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Rebecca Anderson April 12, 2020

Tissue-Specific Analysis of UDP-sugar Levels and Ratios in a Rat Model of Classic Galactosemia

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Classic galactosemia (CG) is a rare, autosomal recessive disorder that results from deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir pathway. Despite improved survival outcomes in neonates following newborn screening and rapid dietary restriction of galactose, a variety of long-term complications continue to persist in many treated patients, including speech delay, cognitive delay, growth delay, and primary ovarian insufficiency within females. One proposed explanation for the long-term outcomes associated with the disease is the perturbation of UDP-sugar pools hypothesized to underlie alterations of glycosylation. However, conflicting reports of how UDP-sugar levels and ratios are impacted by CG, as well as a lack of studies in primary tissues, have made answering this question difficult. Recently, our lab reported a GALT-null rat model that accurately reflects many important long-term phenotypes associated with CG. In the current study, we quantified levels of UDP-sugars in liver and brain homogenate samples from ten-day old GALT-null rat pups as well as controls. Our results indicate that UDP-sugar levels and ratios vary across tissue type as well as genotype. Despite the small sample size limiting the statistical power of this study, our results clearly establish the importance of UDP-sugar analysis in understanding CG, as well as highlight the need for further study within this area of research. In the future, we hope to expand this study to include red blood cells (which are clinically significant due to their use in diagnosis within human patients) and an adult rat cohort in order to test for age and diet-dependent differences.

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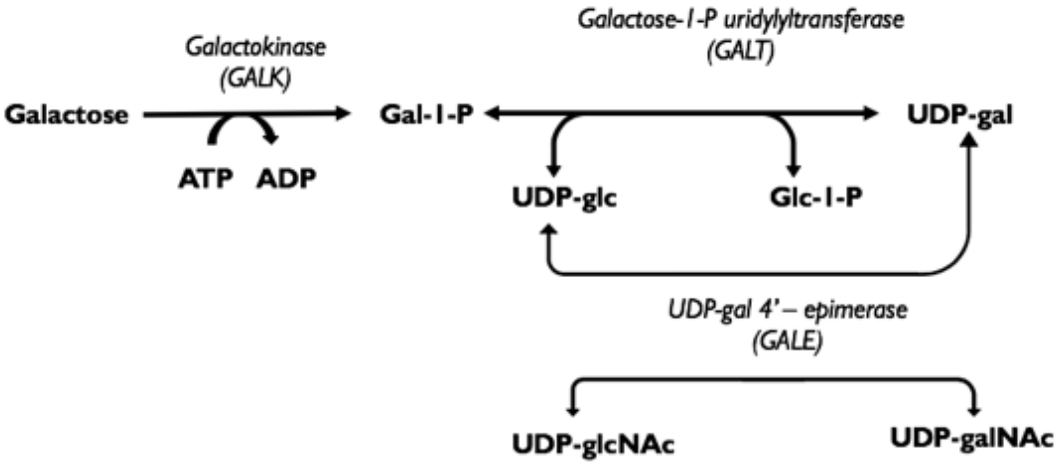
Chapter 1: Introduction to Galactose Metabolism and Classic Galactosemia

Galactose Metabolism and Classic Galactosemia (CG)

Galactose, one of the two monosaccharides that constitute lactose, and its derivatives, have many important biological functions. These functions include, but are not limited to, serving as structural building blocks of glycosylated proteins, extracellular matrix, and the cell membrane[1,2]. Galactose is normally metabolized in three reaction steps via the Leloir pathway. First, galactose is phosphorylated using ATP by galactokinase (GALK), forming galactose-1-phosphate (Gal-1-P). In the second reaction, galactose-1-phosphate uridylyltransferase (GALT) catalyzes the conversion of Gal-1-P plus UDP-glucose to UDP-galactose and glucose-1-phosphate (Glc-1-P) via a ping pong mechanism. Finally, UDP-galactose is converted to UDP glucose by UDP-galactose 4' epimerase (GALE) (see Figure 1). Much of the Glc-1-P produced through this pathway is used to create glucose in the liver[1,3,4].



1. Figure Galactose metabolism via the Leloir pathway. Enzymes are depicted in italics, while substrates and products are bolded.

Classic galactosemia is an autosomal recessive, metabolic disorder that is characterized by the deficiency of GALT, the second enzyme of the Leloir pathway [5–7]. The disorder has an incidence rate of approximately 1 in every 50,000 live births in the United States[8]. Classic galactosemia presents clinically within the first few days of life in infants who are breastfed or fed a lactose-containing formula. Specifically, following exposure to milk, and therefore high levels of dietary galactose, affected infants experience a rapid progression of acute symptoms that include vomiting, failure to thrive, cataracts, *E. coli* sepsis, and potentially neonatal death[5]. Fortunately, these acute symptoms can be prevented or reversed by rapid dietary restriction of galactose, generally achieved by switching the baby from breast milk or a milk-based formula to soy-based or another low-galactose formula[2].

Additionally, classic galactosemia is detectable by newborn screening (NBS), based on a coupled assay detecting enzyme activity of GALT[8]. Newborn screening which enables early diagnosis and intervention, sparing most screened infants from developing the acute symptoms associated with the disease. Despite the reduction or prevention of acute symptoms following dietary galactose restriction, however, most affected individuals still grow to face a wide array of long-term complications, including speech delay, cognitive delay, motor function problems, and primary ovarian insufficiency in females[9–14].

Mixed Prior Literature on Altered UDP-sugars in CG and Implications for Glycosylation

One of the leading hypotheses for the cause of these long-term complications is the buildup of galactose metabolites in blood and tissues due to the blocked Leloir pathway. For example, a deficient GALT enzyme results in the accumulation of chronically elevated levels of

gal-1-p and galactose despite restricted diets. These metabolites are thought to result from endogenous production of galactose in cells, which can also be converted to galactonate or galactitol via alternate pathways (See Figure 2) [1,15–17]. It has also been hypothesized that perturbations in UDP-sugar levels may contribute to the long-term complications associated with CG.

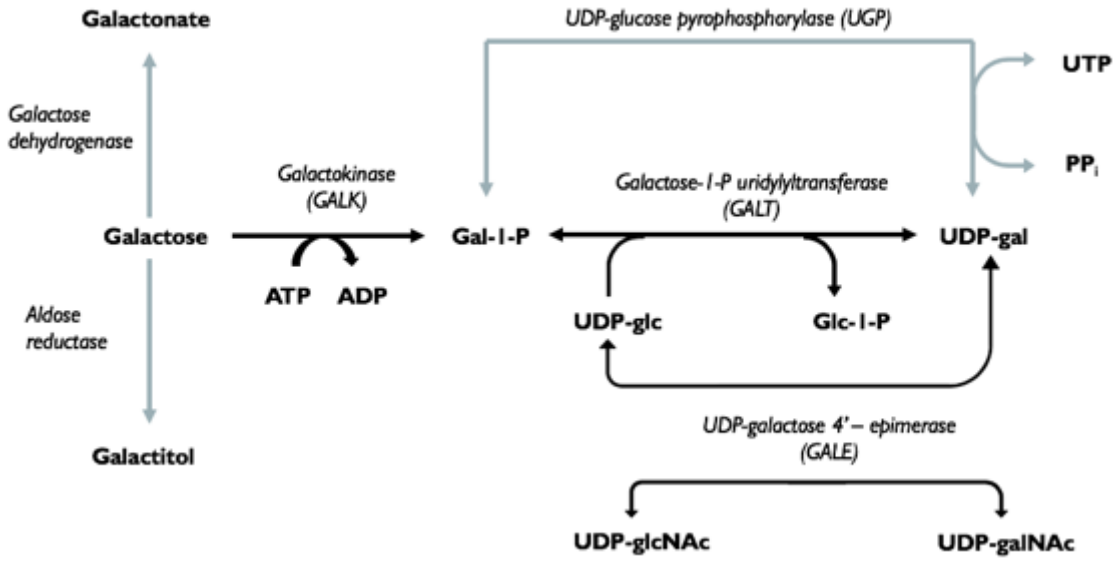


Figure 2. Alternative pathways of galactose metabolism, in blue. Enzymes are depicted in italics, while substrates and products are bolded.

Classic galactosemia has been classified as a congenital disorder of glycosylation (CDG), specifically due to a defect in the N-glycosylation pathway associated with the disease, where glycans are attached to the nitrogen atom of asparagine residues of proteins[18]. It has been reported that over half of all proteins in the human body are N-glycosylated [19,20], making N-glycosylation extremely essential for proper biological functioning. The glycoproteins that result from the process have been linked to a wide array of important biological processes, including

cell-cell signaling, immunity, and fertility[21], which are directly related to outcomes of CG. One of the many roles served by UDP sugars in cells is as a substrate donor for the glycosyltransferase enzymes involved in glycosylation. Altered UDP-sugar concentrations and subsequent impact on glycosylation have therefore been posited as potential contributors to long-term complications of classic galactosemia.

Unfortunately, however, it remains unclear exactly how UDP-sugar levels are affected by the disease due to conflicting reports in the literature. For example, one study reported significant reductions in UDP-galactose levels and significantly higher ratios of UDP-glc/UDP-gal than the expected 3:1 ratio in red blood cells of galactosemic patients [22]. However, a different study reported that neither UDP sugar levels nor ratios were perturbed in leukocyte or fibroblast patient cell lines[23]. The lack of consistent results from studies of cells in culture, or available samples from patients (e.g. blood), which are extremely limited, indicates the need for analysis of other, more relevant tissue types, such as brain and liver, which are associated with the chronic negative outcomes of the disease. Until recently, this was out of reach due to the inaccessibility of these tissues from patients and the lack of an appropriate mammalian model for GALT deficiency.

