Assessing the role of glycosphingolipids in the phenotype severity of Fabry disease mouse model

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Abstract  Fabry disease is caused by deficient activity of α-galactosidase A, an enzyme that hydrolyzes the terminal α-galactosyl moieties from glycolipids and glycoproteins, and subsequent accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3), globotriaosylsphingosine (lyso-Gb3), and galabiosylceramide. However, there is no known link between these compounds and disease severity. In this study, we compared Gb3 isoforms (various fatty acids) and lyso-Gb3 analogs (various sphingosine modifications) in two strains of Fabry disease mouse models: a pure C57BL/6 (B6) background or a B6/129 mixed background, with the latter exhibiting more prominent cardiac and renal hypertrophy and thermosensation deficits. Total Gb3 and lyso-Gb3 levels in the heart, kidney, and dorsal root ganglion (DRG) were similar in the two strains. However, levels of the C20-fatty acid isoform of Gb3 and particular lyso-Gb3 analogs (+18, +34) were significantly higher in Fabry-B6/129 heart tissue when compared with Fabry-B6. By contrast, there was no difference in Gb3 and lyso-Gb3 isoforms/analogs in the kidneys and DRG between the two strains. Furthermore, using immunohistochemistry, we found that Gb3 massively accumulated in DRG mechanoreceptors, a sensory neuron subpopulation with preserved function in Fabry disease. However, Gb3 accumulation was not observed in nonpeptidergic nociceptors, the disease-relevant subpopulation that has remarkably increased isolectin-B4 (the marker of nonpeptidergic nociceptors, the disease-relevant subpopulation that) binding and enlarged cell size. These findings suggest that specific species of Gb3 or lyso-Gb3 may play major roles in the pathogenesis of Fabry disease, and that Gb3 and lyso-Gb3 are not responsible for the pathology in all tissues or cell types.

Supplementary key words  glycolipids • sphingolipids • inborn errors of metabolism • storage diseases • globotriaosylceramide • globotriaosylsphingosine • pathogenesis

Fabry disease is an X-linked metabolic disorder that results from deficiency of the lysosomal hydrolase, α-galactosidase A (α-gal A) (1), which leads to intracellular accumulation of glycosphingolipids (GSLs) with a terminal α-linked galactosyl moiety in various tissues. Known substrates of α-gal A include globotriaosylceramide (Gb3), globotriaosylsphingosine (lyso-Gb3), galabiosylceramide (Gb2), blood groups B and P1 glycolipids, and isoglobotriaosylceramide (iGb3) (2–5). Fabry disease exhibits a variety of clinical manifestations, including stroke, hypertrophic cardiomyopathy, renal insufficiency, and painful small-fiber neuropathy (2). Currently, enzyme replacement therapy (ERT) and pharmacological chaperones are the commercially approved treatments for Fabry disease. Other promising approaches, such as substrate reduction therapy and gene therapy are under development (6–8).

The mechanism through which accumulated GSLs cause the multisystemic damage is poorly understood. Gb3 and lyso-Gb3 are generally thought to be responsible for the pathogenesis of the disease; however, conclusive evidence is lacking. Clinical manifestations and severity in Fabry patients do not correlate with plasma and urinary Gb3 levels (9). Lyso-Gb3 was shown to have high diagnostic sensitivity (10); however, there is no clear correlation of plasma lyso-Gb3 levels with disease manifestations (3). In addition, Gb3 and lyso-Gb3 may not be reliable markers for therapeutic efficacy. The change of serum lyso-Gb3 after ERT did not correlate with a change in left ventricular mass index (11). Serum lyso-Gb3 had a good response to ERT during early stages, but it became insensitive to the treatment at later stages (11).

Fabry mice have been widely used for both mechanistic and therapeutic studies. Previously, we have found that Fabry mice develop cardiac and renal hypertrophy (12). Compared with WT controls, Fabry mice exhibited increased heart weight and left ventricular wall thickness, and had elevated cardiac expression of hypertrophy marker, atrial natriuretic peptide (ANP), but not brain natriuretic peptide and α- and β-myosin heavy chain. Renal hypertrophy in Fabry mice was likely due, at least in part, to hypertrophied proximal tubular epithelial cells. There was no sign of renal insufficiency (12). Fabry mice also develop impaired thermal sensation (13, 14) that resembles small-fiber neuropathy in patients. However, due to the complexity of GSL biology, such as cell type-specific distribution, heterogeneity...
of fatty acid chains, and the balance between related GSLs that compete common precursors, it is methodologically challenging to elucidate the potential pathogenic role of a given GSL by using simple transgenic approaches in animal models. Elevated Gb3 level by overexpression of human Gb3 synthase (A4GALT, which is responsible for synthesis of Gb3 and Gb2) in Fabry mice was associated with signs of kidney dysfunction (15). However, given that Gb3 biosynthesis is precisely controlled in a cell type-specific manner (as suggested in the present study) and is regulated by various factors including cell cycle and cytokines (16, 17), it can be expected that the constitutive overexpression of A4GALT under ubiquitous CAG promoter would produce a different cellular distribution of Gb3 compared with the intrinsic distribution pattern in Fabry disease. These A4GALT-transgenic Fabry mice exhibit neurological symptoms (e.g., spontaneous tremor) that do not develop in Fabry disease (15). Another difficulty in the pathogenesis studies of Fabry disease is the lack of appropriate experimental models in which the effect of storage compounds on the disease phenotype can be investigated. Kamani et al. (18) robustly characterized GSLs in Fabry mice versus WT mice and demonstrated differential accumulation of Gb3 species in Fabry mouse tissues and a unique fold-change of Gb3 in each tissue in Fabry mice relative to WT. However, because all the Gb3 isoforms are increased in Fabry mice compared with WT controls, it remains unclear which Gb3 species are responsible for the disease.

