responsible for the lower levels of GL-3 storage in *Gla* KO mouse ECs. Consequently, the GL-3 load does not increase in the plasma and tissues of *Gla* KO mice, compared with that in classically affected FD patients, despite the murine α -Gal A deficiency. Moreover, constitutive levels of expression of GL-3 in various tissues and cell types differ between mice and humans.^{34,42} In contrast to the high levels of GL-3 in various human cell types, WT mice have lower GL-3 levels, particularly in the collecting duct epithelium and none at all in the glomerular ECs.^{34,42}

This differential GL-3 pattern in WT mice also correlates with their relative resistance to infection by enterohemorrhagic *Escherichia coli*.⁴² These bacteria produce a potent Shiga toxin that binds with high affinity to GL-3 in membranes of human glomerular ECs, eventually resulting in severe glomerulonephritis.⁴³ By contrast, mice infected with enterohemorrhagic *E. coli* exhibit low-grade renal tubular damage and no significant glomerular disease, as is consistent with their low renal GL-3 levels. Interestingly, *Gla* KO mice challenged with lethal doses of enterohemorrhagic *E. coli* remained unaffected and did not show renal histological changes, compared with WT mice.⁴⁴ The very high GL-3 levels in *Gla* KO mice might have acted as a toxin sink. More importantly, there were no renal histological changes in *Gla* KO mice that died after dosing with high doses of enterohemorrhagic *E. coli* or Shiga toxin.⁴⁴ Furthermore, GL-3 synthase expression also appeared to be preferentially localized in collecting ducts and proximal tubule epithelium in murine kidneys.⁴⁵ Taken together, these features of murine biology may account for the lack of vascular endothelial GL-3 expression and storage in the α -Gal A-deficient mice.

Similar to findings from tissue biopsies from FD patients, we observed GL-3 storage inclusions in VSMCs of multiple blood vessels of *Gla* KO mice but with no associated disease symptoms. Presumably, the low GL-3 load in mouse VSMCs caused no or very subtle functional deficits, which did not result in apparent vascular disease (because of the inverted HDL/LDL profile and the lack of concomitant endothelial cell dysfunction).

Pathology of the Sensory and Autonomic Ganglia in *Gla* KO Mice

A striking observation from the present study is the age-dependent changes in histology and function that are indicative of a sensory neuropathy in *Gla* KO mice. Neurological manifestations in males with type 1 classic FD include diffuse involvement of the vascular endothelium and some central neurons, as well as peripheral neuropathy associated with the DRG, other autonomic ganglia, and peripheral nerves. We did not observe any apparent CNS disease or lysosomal GL-3 storage in CNS neurons from *Gla* KO mice. By immunohistochemistry and TEM, substrate inclusions were confined to VSMCs in the brain, spinal cord, and perineurial cells. Consistent with previous studies using the 129/Sv-based mouse model¹⁶ and the C57BL/6-based model,²⁷ we observed distinct thermosensory deficits in *Gla* KO mice, beginning as early as 3 months of age. Interestingly, histological changes in the DRG neurons were also evident at this early age. The progressive increase in the latency period in the hot-plate test, indicative of thermosensory deficits, and the concomitant enlargement (vacuolation) of the DRG neurons demonstrated an excellent structure–function correlation. Indeed, this feature of the *Gla* KO mouse model has been used as a functional and histological indicator of therapeutic efficacy.^{15,18} Sensory neuropathy in type 1 FD has been attributed to lysosomal GL-3 storage in sensory DRG neurons and perineurial cells in conjunction with local ischemic damage resulting from diffuse vascular endothelial involvement.^{6,27} Striking GL-3 storage pathology was observed in the DRG sensory neurons of *Gla* KO mice by HRLM and TEM. By histological and immunohistochemistry assays, we localized lysosomal GL-3 storage predominantly within DRG sensory neurons. We also found rare storage inclusions in perineurial cells of *Gla* KO mice, but (as noted above) no histological changes or GL-3 inclusions were observed in vascular ECs. It is likely, therefore, that the sensory deficits in *Gla* KO mice were caused predominantly by lysosomal storage in the neuron soma. Consistent with this hypothesis, we observed eosinophilic spheroidal swellings in lamina I of the spinal cord gray matter. This area corresponds to axonal terminations of the sensory neurons whose soma are located within the DRG. Thus, the spheroid-like structures most likely indicate axonal degeneration secondary to lysosomal GL-3 accumulation in the DRG neuron soma.