Rat Model of Classic Galactosemia

Prior to the creation of our GALT-null rat, a variety of models were used to study galactose metabolism and the consequences of GALT deficiency, including accessible patient samples, cultured cells, fish, invertebrates, and mice[24]. The GALT-null mouse was created twice.

The first GALT-null mouse showed no representative phenotypes[25], while the second displayed some characteristic phenotypes after dietary galactose intoxication[26,27]

Recently, we created and characterized GALT-null rat model using CRISPR-Cas9 gene editing; this model recapitulates many of the long-term phenotypes observed in patients with classic galactosemia, such as cataracts, cognitive defect, motor disturbance, and growth delay with no dietary galactose manipulation[28]. Because of its accurate representation of these complications associated with classic galactosemia, our model is an excellent candidate for detailed metabolic analysis of relevant tissues.

In this study, we aimed to quantify the levels and ratios of the four UDP sugars directly impacted by enzymes of the Leloir pathway, UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc. Metabolite levels were measured in brain and liver tissues of GALT-null rats as well as controls. In doing so, we expected to define whether or not the levels and/or ratios of these UDP hexoses were significantly altered by GALT-deficiency within and between relevant tissues. Altered absolute metabolite levels could be a mechanism underlying altered glycosylation within the respective tissue, while altered ratios could indicate perturbation of the GALE enzyme.

Chapter 2: UDP-sugar levels and ratios are perturbed on a tissue-specific basis in GALT-null rats.

Methods

Rat Model and Samples

The rat model used was previously described[28]. Briefly, a 2-base pair insertion was made in exon 6 of the rat *Galt* gene via CRISPR-Cas9 gene editing with non-homologous end joining. Genotypes were confirmed via DNA analysis and enzyme assays.

For the purpose of these experiments, ten nursing rat pups of both mutant (M3/M3) and wildtype (WT/WT) genotypes were analyzed. The mutant and wildtype rat cohorts were each comprised of two litters from different parents, with sample sizes of n=5 (wildtype) and n=4 and n=6 (mutant). Both mutant and wildtype cohorts contained a uniform distribution of males and females, and all pups were euthanized for tissue harvest at ten days after birth (Table 1).

TABLE 1. DESCRIPTION OF RAT COHORTS BY GENOTYPE

	WT/WT	**M3/M3**
SAMPLE SIZE	10	10
NUMBER OF INDEPENDENT LITTERS	2	2
AGE AT EUTHANASIA (DAYS)	10	10

Euthanasia, Tissue Collection, and Blood Processing

Ten-day-old pups were euthanized by isoflurane inhalation and exsanguination. Specifically, after euthanasia, each deceased pup was sprayed with 70% ethanol and a U-shaped incision was introduced to expose the abdominal cavity. Blood was collected from the inferior vena cava into a sodium heparin BD Vacutainer tube (#366480) that was inverted 8-10 times to disperse the anticoagulant and stored on ice until processing.

Blood was processed as follows. First, samples were transferred from vacutainer to microfuge tubes and spun in an Eppendorf 5415D centrifuge at 2000 x *g* for 15 minutes at 4°C to separate plasma from red cells. Plasma was removed, aliquoted, and stored at -80°C until use. The remaining red blood cells (RBCs) were washed with a 1:1 volume of 1X PBS (Corning #21-040-CV) and then aliquoted and stored at -80°C until use.

Tissues to be collected for metabolite analyses were removed, cut with a razor blade into small pieces, flash frozen on dry ice, and stored at -80°C until use. The following solid tissues were collected from each animal: liver and brain homogenate. Brain homogenate, which included a combination of cortex, hippocampus, and cerebellum from each animal, was used instead of individual brain parts due to small size of the brain in the young pups.

Metabolite Extraction and Quantification

The metabolites relevant to the present study included: gal-1-p, galactitol, galactose, glc-1-p, as well as the four UDP-sugars: UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc. In order to quantify these metabolites, multiple 100 mg tissue samples were processed for each animal and combined in order to make the final samples more concentrated. The total weight needed to properly concentrate the samples was optimized for both tissue types. Specifically,

three-100 mg pieces of liver were combined to give a total weight of approximately 300 mg, while five-100 mg samples of brain homogenate were combined, totaling approximately 500 mg.

Tissue samples were ground in 125 µL of ice-cold HPLC-grade water using a micropestle. Extraction took place in chloroform and methanol following a procedure previously described by our lab[29]. Before completion of drying, the metabolite extracts from replicate tissue pieces were combined together to create more concentrated samples for each tissue. Upon completion of extraction, drying, and rehydration, the samples were diluted as follows. Liver samples were diluted either 1:1 or 1:2 with HPLC-grade water to allow for optimal visualization of UDP-sugars, while brain homogenate samples were run undiluted. For analysis of gal-1-p, glc-1-p, galactitol, and galactose, brain homogenate samples were diluted 1:10 and liver samples were diluted 1:50 with HPLC-grade water. Following dilution, the final samples were run on a Dionex HPLC to separate and quantify metabolite levels.

Galactose, galactitol, and gal-1P were separated and quantified as described previously[28]. UDP-galNAc, UDP-glcNAc, UDP-gal, and UDP-glc were separated on a CarboPac PA10 column attached to a Dionex ICS-5000 system. The following buffers were used for separation: Buffer A, 15 mM NaOH, and Buffer B, 50 mM NaOH/1M NaOAc. The program utilized an isocratic procedure with a flow rate of 0.5 mL/min and buffer concentrations of 45% A and 55% B (0–61 min), followed by washing with a linear increase of B to 95% (61–80 min). There was also a five minute pre-equilibration at 45% A and 55% B before each sample was injected (-5-0 min). For all samples, 20 µl sample were injected into a 25 µl injection loop. The metabolites were quantified by comparing integrated peak areas to standards using

Chromeleon software. Metabolite values were standardized to total tissue mass of each sample, yielding a final measure of pmol metabolite/mg tissue.

Statistical Analyses

Metabolite levels were compared between the two genotypes within each tissue type. We tested for normality using the Shapiro-Wilk Normality Test and for equal variances using Fischer's F-test. Two-sample t-tests were performed to determine if any observed differences were significant in normally distributed data sets with equal variances, while the Mann-Whitney U test was used for analyzing data sets that were not normally distributed or had unequal variances. The Mann-Whitney U test is a non-parametric test that compares datasets based on ranked-sums, in which values in each dataset are ranked from the smallest value to the largest, summed, and compared to an expected rank-sum. Mean and standard deviation were reported measures of central tendency and spread in normal distributions, while the median and inter-quartile range (IQR) were used to describe those that were not normally distributed.

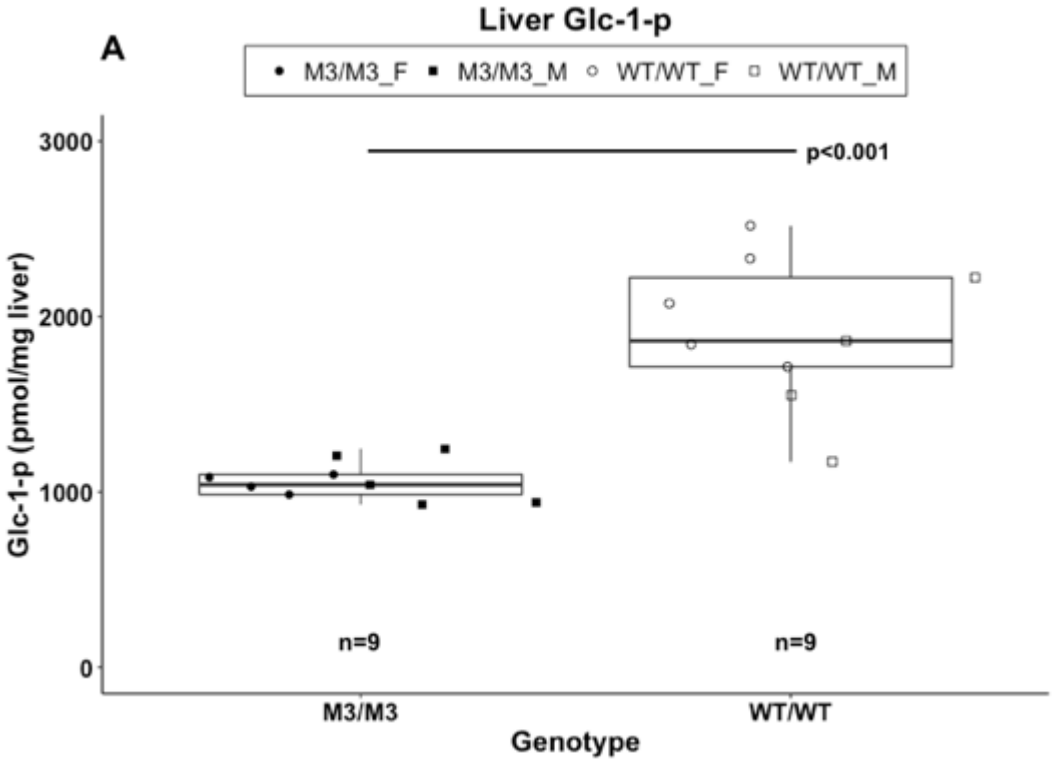
UDP-sugar relationships were analyzed both between litters within a given genotype as well as between the WT/WT and M3/M3 genotypes overall. When comparing between litters, a significance value of $\alpha = 0.025$ was used to account for multiple comparisons. No WT/WT litters showed litter-dependent differences in metabolite levels; however, in the case of a significant difference between animals of the two M3/M3 litters, each M3/M3 litter was compared separately to the WT/WT animals. When both M3/M3 litters were compared separately, an $\alpha = 0.025$ was used to account for multiple comparisons. When no difference