The genetic background of Fabry mice that originated from the National Institutes of Health (19) was a mixture of C57BL/6 (B6) and 129 strains (12). To obtain stable phenotype and to facilitate molecular studies, we backcrossed Fabry mice to the B6 strain. We found that Fabry mice in a pure B6 strain develop much milder phenotypes compared with those in a mixed background. The significantly different severity of disease phenotypes and relatively close genetic backgrounds (~100% vs. ~78% of B6 strain) make these two strains of Fabry mice a good model system to study the potential relationship of GSLs and disease severity. In this study, we investigated the correlations between tissue levels of various Gb3/lyso-Gb3 species and Fabry disease phenotypes in these two mouse strains. In addition, to evaluate the potential involvement of Gb3 in small-fiber neuropathy, we investigated patterns of Gb3 distribution in sensory neuron subpopulations of dorsal root ganglion (DRG) in Fabry mice.

**Correlation between GSLs and Fabry disease phenotypes**

**MATERIALS AND METHODS**

**Mouse colonies**

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor Research Institute. The Fabry mouse colony was originally from the National Institutes of Health (19), which was on a B6:129 mixed background (12). Fabry mice were produced by breeding pairs of hemizygous males and homozygous females. To reduce the genetic background (12), Fabry mice were produced by breeding pairs of C57BL/6 (B6) and 129 strains (12). To obtain stable genetic background (100% vs. 99.7%) was confirmed by 384-SNP analysis. Backcrossed Fabry mice to the B6 strain for 10 generations, and their B6 background (~99.7%) was confirmed by 384-SNP analysis. Previously, we found that both hemizygous male mice and homozygous female mice develop cardiac and renal hypertrophy with no significant sex difference (12). Therefore, in the present study, we focused on male mice only. A total 159 mice were used in this study (WT-B6/129, Fabry-B6/129, WT-B6, and Fabry-B6; n = 27, 39, 39, and 54, respectively).

All animal procedures (dissection and organ weight measurements, hot-plate tests, and Gb3 and lyso-Gb3 assays were done in a blinded manner.

**Hot plate tests (conventional)**

Mice were placed on a heated surface (hot plate analgesia meter; IITC Life Science) equilibrated at 55°C, and the time interval between placement and shaking (or licking) of a hind paw was recorded as the response time. To avoid injury, cut-off time was set at 50 s. To minimize the effect of learning by the mice, the hot plate test was not repeated in the same animal within 2 months.

**Hot plate test (incremental)**

The incremental hot plate test was performed using an incremental analgesia meter (IITC Life Science). The starting temperature was set at 30°C; after the mouse was placed on the plate, the temperature increased at a rate of 6°C/min. The temperature at which the mouse responded (shaking or licking of hind paw) was recorded as the threshold (degrees Celsius). The cut-off temperature was set at 55°C.

**Gb3 and lyso-Gb3 analysis**

Gb3 was measured in two different laboratories. For the data shown in Fig. 2, eight Gb3 isoforms were measured using MS at the Institute of Metabolic Disease at Baylor Research Institute as described previously (20). Gb3 levels were normalized according to total protein levels. For the more comprehensive Gb3 analysis (data shown in Fig. 3), the assay was performed in the Division of Medical Genetics, Université de Sherbrooke, as described previously (21). Lyso-Gb3 was measured as described previously (22–25).

**Immunofluorescence staining**

Mice were fixed by transcardial perfusion of 4% paraformaldehyde in PBS. Tissues were postfixed in the same fixative overnight at 4°C (cryoprotected in 15% sucrose) and were embedded in OCT compound. Cryosections were cut at 7 μm, permeabilized in 0.3% Triton X-100 in PBS (PBST) and were blocked in 10% normal goat serum in PBST for 1 h. After incubation with primary antibodies diluted in 5% normal goat serum/PBST overnight at 4°C, the antibody binding was visualized by incubation with Alexa 594- or Alexa 488-conjugated secondary antibodies (Invitrogen). Slides were mounted in ProLong Gold with DAPI (Invitrogen). Primary antibodies used were: mouse anti-Gb3 (Amsbio), rabbit anti-PG9.5 (Thermo Fisher Scientific), guinea pig anti-Substance P (Abcam), mouse anti-calcitonin gene-related peptide (CGRP)
(Abcam), mouse anti-NF200 (RT97; Millipore), rabbit anti-NF200 (Millipore), guinea pig anti-Parvalbumin (Swant), and rabbit anti-Ret (Abcam). For isoelectric B4 (IB4) staining, sections were incubated with Alexa 488-conjugated IB4 (Invitrogen) overnight at 4°C (in primary antibody step), with 0.1 mM MgCl2/CaCl2 in the buffer.

After testing various concentrations of Triton, we confirmed that the above procedures gave the satisfactory detection of lysosomal accumulation of Gb3 in Fabry mouse tissues with respect to that the above procedures gave the satisfactory detection of lysosomal accumulation of Gb3 in Fabry mouse tissues with respect to intensity of specific signal and signal/noise ratio. Triton was also needed for optimal staining of other antigens that were double stained with Gb3 in this study. However, Triton might extract Gb3 distributed in non-raft plasma membrane and other organelles. To test whether the use of Triton in the staining altered the Gb3 localization pattern, we compared Gb3 staining with or without Triton. In the latter case, Triton was not included in any steps, i.e., PBS was used in place of PBST in the protocol.

Quantitative real-time RT-PCR
Quantitative RT-PCR for ANP was performed as described previously (12), using predesigned TaqMan probe/primers (Applied Biosystems).

Statistical analysis
Data are presented as the mean ± SEM. Significance was determined using Student’s t-test.

RESULTS
Fabry-B6/129 mice have earlier onset, more prominent cardiac and renal hypertrophy, and greater thermosensation deficit than Fabry-B6
In this study, we characterized Fabry mice on a B6:129 mixed background (~78% of B6 strain; referred to as Fabry-B6/129) and Fabry mice on a purely B6 strain (referred to as Fabry-B6), compared with their WT counterparts with an identical genetic background (referred to as WT-B6/129 and WT-B6, respectively). Only male mice were used in this study (WT-B6/129, n = 27; Fabry-B6/129, n = 39; WT-B6, n = 39, and Fabry-B6, n = 54) (see the Materials and Methods for details).