The distinct anatomical location and unique structural and functional features of DRG neurons may explain their increased susceptibility to progressive GL-3 accumulation. Unlike CNS neurons and peripheral nerve axons, which are isolated from the systemic circulation by the blood–brain barrier and the blood–nerve barrier, respectively, DRG are located in a highly permeable vascular compartment. This anatomical feature likely exposes DRG neurons to circulating GL-3. Consistent with this notion, GL-3 storage in certain CNS tissues from FD autopsy cases has been reported within certain brain areas (eg, choroid plexus, certain brain nuclei) where the blood–brain barrier is either absent or most permissive. Furthermore, high metabolic demand, unique structural features, and the post-mitotic state of the DRG neuron soma contribute to their vulnerability to progressive GL-3 accumulation. Each DRG comprises thousands of pseudo-unipolar sensory neurons whose peripheral axons serve as afferent sensory nerve fibers and whose central axons synapse with interneurons in the dorsal gray matter of the spinal cord.⁴⁶ Unlike a typical animal cell, the DRG neuron cell soma represents only 0.2% of the cell cytoplasm (the remaining 99.8% being present in the axon).⁴⁶ The soma is responsible for meeting the energy demand of the axon, overall protein synthesis, and conduction of peripheral impulses to the CNS. In light of these unique features of the DRG neurons, it is reasonable to expect structural changes indicative of lysosomal GL-3 accumulation in the soma. Of course, the role of constitutive GL-3 production by DRG neurons and pathways used by these cells for uptake, storage, and clearance of GL-3 from the circulation probably also contribute to their increased susceptibility for GL-3 storage. Further investigations are needed to elucidate the mechanisms of DRG sensory neuron involvement in thermosensory deficits in *Gla* KO mice.

Similar to DRG, trigeminal ganglia are also located outside the CNS. Neurons within the trigeminal ganglia of *Gla* KO mice were seen as markedly enlarged and vacuolated under routine LM (see *Light Microscopy* section), and TEM revealed characteristic lysosomal GL-3 storage inclusions. Neurons in trigeminal ganglia receive afferent sensory fibers from the face and whiskers. Lysosomal GL-3 storage has been documented in trigeminal ganglia of FD patients.^{6,27} It would be interesting to further investigate these histological changes in *Gla* KO mice and identify relevant functional correlates that can be used as clinical endpoints in animal studies. *Gla* KO mice thus appear to be an attractive tool for exploring the sensory neuropathy of type 1 classic FD.

Neurons in intestinal autonomic ganglia (myenteric plexus) also showed GL-3 storage, as evidenced by characteristic zebra-body inclusions that stained positively by anti-CD77 IFA. Diffuse involvement of myenteric plexus neurons, in conjunction with intestinal smooth muscle cell GL-3 storage, may result in intestinal dysfunction. The underlying pathophysiology has not been extensively investigated, but existing histopathological and clinical data suggest involvement of the enteric nervous system (myenteric plexus), smooth muscle cells, and vascular ECs.^{30,31} Our present observations in *Gla* KO mice

indicate the potential usefulness of this mouse model for investigating type 1 classic FD enteropathy.

Conclusions

In the present study, we systematically analyzed the histopathological changes associated with GL-3 accumulation in 129/Sv-based *Gla* KO mice. Our results should help guide the appropriate incorporation of histologic, functional, or biochemical endpoint assays when using these mice to evaluate potential therapies for FD. *Gla* KO mice recapitulated several histopathological features of FD patients, especially those with the type 2 later-onset phenotype, which is the more prevalent FD phenotype.^{47,48} The most striking histological difference between FD patients and *Gla* KO mice was the lack of lysosomal GL-3 storage in vascular ECs, cardiomyocytes, and glomerular mesangial cells, the cell types most commonly affected in FD patients with the type 1 classic phenotype. We also delineated a detailed histological basis for the thermosensory deficits previously reported in this mouse model. Our observations of early and widespread lysosomal storage in neurons of sensory and autonomic ganglia and intestinal smooth muscle cells of *Gla* KO mice indicate their potential utility as a tool for investigating FD neuropathy and enteropathy.

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