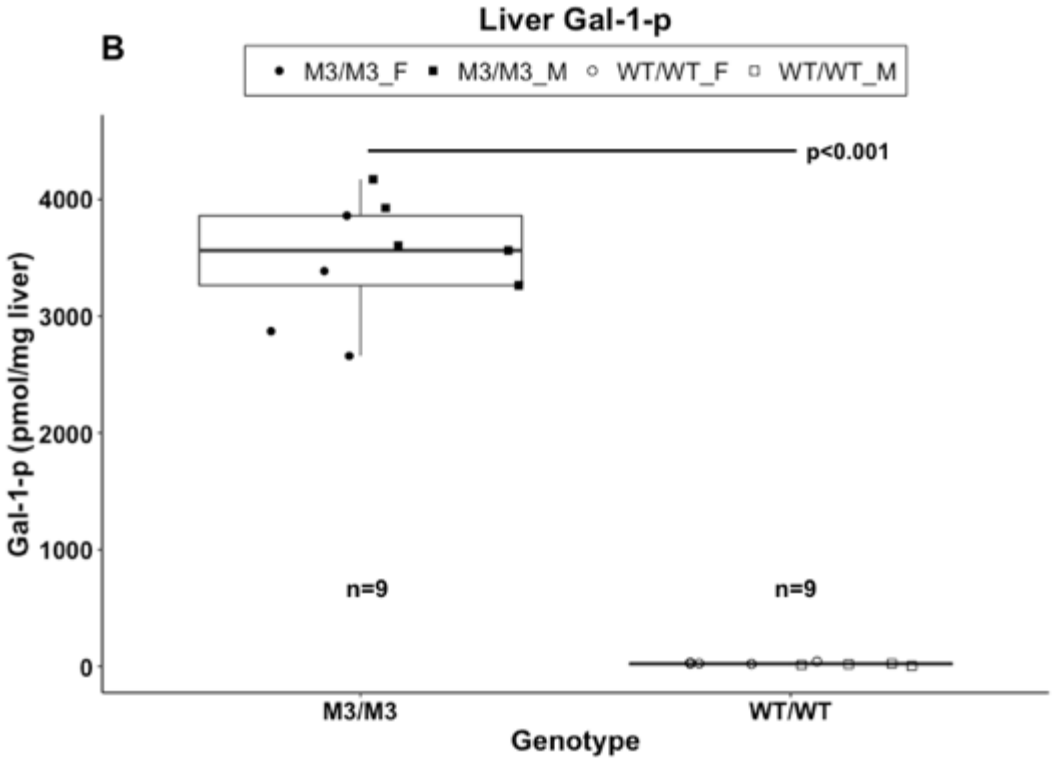
between M3/M3 litters was found, all M3/M3 animals were compared to WT/WT animals using $\alpha = 0.05$.

Results

Galactose metabolites

The galactose, galactitol, and gal-1-p metabolite levels seen in this study resembled the general profiles previously observed in our rat model[28]. As expected, liver gal-1-p levels were extremely elevated in mutants, with an average concentration of 3,478 pmol/ mg liver, compared to 22.8 pmol/mg liver in wildtype rat livers (Table 2). Likewise, Gal-1-p levels in the brain were markedly increased in mutants, however, overall brain gal-1-p levels were much lower than in the liver, regardless of genotype (Table 2). Boxplot analysis showed virtually no overlap between mutant and wildtype gal-1-p distributions in either of the tissues analyzed (Figures 3B and 4B).


A
Liver Glc-1-p
M3/M3_F
M3/M3_M
WT/WT_F
WT/WT_M
p<0.001
Glc-1-p (pmol/mg liver)
3000
2000
1000
0
n=9
n=9
M3/M3
WT/WT
Genotype


B
Liver Gal-1-p
M3/M3_F
M3/M3_M
WT/WT_F
WT/WT_M
p<0.001
Gal-1-p (pmol/mg liver)
4000
3000
2000
1000
0
n=9
n=9
M3/M3
WT/WT
Genotype

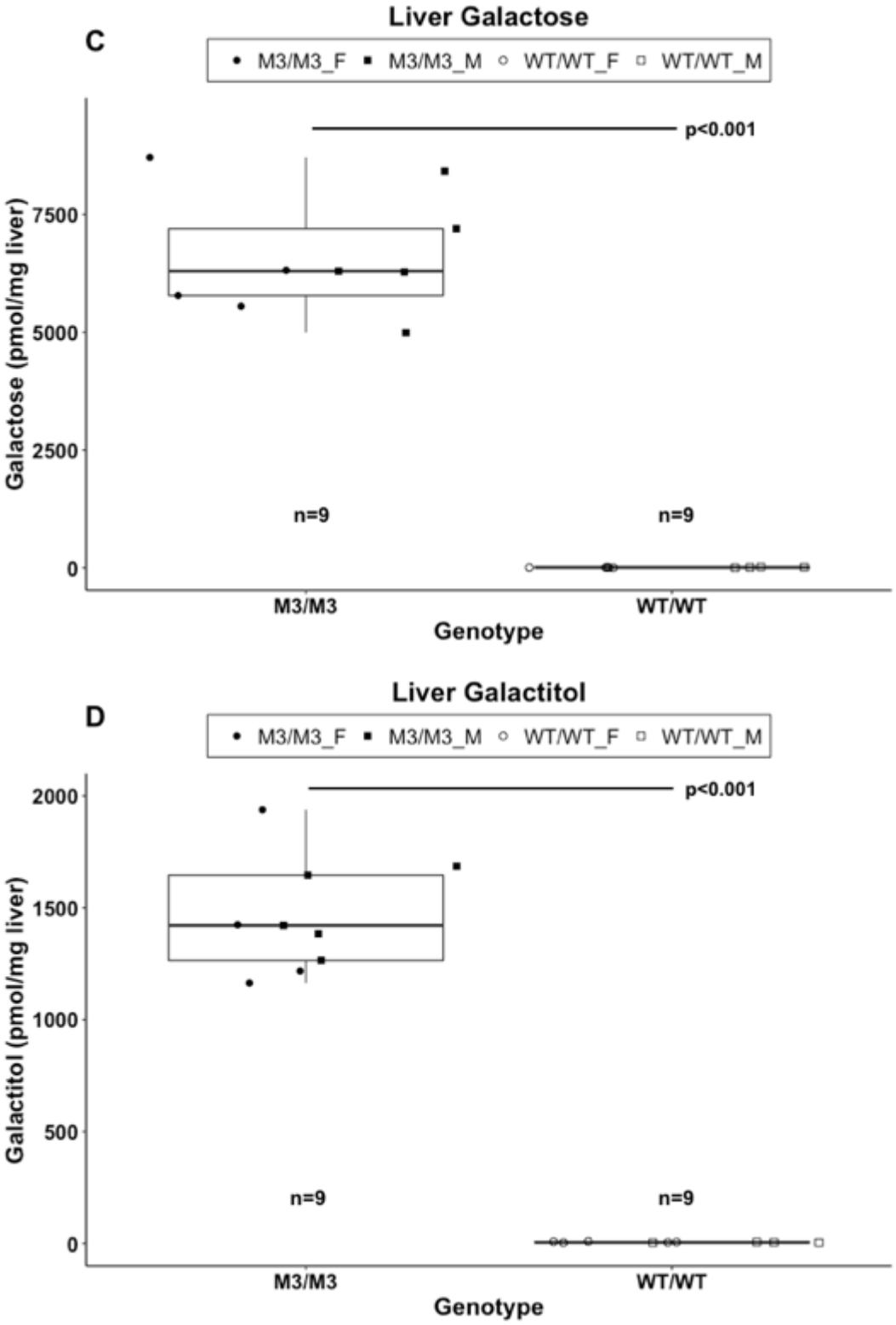
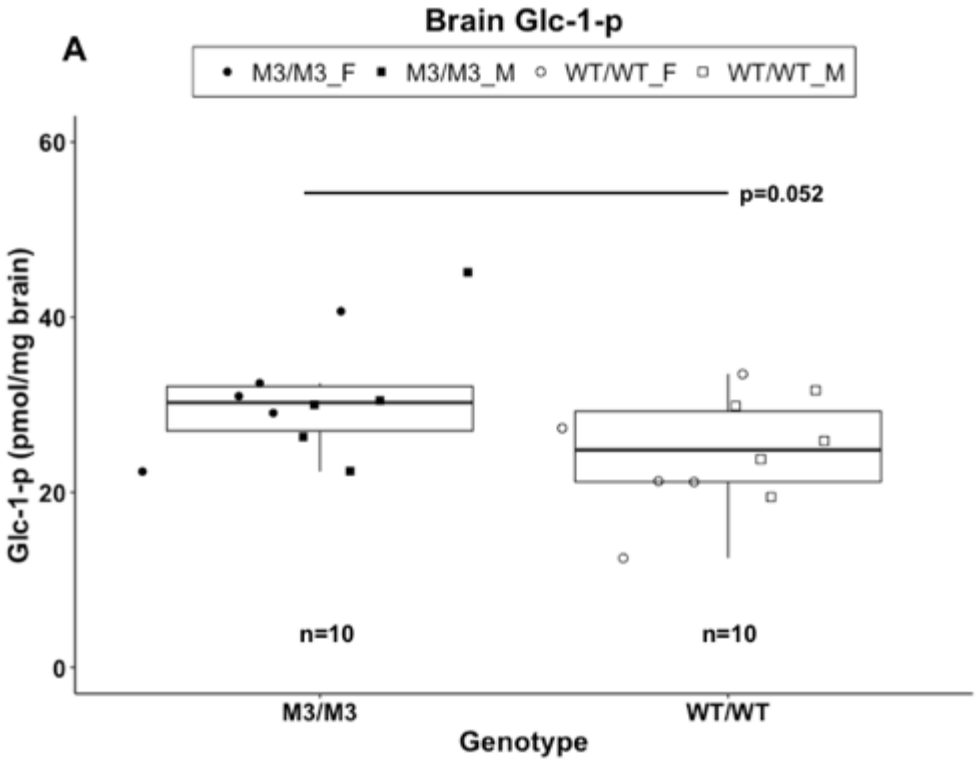
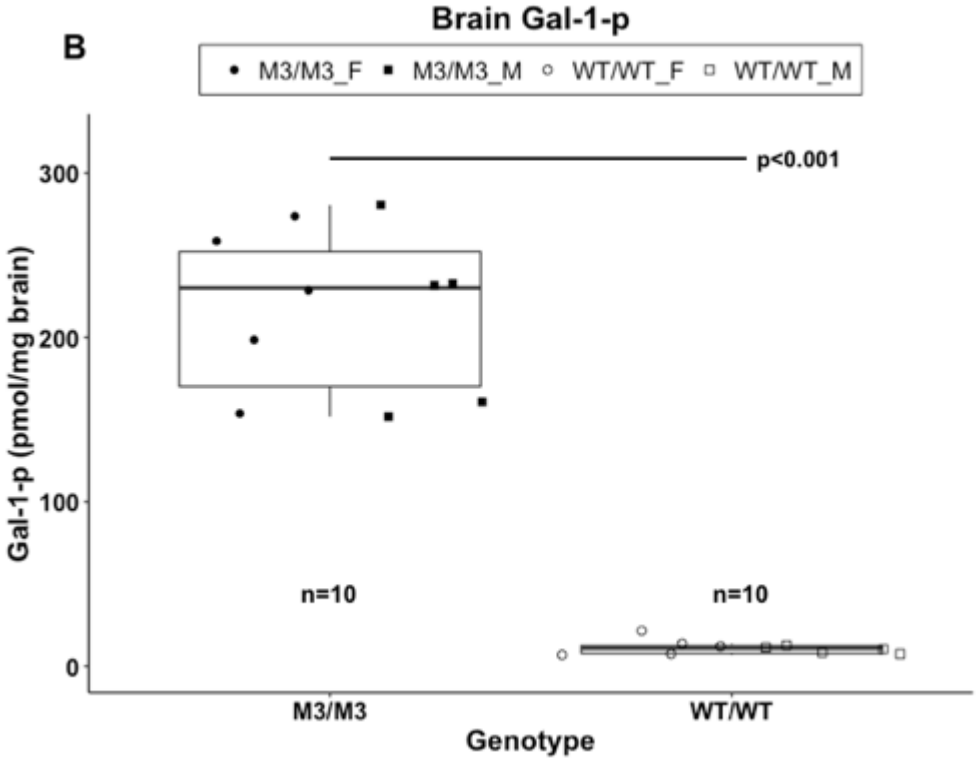