Consistent with our previous study (12), Fabry mice in both strains developed progressive cardiac and renal hypertrophy. However, Fabry-B6/129 exhibited earlier onset and more severe phenotypes than Fabry-B6 (Fig. 1A-E). Cardiac and renal hypertrophy appeared at age 6 months in Fabry-B6/129 but at age 12 months in Fabry-B6 (Fig. 1A, D). At 12 months, heart- or kidney-to-body weight ratio increased by ~60% in Fabry-B6/129 (compared with WT) but only ~15% in Fabry-B6 (Fig. 1B, E). Although the increased heart and kidney weight in Fabry-B6 relative to WT controls was statistically significant, the difference was marginal (Fig. 1A, D). Cardiac expression of hypertrophy marker ANP, was upregulated in 12-month-old Fabry-B6/129 but not in Fabry-B6 (Fig. 1C). In contrast to the heart and kidney, the weight of the liver, an unaffected organ in Fabry disease, was not different between Fabry and WT mice in both strains (data not shown).

Compared with WT controls, both strains of Fabry mice exhibited delayed response to heat stimuli when assessed using a conventional hot plate that was equilibrated to 55°C (Fig. 1F, G). The delayed response in Fabry-B6/129 appeared at as early as 1 month of age (Fig. 1F), although there was large overlapping between WT and Fabry mice until ~5 months of age (see supplemental Fig. S1 for details). In contrast, delayed heat response in Fabry-B6 was first observed at age 7 months (Fig. 1F). The delayed response was more severe in Fabry-B6/129 than in Fabry-B6 at 7 months of age (Fig. 1G). We also tested thermosensation by using an incremental hot plate in 5-month-old mice (Fig. 1H). Unlike the conventional hot plate that measures the response latency to a constant and harsh temperature (55°C), the incremental hot plate measures the heat detection threshold. WT-B6/129 and WT-B6 had different thresholds (46.3°C and 48.1°C, respectively). Both strains of Fabry mice exhibited a higher threshold compared with WT controls (Fig. 1H). The increment of the threshold in Fabry-B6/129 was higher than that in Fabry-B6 (4.3°C vs. 2.5°C).

Both strains of Fabry mice had enlarged DRGs compared with WT controls of the same genetic background (Fig. 1I). The enlargement of DRGs in Fabry mice was noticeable as early as 2–3 months of age and progressed with age. Although no quantitative comparison was done, it appeared that the DRG enlargement in Fabry-B6/129 was more obvious and consistent than that in Fabry-B6 mice.

The relationship between Gb3 isoforms and disease phenotype
Gb3 has various isoforms (different lengths of fatty acid chain) and analogs (modifications of the sphingosine or the fatty acyl moiety, e.g., extra double bonds, methylation, and hydroxylation). As described previously (20), we measured eight Gb3 species [C16:0, C18:0, C20:0, C22:0, C24:0, C22:1, C24:1, and C24:0(OH)], and the sum of these was used to present total Gb3 level. As expected, compared with WT controls, Gb3 accumulated in both strains of Fabry mice in an age-dependent manner (Fig. 2A). Total Gb3 levels in the heart, kidney and lumbar DRGs were similar between Fabry-B6/129 and Fabry-B6 at both 6 and 12 months (Fig. 2A). However, total Gb3 level in the liver was significantly higher (~24-fold) in Fabry-B6/129 compared with Fabry-B6 (Fig. 2A). These results suggested that total Gb3 level does not correlate with disease severity in Fabry mice, especially the severity of cardiac hypertrophy that is clearly different in the two strains.

It was suggested that different fatty acid isoforms of Gb3 might have different biological functions (26). Therefore, we compared individual Gb3 isoforms between two strains of Fabry mice. In the heart, levels of C18:0- and C20:0-containing Gb3 were significantly higher in Fabry-B6/129 compared with Fabry-B6 at both 6 and 12 months (Fig. 2B). C22:0 trended toward a higher level in Fabry-B6/129 mouse heart. Other isoforms were similar between the two strains (Fig. 2B).

In contrast to the heart, there was no consistent and substantial difference in Gb3 isoform levels in the kidneys between the two strains of Fabry mice, except that C24:1 was moderately lower in Fabry-B6/129 than Fabry-B6 at ages 6
Fig. 1. Disease phenotypes in Fabry-B6/129 and Fabry-B6 mouse strains. A: Heart-to-body weight ratio in 6- and 12-month-old mice (n = 8–45). Note that there were two potential outliers in Fabry-B6 at 12 months with higher heart weight than the rest of the group. The difference between this group and WT-B6 was significant regardless of inclusion or exclusion of these two animals (P = 0.010 and 0.013, respectively). B: The same data as shown in A, but expressed as percent of WT controls. C: ANP mRNA levels in heart tissues measured by real-time RT-PCR (n = 5–8). D: Kidney-to-body weight ratio in 6- and 12-month-old mice (n = 8–45). E: The same data as shown in D, but expressed as percent of WT controls. F: Response time to heat (55°C) at various time points, assessed by using a conventional hot plate (n = 7–28). *P < 0.05 determined via the t-test (Fabry vs. WT at each time point). G: Detailed data of response time to 55°C at 7 months of age (n = 10–25). H: Heat detection threshold at 5 months of age, assessed by using an incremental hot plate (n = 9–22). I: Fabry mice have enlarged DRG compared with WT controls. The spinal cords with DRGs (arrows) (upper); dissected DRGs (lower). D, DRG; N, nerve. Scale bars: 3 mm in spinal cords, 0.5 mm in DRGs.
and 12 months (supplemental Fig. S2A). Similarly, there was no difference in DRG, except that C24:0(OH) was lower in Fabry-B6/129 compared with Fabry-B6 (supplemental Fig. S2B).

In the liver, all the isoforms, except C24:0(OH), were higher in Fabry-B6/129 compared with Fabry-B6 (supplemental Fig. S3). Therefore, unlike in the heart, the difference in Gb3 isoforms in the liver between the two strains was not fatty acid length dependent.

The heterogeneity of fatty acid chains in GSLs is known to be caused by a family of fatty acid-selective ceramide synthases (27). To test whether the higher levels of certain isoforms in the heart or liver of Fabry-B6/129 is due to increased synthesis of these isoforms in the mixed B6/129 compared with the B6 mouse strain, we compared isoform profiles in WT controls. There was no difference between WT-B6/129 and WT-B6, except C22:1 that was decreased in WT-B6/129 heart and liver at 12 months (supplemental Fig. S4), suggesting that the increased isoform levels in Fabry-B6/129 are likely related to impaired glycolipid metabolic pathways in Fabry disease rather than merely to a different synthesis rate in different mouse strains.

The increased cardiac level of C18- and C20-containing Gb3 in Fabry-B6/129 (Fig. 2B) was intriguing. Thus, we focused on the heart and tested a more comprehensive panel of Gb3 isoforms (21, 28) that consisted of 21 species grouped in four categories:

1. Gb3 isoforms with saturated fatty acid,
2. Gb3 isoforms with unsaturated fatty acid (one or two extra double bonds),
3. methylated Gb3 isoforms,
4. hydroxylated Gb3 isoforms (Fig. 3A).

Sixteen out of 21 Gb3 species were significantly higher in 12-month-old Fabry-B6/129 mouse heart than Fabry-B6 (Fig. 3A). Importantly, among five different lengths of fatty acid (C16, C18, C20, C22, and C24), the greatest difference was in C20-containing Gb3 (∼2-fold) regardless of the category (Fig. 3A). When the level of each Gb3 species in Fabry-B6/129 mouse heart was expressed as a ratio to Fabry-B6, the difference between the two strains was clearly fatty acid length

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**Fig. 2.** Total Gb3 and isoforms/analogs in Fabry-B6/129 and Fabry-B6 mouse tissues. A: Total Gb3 levels in the heart, kidney, DRG, and liver (n = 5–10). Note that there was an apparently higher kidney Gb3 level in WT-B6 than in WT-B6/129 at 12 months. This is likely due to the large variability of this cohort of mice, and also partly due to the relatively small sample size (n = 6). B: Gb3 isoforms/analogs in the heart (n = 7–10).
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dependent, with a bell-shaped distribution peaking at C20 (Fig. 3B). The levels of C20-containing Gb3 isoforms (saturated, unsaturated, or methylated) were closely correlated with heart-to-body weight ratio in Fabry-B6/129 mice (Fig. 3C, Table 1). By contrast, in Fabry-B6 mice, there was no correlation between heart weight and any Gb3 species except C16:1 (Fig. 3C, Table 1). There was no correlation between total Gb3 and heart size.

The relationship between lyso-Gb3 analogs and disease phenotype

We also measured lyso-Gb3 and its analogs (−28, −2, +16, +18, +34, and +50) in the heart, kidney, and DRG. These analogs are lyso-Gb3 variants with modifications in the sphingosine moiety (Fig. 4A) (22–25). In the heart, levels of lyso-Gb3, lyso-Gb3 (−28), and lyso-Gb3 (−2) were similar between Fabry-B6/129 and Fabry-B6; however, analogs (+18) and (+34) were significantly higher in Fabry-B6/129 mouse heart compared with Fabry-B6 at both 12 and 18 months of age (Fig. 4B). There was no significant correlation between these GSLs and heart-to-body weight ratio (supplemental Fig. S5).

In the kidneys, lyso-Gb3 (Fig. 4C) and the analogs (−28, −2, +16, +18, and +34) (supplemental Fig. S6A) were similar between Fabry-B6/129 and Fabry-B6 mice. Due to the low abundance of lyso-Gb3 and the small size of mouse DRG, we pooled DRGs (≈100) collected from five mice in the same group (i.e., one analyte per group). Lyso-Gb3 was

**Fig. 3.** Comprehensive panel of Gb3 isoforms/analogs in 12-month-old mouse heart. A: Levels of Gb3 isoforms/analogs (21 species) in the heart tissues, grouped in four categories (n = 7–8). *P < 0.05, **P < 0.0001 determined via the t-test (Fabry-B6/129 vs. Fabry-B6). B: Levels of Gb3 isoforms/analogs in Fabry-B6/129 heart expressed as the ratio to Fabry-B6. C: Correlations between cardiac levels of C20:0- and C20:0-Me-containing Gb3 and heart weight.
markedly accumulated in 6-month-old Fabry mouse DRGs compared with WT controls (Fig. 4D). Lyso-Gb₃ appeared to be higher in Fabry-B6/129 DRGs than Fabry-B6 (Fig. 4D); however, the analogs were generally lower in Fabry-B6/129 (supplemental Fig. S6B). Thus, it is likely that levels of lyso-Gb₃ and its analogs in DRG are not different between the two strains of Fabry mice.

Cellular localization of Gb₃ in Fabry-B6/129 and Fabry-B6 mouse tissues

To exclude the possibility that Gb₃ is accumulated in different cell types in the two strains of Fabry mice, the cellular distribution of Gb₃ was analyzed by immunofluorescence staining in the heart, kidney, lumbar (L4/5) DRG, and liver from 12-month-old Fabry mice. No specific signal was detected in WT controls; in contrast, all Fabry mouse samples had distinct positive signals with typical punctuate lysosomal-like distribution (supplemental Fig. S7). No difference was found in Gb₃ localization in the heart, kidney, and DRG between Fabry-B6/129 and Fabry-B6. In the heart from both strains of Fabry mice, Gb₃ was detected in interstitial cells but not in cardiomyocytes (Fig. 5A). In the kidneys, specific signal was predominantly detected in tubular epithelial cells (Fig. 5B). In DRGs, Gb₃ was mainly detected in neurons (somas) and less extensively in nerve fibers and nonneuronal cells (Fig. 5C).