Figure 3. Differences in rat liver glucose-1-phosphate, galactose-1-phosphate, galactitol, and galactose levels, by genotype. Mann-Whitney U tests were conducted for all comparisons, α =0.05. Gal-1-p, galactitol, and galactose were significantly elevated in mutant pups, confirming previous observations[28]. N=9 instead of n=10 due to loss of sample. A, Liver glc-1-p. B, Liver gal-1-p, C, Liver galactitol. D, Liver galactose.


A
Brain Glc-1-p
M3/M3_F
M3/M3_M
WT/WT_F
WT/WT_M
p=0.052
Glc-1-p (pmol/mg brain)
60
40
20
0
n=10
n=10
M3/M3
WT/WT
Genotype


B
Brain Gal-1-p
M3/M3_F
M3/M3_M
WT/WT_F
WT/WT_M
p<0.001
Gal-1-p (pmol/mg brain)
300
200
100
0
n=10
n=10
M3/M3
WT/WT
Genotype

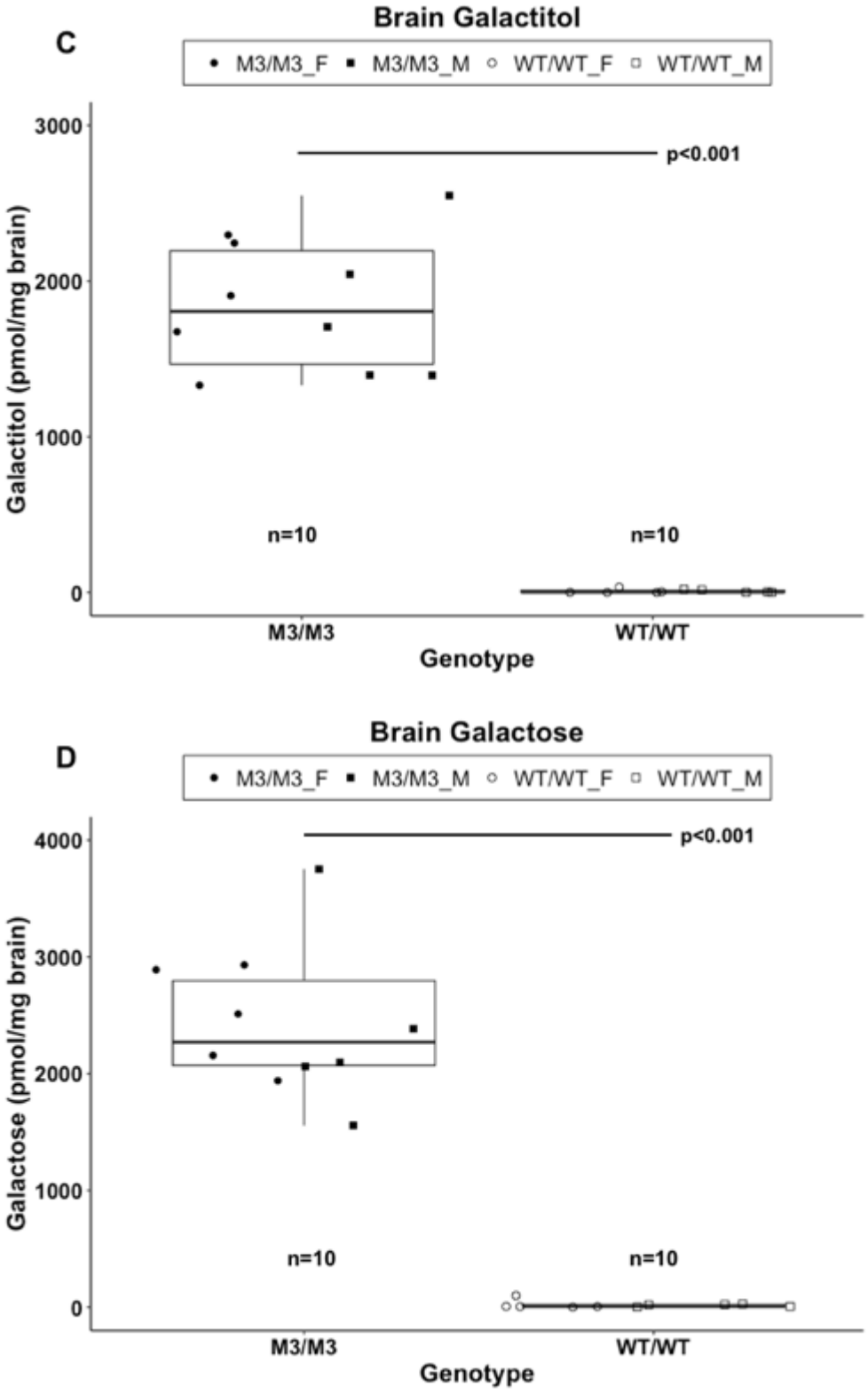


Figure 4. Differences in rat brain homogenate glucose-1-phosphate, galactose-1-phosphate, galactitol, and galactose levels by genotype. Trends seen in B, C, and D confirm previous observations[28]. Unless otherwise noted, Mann-Whitney U tests were conducted for comparisons, α =0.05. A, Brain Glc-1-P levels were analyzed by a two-sample t-test , α =0.05. B, Brain Gal-1-p. C, Brain galactitol. D, Brain galactose.

Table 2. Summary statistics and tests for metabolite concentrations (pmol/mg tissue) for M3/M3 and WT/WT rat brain homogenate and liver tissues.