In the liver of both Fabry-B6/129 and Fabry-B6, Gb₃ was mainly detected in putative Kupffer cells (Fig. 5D). Among the tissues tested, the only cell type that had strain-dependent differences was hepatocytes. Gb₃ was detected in the hepatocytes in Fabry-B6/129 but not Fabry-B6 (Fig. 5E). However, the intensity of the positive signal in Fabry-B6/129 hepatocytes was much lower than that in putative Kupffer cells.

Triton was used in the majority of immunofluorescence staining presented in this study. To test whether the use of Triton altered Gb₃ localization pattern, we compared Gb₃ staining with or without Triton (see the Materials and Methods for more details). There was no difference in

### Table 1. Correlation (Pearson r) between Gb₃ isoforms and heart-to-body weight ratio

| Fatty Acid Chain Length | C16 | C18 | C20 | C22 | C24 |
|------------------------|-----|-----|-----|-----|-----|
| Saturated fatty acid   | 0.722 | 0.710 | 0.864 | 0.479 | 0.281 |
| Unsaturated (one extra double bond) | 0.691 | 0.799 | 0.769 | 0.321 | 0.601 |
| Unsaturated (two extra double bonds) | - | - | - | 0.547 | 0.449 |
| Methylated (saturated fatty acid) | 0.785 | 0.778 | 0.816 | 0.427 | 0.047 |
| Methylated (one extra double bond) | - | - | - | 0.551 | 0.773 |
| Hydroxylated (saturated fatty acid) | 0.828 | 0.221 | - | - | - |

Numbers are correlation coefficients (Pearson r); bold numbers are P < 0.05; others are not significant.

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**Fig. 4.** Lyso-Gb₃ and its analogs in the heart, kidney, and DRG. A: Sphingosine modifications of lyso-Gb₃ analogs. B: Lyso-Gb₃ and its analogs in heart tissues from 12- and 18-month-old mice (n = 7–8). C: Lyso-Gb₃ levels in the kidneys (n = 7–8). D: Lyso-Gb₃ levels in the DRGs from 6-month-old mice. Each group had one analyte (pooled ~100 DRGs from five mice).
Gb3 localization in the heart and kidneys between the two staining conditions (supplemental Fig. S8).

**Subpopulation-specific Gb3 accumulation and aberrant IB4 binding in Fabry mouse DRG neurons**

DRG neurons are highly diversified and are classified into three major groups according to the sensory modalities that they subserve: nociceptors, mechanoreceptors, and proprioceptors (29). Nociceptive sensory neurons are further divided into two subgroups based on their expression of neuropeptides and on their growth factor dependence (30). Compared with mechanoreceptors and proprioceptors, nociceptive neurons are of small diameter and give rise to thinly myelinated (Aδ) or unmyelinated (C) fibers. We found that Gb3 accumulation in Fabry mouse DRG neurons was highly heterogeneous, with some neurons strongly positive while others were weakly positive or negative. We double stained Fabry-B6/129 mouse DRGs for Gb3 and subpopulation markers, and found that Gb3 accumulation has a distinct subpopulation-specific pattern (Table 2). Almost all IB4-positive neurons were Gb3 negative (Fig. 6A). Most substance P-positive (marker of peptidergic nociceptors) neurons were Gb3 positive (Fig. 6B). Many (>50%) NF200-positive (marker of mechanoreceptors) neurons were Gb3 positive (Fig. 6C). In fact, neurons with the most massive Gb3 accumulation in DRGs were often NF200+. Almost all parvalbumin-positive (marker of proprioceptors) neurons were Gb3 negative (Fig. 6D). From the number and

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**Fig. 5.** Cellular localization of Gb3 in Fabry-B6/129 and Fabry-B6 mouse tissues. Gb3 was detected by immunofluorescence staining (red). A: Heart. Arrows, Gb3+ interstitial cells. Phalloidin (green) was used to label cardiomyocytes. (B) Kidney. Arrows, Gb3+ tubular epithelial cells. T, cortical tubules. Wheat germ agglutinin (WGA) was used to visualize the structure. (C) DRG. Arrows indicate Gb3+ neurons. PGP9.5, a pan-neuronal marker, was used to label neurons. A horizontal arrow in Fabry-B6/129 marks a PGP9.5-negative nonneuronal cell that is Gb3+. D: Liver. Arrows indicate Gb3+ putative Kupffer cells. Asterisks indicate nuclei of hepatocytes. Phalloidin was used to visualize the structure. E: Liver. Arrows indicate Gb3+ hepatocytes in Fabry-B6/129. Asterisks indicate nuclei of hepatocytes. Note that the intensity of Gb3-positive signals in hepatocytes was much lower than that in putative Kupffer cells. Longer exposure time was used to visualize Gb3 signals in hepatocytes. Scale bar: 20 μm (same for all panels).
intensity of Gb3-positive signals in the DRG sections, it appeared that Gb3 content in DRG homogenates measured with LC-MS/MS mainly reflected that in mechanoreceptors.

IB4 is the most widely used marker for nonpeptidergic nociceptors. IB4 selectively binds to glycoconjugates with terminal α-D-galactose. We found that IB4 binding was remarkably increased in Fabry-B6/129 mouse DRG neurons compared with WT controls (Fig. 7A). In contrast to punctate IB4+ signals in WT mice, Fabry mice exhibited granular signals that diffusely filled the entire cytoplasm (Fig. 7B). Because IB4 binds to α-D-galactose, theoretically, Gb3 and other substrates of α-gal A should be recognized by IB4. Indeed, intense Gb3+ signals in some neurons were weak IB4+ (Fig. 7C, arrows). However, most Gb3+ signals did not overlap with IB4, and conversely, strong IB4+ neurons (with diffuse cytoplasmic signal) were not Gb3+ (Figs. 6A, 7C). The relationship between IB4 and Gb3 was also examined in heart sections. In agreement with previous reports (31), IB4 selectively labeled capillaries in WT mouse heart. The pattern and intensity of IB4 binding in Fabry mouse heart were similar to those in WT mice (supplemental Fig. S9). Similar to the findings in DRG, IB4 did not label Gb3+ interstitial cells in Fabry mouse heart (Fig. 7D). These data suggested that IB4 has relatively low affinity to Gb3, at least under the histology conditions used in this study. Nevertheless, it is clear that the IB4-binding

### Table 2. Gb3 accumulation and morphological changes in DRG neuron subpopulations

| Subpopulations       | Physiological Properties | Findings in Fabry Mice |
|----------------------|--------------------------|------------------------|
|                      |                          | Gb3 Staining | Marker Staining and Cell Size |
| Nociceptors          | Small                    | IB4, Ret      | Gb3, negative | IB4-binding ↑, cell size ↑ |
| Nonpeptidergic       | Small                    | Substance P, CGRP | Many are Gb3+ | No obvious change |
| Peptidergic          | Large                    | NF200         | Strong Gb3+   | No obvious change |
| Mechanoreceptors     | Large                    | Parvalbumin   | Gb3, negative | No obvious change |

Fig. 6. Subpopulation-specific Gb3 accumulation in Fabry-B6/129 mouse DRGs. A–D: L4/5 DRGs from 12-month-old Fabry-B6/129 mice were double stained for Gb3 (middle column; positive signals in red or green color) and various sensory neuron markers (left column). The arrows mark examples of double-labeled cells. Scale bar: 100 μm (same for all panels).

Fig. 7. Morphological changes of IB4+ DRG neurons in Fabry-B6/129 mice. A: L4/5 DRGs from 12-month-old Fabry-B6/129 mice and WT controls were stained with IB4. The arrows mark IB4+ neurons in WT mouse DRG heart. The exposure time that allows IB4+ cells in WT to be visible (0.6 s) led to overexposure for IB4+ cells in Fabry mouse DRG, thus 0.3 s was also shown. B: Higher magnification shows punctate IB4+ signals in WT and the diffuse granular cytoplasmic signals in Fabry DRG neurons. IB4+ cells in Fabry mice were enlarged compared with WT mice. C: An example of a small number of Gb3+ DRG neurons labeled by IB4 (arrows, double positive signals). The asterisks mark IB4+ (diffuse cytoplasmic) neurons that are Gb3 negative. D: Heart section from 12-month-old Fabry-B6/129 mouse was double stained for Gb3 and IB4. IB4 did not label Gb3+ interstitial cell. Scale bars: 50 μm in A, 20 μm in B–D.
glycoconjugate in those intense IB4+ DRG neurons in Fabry mice is not Gb3.

In addition to increased IB4-binding, the size of IB4+ neurons in Fabry mice tended to be larger than in WT controls (Fig. 7B). In contrast to the small soma size of peptidergic neurons, the overall size of IB4+ neurons in Fabry mice was similar to that of mechanoreceptors and proprioceptors (Fig. 6A–D). There was no clear difference in marker expression and cell size between Fabry-B6/129 mice and WT controls in peptidergic nociceptor, mechanoreceptor, and proprioceptor neurons.

Although the increased IB4 binding in Fabry mouse DRGs is not due to the binding to Gb3, IB4 may bind to other substrates of α-gal A (e.g., iGb3) that might accumulate in Fabry mouse DRG neurons. Therefore, it was important to determine whether, in Fabry mice, IB4 is still a useful marker to distinguish nonpeptidergic nociceptors from other neuronal subpopulations. To this end, we double stained Fabry-B6/129 mouse DRGs with IB4 and subpopulation markers. The results showed that there was no overlap between intense IB4+ signal and other markers (substance P, NF200, or parvalbumin) (Fig. 8A–C). In addition, many IB4+ neurons in Fabry mice were also positive for Ret (another marker of nonpeptidergic nociceptors) (Fig. 8D). We further assessed the central projection pattern of IB4+ neurons. Axons of peptidergic and nonpeptidergic nociceptors terminate in the superficial laminae (lama I and outer lamina II) and inner lamina II in the dorsal horn of the spinal cord, respectively (32). The laminar distribution of IB4+ fibers and CGRP-positive (marker of peptidergic nociceptors) fibers in the Fabry mouse spinal cord was in agreement with this pattern, and there was no difference in distribution pattern between Fabry and WT mice (supplemental Fig. S10). Collectively, these data suggest that the neurons with intense IB4 labeling in Fabry mouse DRGs are nonpeptidergic nociceptors.

The main findings of Fabry mouse DRGs were summarized in Table 2. Fabry-B6/129 mice were used in these characterization studies (Figs. 6–8). However, we confirmed that the key findings were also present in Fabry-B6 mice (i.e., the markedly increased IB4-binding and the observation that these IB4+ neurons are Gb3 negative and have increased cell size).

**DISCUSSION**

From the pathophysiological point of view, Fabry disease is one of the most complex lysosomal disorders. Different substrates of α-gal A may have different cellular distributions because of their different biosynthetic pathways. Each substrate and its variants may have different biological functions or pathological effects. Therefore, in Fabry disease, each affected tissue may have different underlying disease mechanisms, determined by the type of storage material, cell type in which the substrate accumulates, and the basic cellular processes that are compromised by the storage. In this study, we showed novel findings regarding tissue- and cell type-specific distribution of Gb3/lyso-Gb3 species and their relationship with disease phenotypes in Fabry mouse models.

In the heart, C20-containing Gb3, but not the rest of the isoforms or total Gb3, had a strong correlation with cardiac hypertrophy. Also, only particular lyso-Gb3 analogs (+18 and +34) were increased in Fabry-B6/129 heart compared with Fabry-B6. These data suggest that particular species of Gb3 and lyso-Gb3 may play roles in the pathogenesis of cardiac hypertrophy. The changes of both Gb3 and lyso-Gb3 species were seen in the heart, but not in kidneys and DRGs, suggesting that these changes are not coincidental. These molecular species may also serve as better biomarkers compared with total Gb3 or lyso-Gb3. Consistently with this, a clinical study showed that urinary levels of lyso-Gb3 analogs (+16, +34, +50) were positively associated with the left ventricular mass index in Fabry patients with a late-onset IVS4+919G>A cardiac variant mutation (22).