	Brain measure of center* (measure of spread)**		**Liver measure of center* (measure of spread)****	
Metabolite	**M3/M3**	**WT/WT**	**M3/M3**	**WT/WT**
Glc-1-p	30.99(7.23)	24.64(6.35)	1062.22(109.90)	1921.10(416.734)
Gal-1-p	217.16(48.80)	11.26(4.36)	3478.76(493.08)	22.81(10.71)
Galactitol	1806(728) †	2.426(13.106) †	1461(252)	6.01(2.05)
Galactose	2271(725) †	6.886(17.132) †	6616(1263)	9.04(4.73)

***Mean or Median reported as measures of center for each group based on normality of distribution. ** Standard deviation (SD) or interquartile range (IQR) reported as measures of spread for each group based on the normality of distribution. Median(IQR) is reported for non-normal distributions and is marked with †.**

Substantial increases in both galactose and galactitol levels were also observed. Brain and liver galactose and galactitol levels increased significantly in mutants compared to wildtypes, with no overlap between the genotype distributions (Figure 3C & Figure 3D; Figure 4C & 4D). Significant differences were detected in liver galactitol level between the two wildtype litters, however, this did not impact the overall relationship between the two genotypes (data not shown). These results confirm the clear distinctions between mutant and wildtype galactose, galactitol, and Gal-1-p levels in the rat pups used in this study.

Glc-1-p levels, which have not previously been analyzed in our rat model, were also compared between genotypes and tissues. Overall, absolute levels of Glc-1-p were much lower in brain homogenate for both wildtype and mutant rats than in liver tissue. In liver tissue specifically, Glc-1-p significantly decreased by 45% from an average 1921.1 pmol/ mg liver in wildtypes to 1062.2 pmol/ mg liver in mutants (Table 2). Interestingly, brain glc-1-p values demonstrated the opposite trend, increasing by 25% in mutants (Table 2). However, there was still substantial overlap between mutant and wildtype Glc-1-p distributions, and therefore, the observed increase in mutant brains was not statistically significant (Figure 3A).

UDP-sugars in the Liver

Significant differences were found between wildtype and mutant genotypes in all but two UDP-sugar relationships analyzed; only the ratio between UDP-galNAc and UDP-glcNAc and the level of UDP-glc did not differ significantly between *Galt* genotypes.

Table 3. Summary statistics for UDP-sugar concentrations (pmol/mg liver) and ratios for M3/M3 and WT/WT rat liver tissue.

	Measure of Center* **(Measure of Spread)****	
Metabolite	**M3/M3**	**WT/WT**
UDP-gal	19.19(5.76)	29.40(6.88)
UDP-glc	139.73(73.94)	75.19(34.31)
UDP-glc/UDP-gal	7.81(2.42)	2.54(0.94)
UDP-galNAc	27.28(2.99)†	42.95(12.61) †
UDP-glcNAc	48.6(9.44)	86.91(26.35)
UDP-glcNAc/UDP-galNAc	1.89(0.18) †	1.87(0.26)†

***Mean or Median reported as measures of center for each group based on normality of distribution. ** Standard deviation (SD) or interquartile range (IQR) reported as measures of spread for each group based on the normality of distribution. Median(IQR) is reported for non-normal distributions and is marked with †**

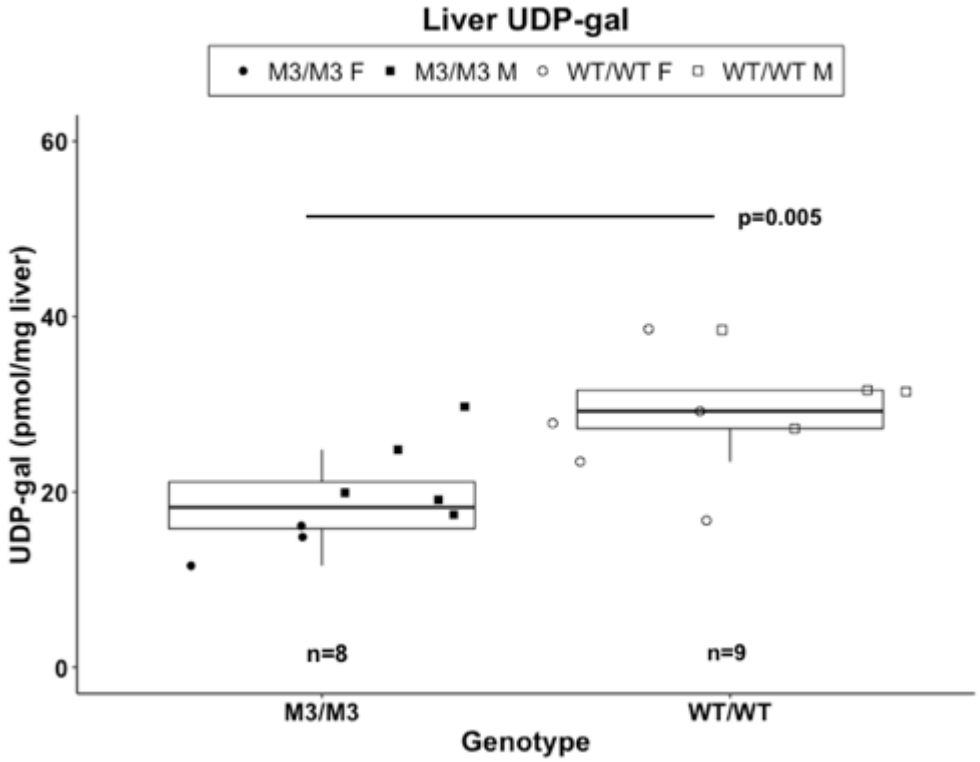


Figure 5. Genotype-specific differences in rat liver UDP-galactose absolute levels. After confirming equal variances and the normality of the distributions, a two-sample t-test was conducted, α=0.05. N=8/N=9 due to lost samples and the inability to quantify metabolite levels.

UDP-galactose levels in liver samples from GALT-null pups were decreased by 35% when compared to samples from wildtype pups, with averages of 19.2 pmol/mg liver and 29.4 pmol/liver, respectively (Table 3). Both distributions were relatively normal, with minimal overlap between the two, yielding a statistically significant difference in UDP-galactose levels between genotypes (Figure 5). No litter-specific differences were observed (data not shown), meaning that UDP-galactose levels were consistent within mutant and wildtype genotypes, regardless of litter.

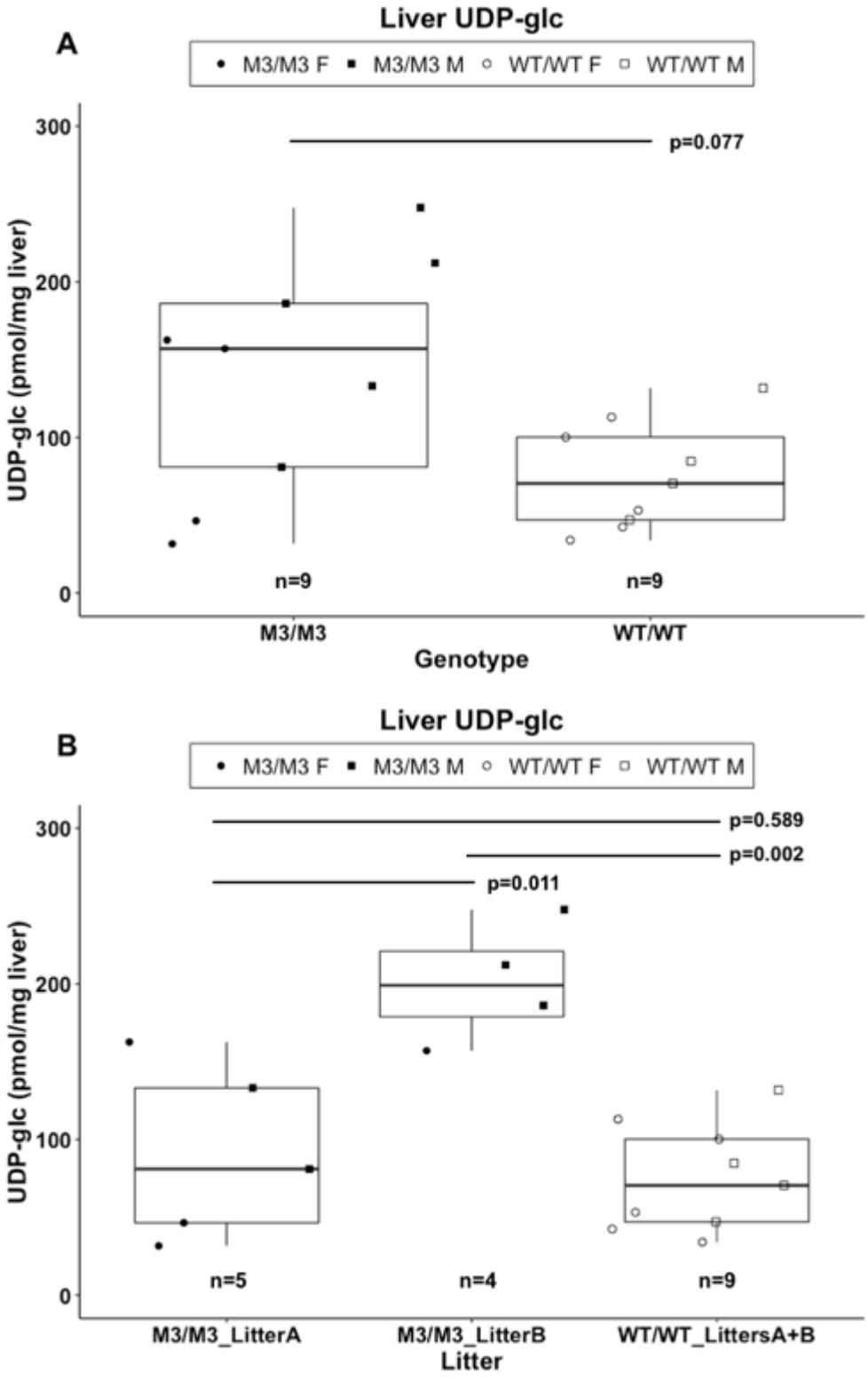


Figure 6. Differences in rat liver absolute UDP-glucose concentrations by genotype and litter. A, Due to unequal variances, a Mann-Whitney U test was conducted, using α =0.05. B, Due to a significant difference in UDP-glucose between the two mutant litters, both litters were compared to the wildtype cohort with 2-sample t-tests, using α' =0.025 as a result of multiple testing correction. N=9 and not n=10 due to loss of samples.

UDP-glucose, however, displayed the opposite trend, with mutant levels increasing by 85.8% when compared to wildtype levels (Table 3). The spread of the data was quite large (Figure 4), especially in the mutant distribution, which had a standard deviation of approximately 74 (Table 3). Upon analysis of the four litters separately, statistically significant differences between the two mutant litters were discovered, with almost no overlap in distribution between them (Figure 6). One mutant litter reported a mean of 90.9 pmol UDP-glc/mg liver while the other had a mean of 200.7 pmol/mg liver, a 121% increase. Because no differences in UDP-glc levels were observed in the two wildtype litters (Figure 6), they remained combined for the remainder of the analysis. When the two mutant litters were considered separately, it became clear that only one had a statistically significant increase in UDP-glc level when compared to the wildtypes, while the other mutant litter closely resembled the sugar distribution seen in wildtype livers (Figure 6).

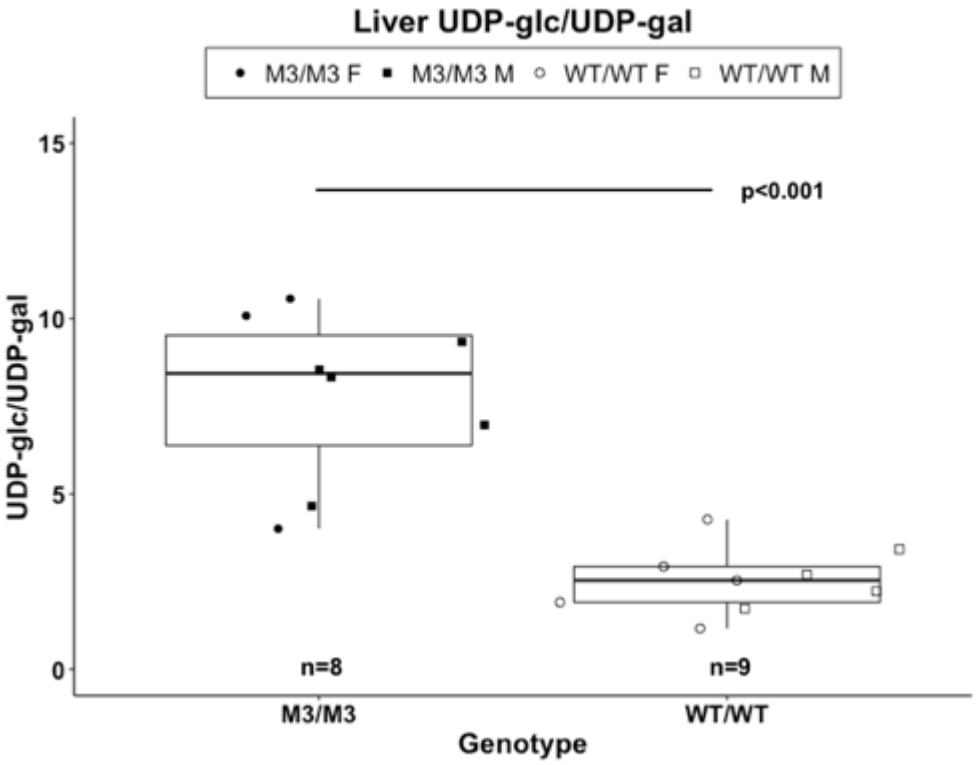
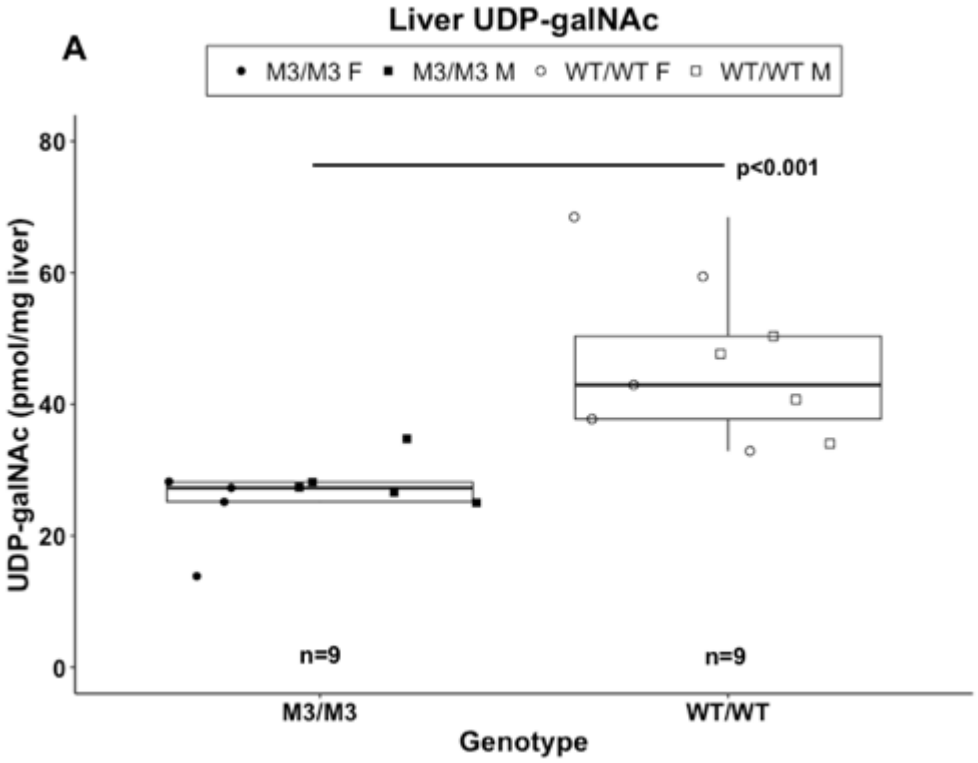
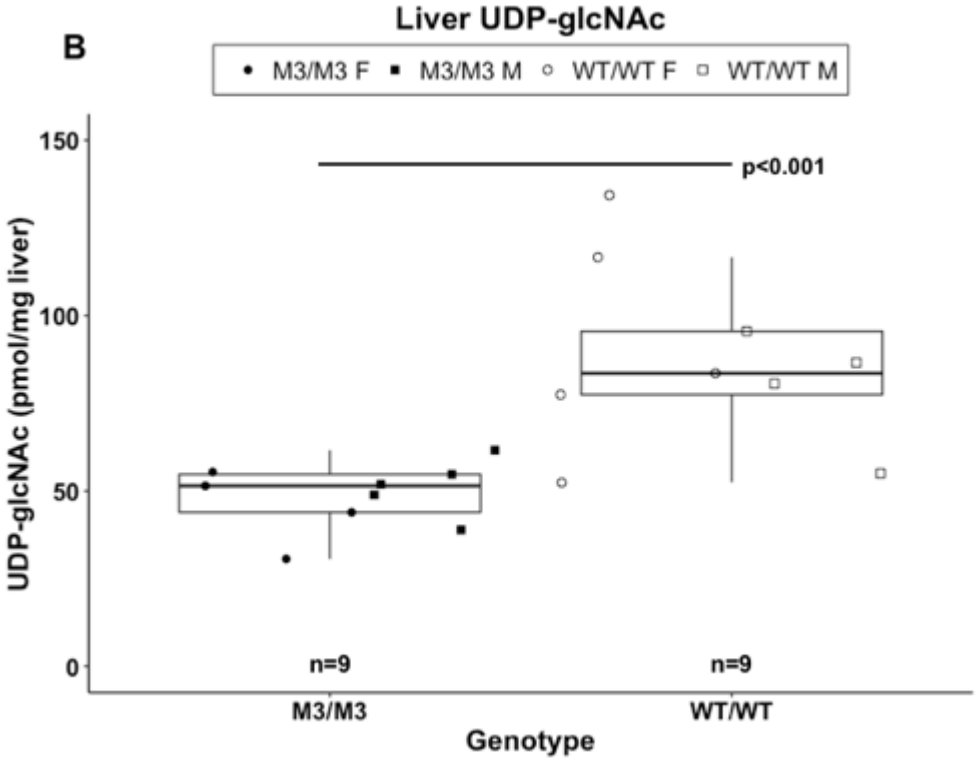


Figure 7. Genotype-specific differences in rat liver UDP-glucose/UDP-galactose relative ratios. Ratios were calculated by dividing the absolute concentration of standardized UDP-glucose by UDP-galactose within the liver. A Mann-Whitney U test was conducted to compare ratios, $\alpha = 0.05$.