The significant effect of fatty acid chain length on biological function of Gb3 has been suggested by investigations of verotoxins (33). Gb3 within lipid rafts functions as a receptor for verotoxins. The binding of verotoxins to Gb3 is markedly dependent on fatty acid chain length (26, 33).
Interestingly, similarly to the bell-shaped distribution in our study (Fig. 3B), the binding capacity of Gb₃ isoforms to verotoxin-1 increased with increasing fatty acid length, reaching maximum at C20, and declined for further longer fatty acid species (26). It is likely that fatty acid lengths of GSLs are an important factor in the organization of lipid rafts. Lipid rafts contain high concentrations of GSLs and cholesterol as well as a variety of membrane proteins involved in signal transduction (34). Some raft-associated proteins (e.g., GPI-anchored proteins, Src family kinases, and eNOS) partition into lipid rafts via fatty acid chains attached to these proteins (35, 36). It was suggested that acyl chains of GSLs participate in the localization of these proteins in lipid rafts in a chain length-dependent manner (37). In human neutrophils, LacCer with C24 (but not shorter chains) was specifically necessary for the association of the Src family kinase Lyn with lipid rafts (37). As one of the potential mechanisms, it was speculated that the long chain (C24) of LacCer inserted into the outer layer of the membrane might protrude in part into the cytoplasmic leaflet, and thus could interdigitate with fatty acid chains of Lyn anchored in the cytoplasmic leaflet of lipid rafts. Collectively, it appears that the composition of different isoforms of GSLs within the membrane and the preferential interactions between particular lengths of fatty acids of GSLs and membrane proteins (and cholesterol) are fundamental to the proper structure and functions of lipid rafts.

Accumulating evidence suggests abnormal composition and function of the plasma membrane in Fabry disease. Caveolar fractions from cultured Fabry mouse endothelial cells had increased Gb₃ and decreased cholesterol levels, and these changes were reversed after substrate reduction using an inhibitor of glucosylceramide synthase (D-β-EtDO-P4) (38). An in vitro study with a fluorescent analog of LacCer suggested that intracellular trafficking of GSLs and cholesterol homeostasis are disrupted in Fabry disease and other glycosphingolipidoses (39). In another study, despite the elevated tissue Gb₃ levels, Fabry mice were less, not more, sensitive to verotoxins than WT mice (40), suggesting that the function of Gb₃ within lipid rafts is compromised in Fabry mice. Our previous study showed that the clearance of accumulated Gb₃ in both plasma membrane and lysosomes (but not depletion of lysosome-accumulated Gb₃ alone) could restore the tetrahydrobiopterin deficiency in cultured Fabry patient endothelial cells (41), suggesting that the Gb₃ fraction accumulating in plasma membrane may be more directly relevant to the pathogenesis than lysosomal accumulation. Taking these observations together, we hypothesize that, in the Fabry-B6/129 mouse heart, the increased content of C20-containing Gb₃ in lipid rafts may affect proper localization of membrane proteins, especially those that utilize fatty acids for their membrane targeting, thus leading to aberrant signaling pathways and subsequent cardiac hypertrophy. eNOS is one of the candidates that might be involved in this disease process. eNOS is dually acylated by myristoylation and palmitoylation and is targeted to caveolae through these fatty acid moieties (36). Decreased expression, activity, and dimerization of eNOS in Fabry mouse arteries and the heart have been reported (41–43).

Further studies are needed to investigate the potential link between Gb₃ isoforms and abnormalities in eNOS and other membrane proteins. Immunostaining suggests that most of the Gb₃ in mouse heart homogenates comes from perivascular cells and fibroblasts. It remains unclear what cell types and cellular compartments (lipid rafts vs. lysosomes) contribute to the differential Gb₃ and lyso-Gb₃ profiles between the two strains of Fabry mice. LC-MS/MS analysis of subcellular fractions should be conducted to further answer the question.

In contrast to the heart, there was no difference in the levels of Gb₃ and lyso-Gb₃ species in the kidneys and DRG homogenates between the two strains of Fabry mice, despite the significant difference in severity of renal hypertrophy and neuropathic phenotype. Although we cannot rule out the potential effects of other genetic and epigenetic factors on these phenotypes, our data suggested that the pathogenic role of GSL species is highly tissue-specific. The disconnection between Gb₃ accumulation and functional abnormality was further suggested by immunohistochemistry findings of sensory neuron subpopulations.

The neuropathy in Fabry disease is a length-dependent small-fiber neuropathy primarily affecting thinly myelinated Aδ fibers and unmyelinated C fibers (pain and temperature perception), while large myelinated Aα and Aβ fibers (position and vibration sensation) are generally preserved (44, 45). IB4+ sensory neurons are C-fiber nociceptors that sense noxious heat stimuli (50, 46). In agreement with these, the significant morphological changes of IB4+ neurons found in our study suggest that nonpeptidergic nociceptors are affected. The increased IB4-binding and swollen cell body were present in both strains of Fabry mice and were also reported in a rat model of Fabry disease (47), suggesting that these are strong and reproducible phenotypes. The increased size of IB4+ neurons may also contribute to the enlarged DRG size in mice (as shown here) and patients (48) with Fabry disease. It has been shown that the transient receptor potential ankyrin 1 (TRPA1) is sensitized in Fabry rat sensory neurons (47). TRPA1 is functionally expressed primarily in IB4+, CGRP-negative small neurons in both mouse and rat (49), further suggesting that nonpeptidergic nociceptors are functionally affected in Fabry disease.

Strikingly, however, IB4+ nonpeptidergic nociceptors had no Gb₃ accumulation. On the other hand, although both mechanoreceptors and proprioceptors are not affected functionally, the former had massive Gb₃ accumulation, while the latter was completely Gb₃ negative. Thus, there seems to be no apparent correlation between Gb₃ accumulation and functional abnormalities in these cell types, suggesting that other substrates of α-gal A may play pathogenic roles in DRGs.

In this context, iGb₃ is of particular interest. Previous studies suggested that iGb₃ distribution is limited to DRG neurons. In WT mice, DRG was the only tissue where iGb₃ could be detected; and relative to WT mice, iGb₃ was significantly accumulated in Fabry mouse DRG, but not in other tissues (5). Moreover, lysosomal inclusions (under electron microscopy) in Fabry mouse DRG neurons were cleared.
only when Gβ3 and iGb3 were simultaneously depleted (by crossing Fabry mice with the mice deficient for both Gb3 and iGb3 synthases), but not by depletion of Gb3 alone (50). In contrast, in the heart, kidney, and liver, the lysosomal inclusions were eliminated by depletion of Gb3 alone. It is therefore possible that iGb3 may be selectively accumulated in nonpeptidergic nociceptors, leading to pathological changes of this subpopulation.

Another important aspect that needs to be considered is the identity of IB4-binding material in Fabry mouse DRG neurons. The identification of IB4-binding material that distinguishes nonpeptidergic neurons from other subpopulations remains elusive (that can be glycolipids, glycoproteins, or oligosaccharides); however, it has been suggested that IB4-binding material can be critical for biological functions of these nociceptors (46, 51). Thus, the markedly increased level of IB4-binding material in Fabry mice may relate to the pathogenesis of the small-fiber neuropathy in this disease. We showed here that IB4-binding material in Fabry mouse DRG is not Gb3. Also, from the diffuse cytoplasmic distribution of the IB4 signal, which differs from the typical lysosomal storage pattern, the increased IB4 signal in Fabry mouse DRG is not likely due to IB4 binding to other α-gal A substrates accumulated in the lysosomes. One possibility is that the IB4-binding material is increased in Fabry mice as a secondary storage for α-gal A substrate deposit. Many lysosomal storage disorders exhibit secondary accumulation of compounds in lysosomes or nonlysosomal compartments, which cannot be explained by the underlying enzymatic defect. As an example, in MPSIIIA mouse brain, lysosomal accumulation of heparan sulfate is associated with storage of Gα2 and Gα3 gangliosides in lipid rafts (52, 53). Miller et al. (47) found significantly changed protein N-glycan profile in Fabry rat DRGs relative to WT controls. In future studies, analysis of glycolipids, glycoproteins, and oligosaccharides in IB4+ neurons enriched from dissociated DRGs (instead of whole DRG homogenates) from Fabry mice will likely facilitate the identification of the IB4-binding material and/or other compounds responsible for cellular damage of nonpeptidergic nociceptors.

The different cardiac Gb3 isoform profile between two strains of Fabry mice is not likely due to the intrinsic difference in the synthesis in different genetic backgrounds because there was no difference in the isoforms between two strains of WT mice. However, storage of certain lipids can affect the metabolism of other lipids. For example, accumulated GlcCer in Gaucher disease increases synthesis of phosphatidylcholine through activation of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in phosphatidylcholine synthesis (54). Thus, a potential explanation is that the primary enzyme deficiency and GSL storage in Fabry mouse heart might have affected particular ceramide synthases, and mice in different genetic backgrounds may have different susceptibility to this alteration. If this is the case, it is possible that the different fatty acid profile, i.e., the higher C20 in Fabry-B6/129 than in Fabry-B6 shown in the present study (Figs. 2, 3), may also exist in other relevant GSLs (e.g., GlcCer, LacCer, and their derivatives). This may provide another explanation for the lack of connection between Gb3 isoforms and disease severity in kidney and DRGs; the potentially altered isoform profile in GSLs other than Gb3 may play pathogenic roles in these organs, through aberrant GSL composition in lipid rafts. Further studies should be conducted to analyze wider arrays of GSLs and gene expression in these two Fabry mouse strains. If the secondary changes in GSL biosynthesis are identified (e.g., specific ceramide synthases), these pathways can be manipulated in animal and cellular models to further elucidate the pathogenic roles of particular GSL isoforms in Fabry disease.

In conclusion, our findings provide new insights into the pathophysiology of Fabry disease. First, certain Gb3 and lyso-Gb3 species with particular fatty acid lengths and sphingosine modifications, but not total Gb3 or lyso-Gb3, correlate with cardiac hypertrophy, suggesting that minor storage compounds may play major roles in the disease pathogenesis, and that total Gb3 or inclusion bodies may not always be considered as pathogenic just because of their large quantities. Second, the relationship between GSL species and disease phenotype can be highly tissue- and cell type-specific. Our data do not support the relevance of Gb3 and lyso-Gb3 in the renal and neuropathic phenotypes in Fabry mice, suggesting that other substrates may play pathogenic roles in these tissues. Third, the accumulated IB4-binding material in DRG neurons might be an important mediator in the pathological cascade of neuronal dysfunction, and identification of this material may provide a clue for unraveling the molecular basis of peripheral neuropathy in Fabry disease.

Data availability
All data are contained within the article and supplemental data.

Acknowledgments
The authors are grateful to Waters Corporation for their continued scientific support and partnership and to Marie-Anne Schiffmann for proofing and editing the manuscript.

Author contributions
S.-T.-T., M.B., T.S.D., and M.T. methodology; R.S. and C.A.-B. writing-review and editing; C.A.-B. and J.-S.S. formal analysis; J.-S.S. supervision; J.-S.S. conceptualization; J.-S.S. writing-original draft.

Funding and additional information
This work was supported by Baylor Scott & White Research Institute.

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
ANP, atrial natriuretic peptide; B6, C57BL/6; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; ERT, enzyme replacement therapy; α-gal A, α-galactosidase A; Gb3, galabiosylceramide; Gb2, globotriaosylceramide; Gb3, globotriaosylceramide; GSL, glycosphingolipid; IB4, isolectin B4; iGb3, isoglobotriaosylceramide; lyso-Gb3, globotriaosylsphingosine; PBST, 0.3% Triton X-100 in PBS.
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