Due to the relative increase in UDP-glc level and decrease in UDP-gal level in mutants, there was a significant increase in the ratio of UDP-glc/UDP-gal in mutants as well (Figure 7). The average ratio in mutants was 7.9:1, while the wildtype average ratio was around 2.5:1 (Table 3), which closely resembled the expected 3:1 ratio. Additionally, mutant livers had a much larger spread (SD=2.421) than wildtype tissues (SD=0.936) (Table 3), however the wide spread could not be explained by litter-specific differences in ratio.


A
Liver UDP-galNAc
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
p<0.001
UDP-galNAc (pmol/mg liver)
80
60
40
20
0
n=9
n=9
M3/M3
WT/WT
Genotype


B
Liver UDP-glcNAc
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
p<0.001
UDP-glcNAc (pmol/mg liver)
150
100
50
0
n=9
n=9
M3/M3
WT/WT
Genotype

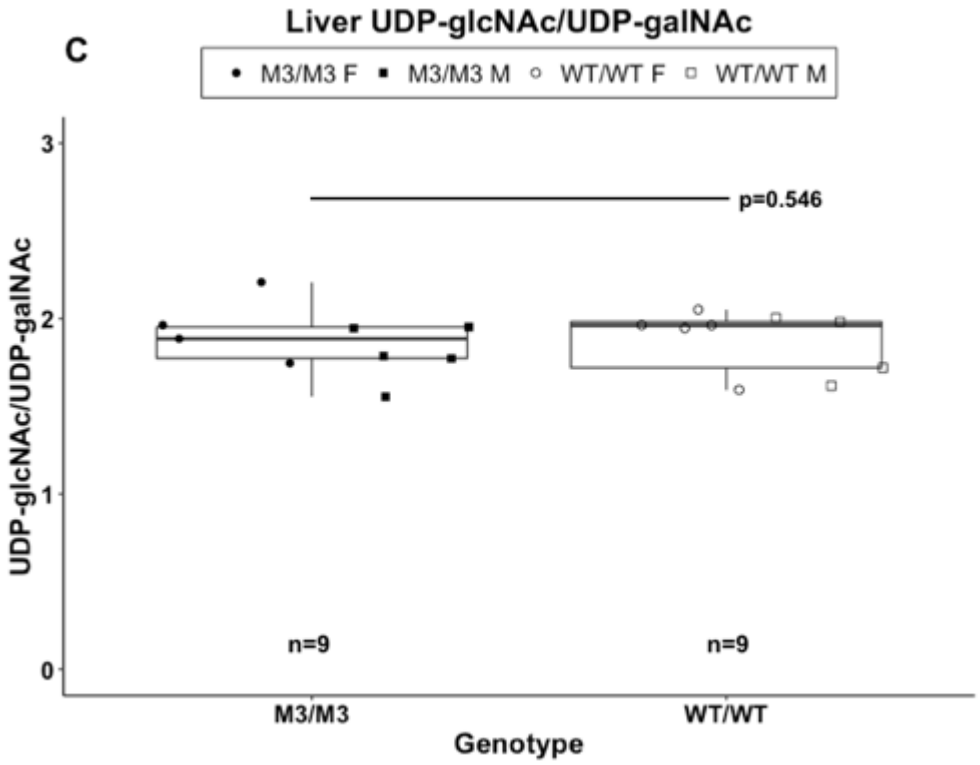


Figure 8. Differences in rat liver UDP-N-acetylglucosamine levels, UDP-N-acetylgalactosamine levels, and UDP-glcNAc/UDP-galNAc by genotype. Mann-Whitney U tests were conducted for all three comparisons, α =0.05. A, UDP-galNAc. B, UDP-glcNAc. C, UDP-glcNAc/UDP-galNAc.

UDP-galNAc and UDP-glcNAc levels both significantly decreased in mutant livers. The mean UDP-galNAc level decreased approximately 36%, from 42.95 pmol/mg liver in wildtype rats to 27.3 pmol/mg liver in mutants, while the level of UDP-glcNAc decreased by 44% (Table 3). Interestingly, the spread in wildtype UDP-galNAc (SD=12.61) and UDP-glcNAc (SD=26.35) distributions were much larger than in their counterpart mutant distributions, SD=2.99 and 9.44, respectively (Figure 8). There were no statistically significant differences observed on a litter-specific basis for either UDP-galNAc or UDP-glcNAc (data not shown).

Because UDP-galNAc and UDP-glcNAc levels decreased in a similar manner, the overall ratio between these two sugars did not differ significantly between wildtype and mutant genotype (Figure 8). Spread was consistent between genotype distributions and the overlap between them was substantial (Figure 8).

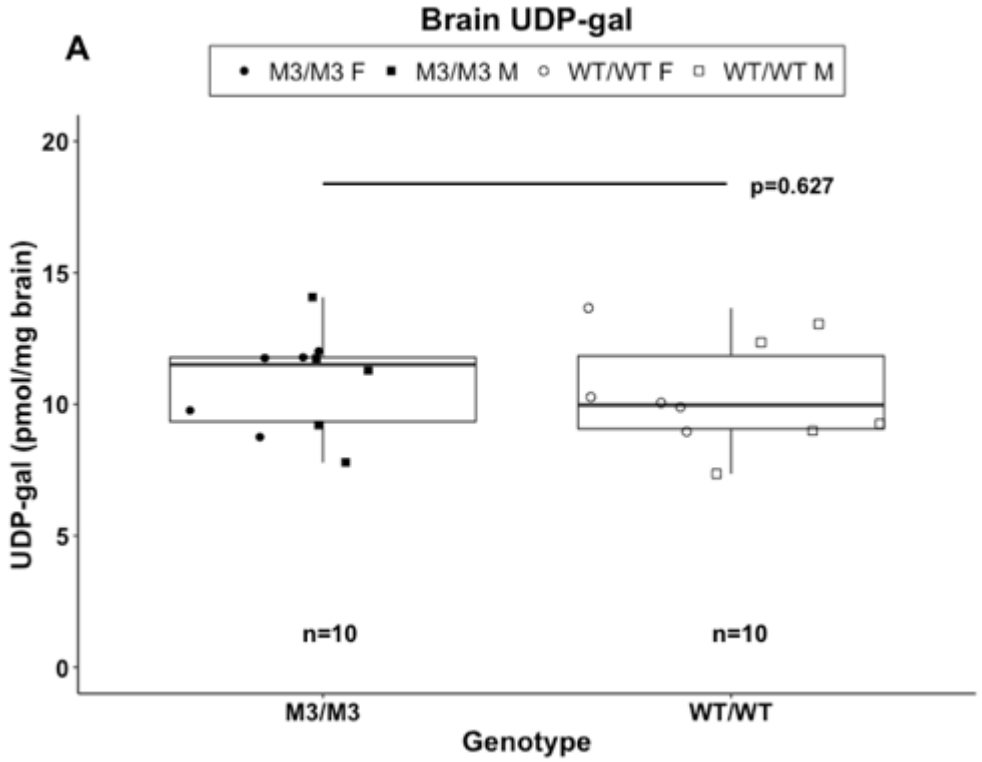
UDP-sugars in Brain Homogenate

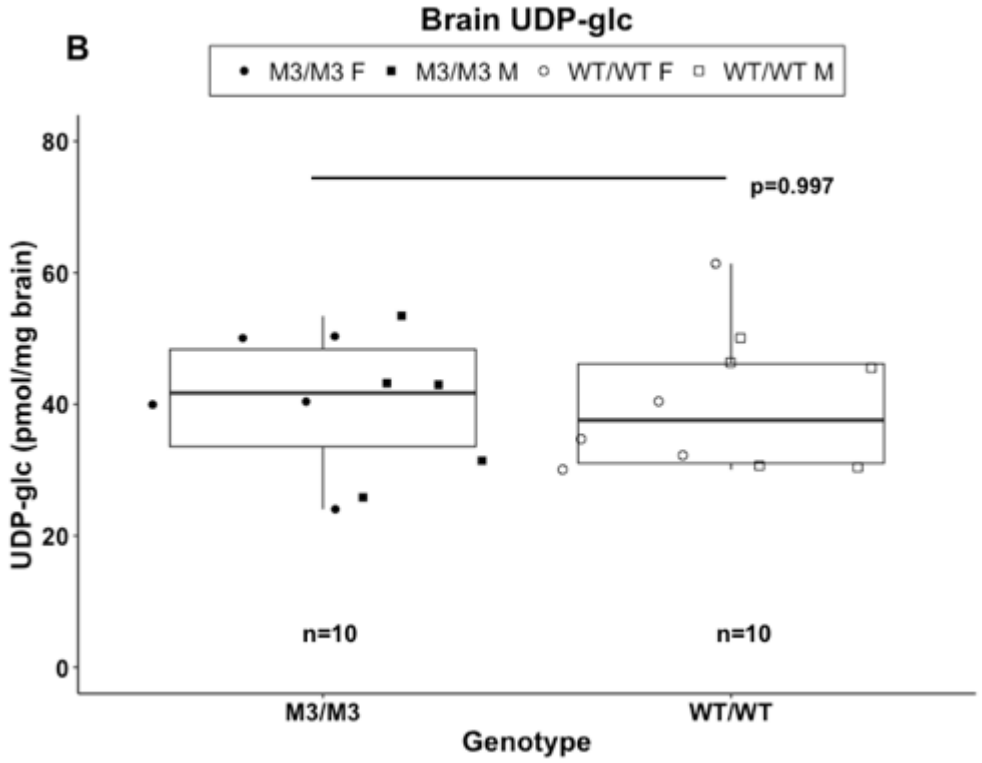
In brain homogenate samples, significant differences between wildtype and mutant rats were found only in levels of UDP-galNAc and UDP-glcNAc.

Table 4. Summary statistics for UDP-sugar concentrations (pmol/mg brain) and ratios for M3/M3 and WT/WT rat brain homogenate tissue

	Mean (standard deviation)*	
Metabolite	**M3/M3**	**WT/WT**
UDP-gal	10.82(1.89)	10.09(1.89)
UDP-glc	40.17(10.21)	39.09(10.53)
UDP-glc/UDP-gal	3.85(1.21)	4.05(1.45)
UDP-galNAc	15.92(2.85)	19.45(3.42)
UDP-glcNAc	37.9(6.40)	45.77(7.59)
UDP-glcNAc/UDP-galNAc	2.39(0.06)	2.36(0.05)

*** Mean(sd) reported for each group because all had normal distributions.**


A
Brain UDP-gal
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
p=0.627
UDP-gal (pmol/mg brain)
20
15
10
5
0
n=10
n=10
M3/M3
WT/WT
Genotype


B
Brain UDP-glc
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
p=0.997
UDP-glc (pmol/mg brain)
80
60
40
20
0
n=10
n=10
M3/M3
WT/WT
Genotype

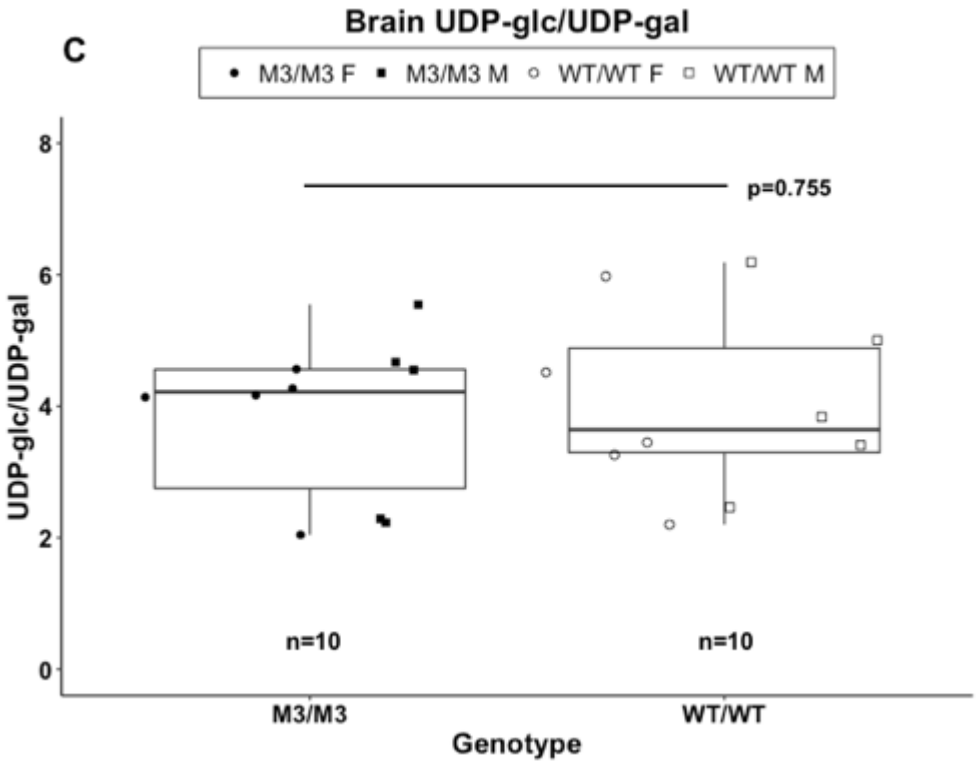
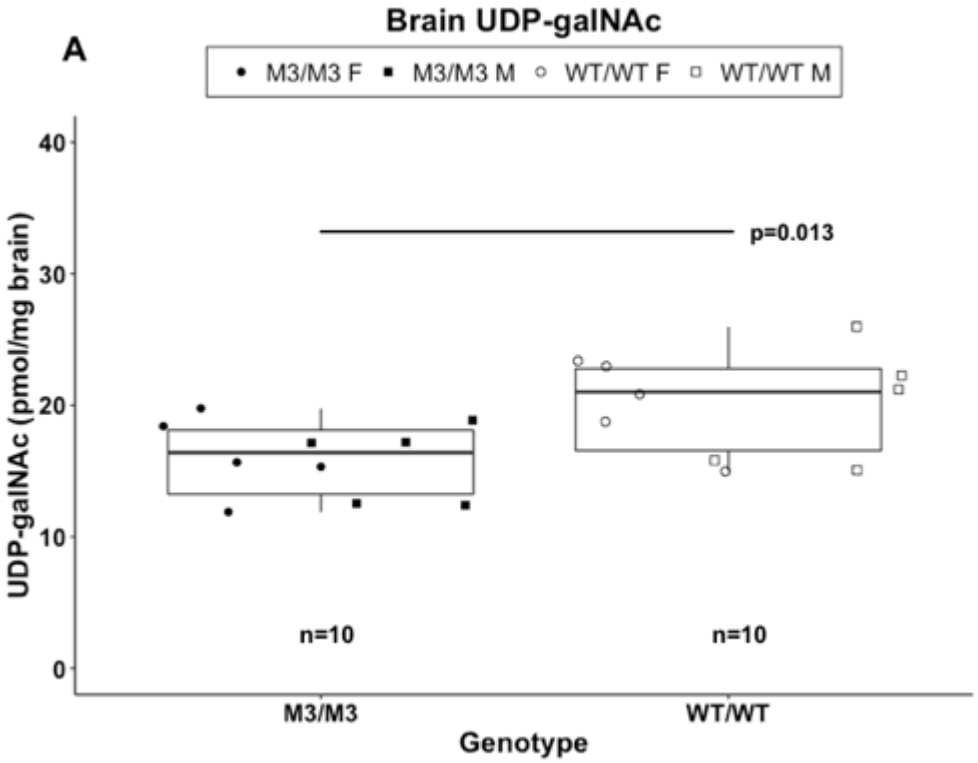
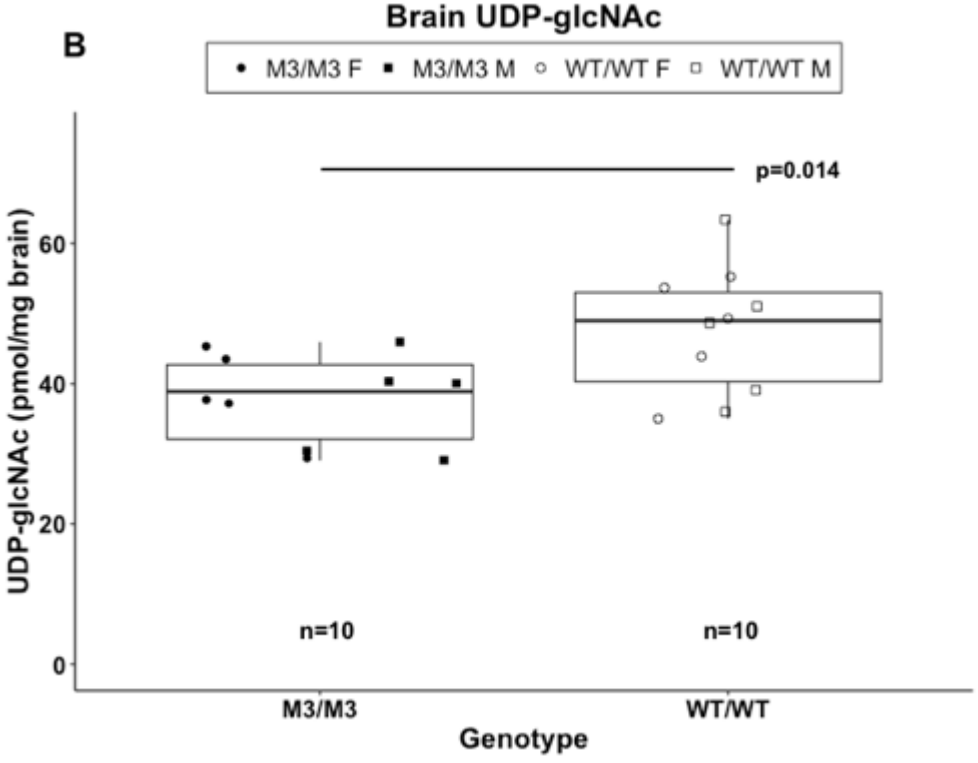


Figure 9. Differences in rat brain homogenate UDP-galactose levels, UDP-glucose levels, and UDP-glucose/UDP-galactose ratio. Two-sample t-tests were conducted for all three comparisons, α =0.05. A, Brain UDP-galactose. B, Brain UDP-glucose. C, Brain UDP-glucose: UDP-galactose.

No significant differences were observed in either UDP-gal levels or UDP-glc levels between wildtype and mutant rat brains (Figure 9). Average UDP-gal levels were 10.82 pmol/mg brain and 10.09 pmol/mg brain, while average UDP-glc levels were 40.17 pmol/ mg brain and 39.09 pmol/brain, in mutants and wildtypes, respectively (Table 4). Accordingly, the reported ratios of 3.85 and 4.05, in mutants and wildtypes, respectively, confirm the lack of significant differences based on genotype (Figure 9). Additionally, the spread of the data for each of the three comparisons were consistent across genotypes (Figure 9), and no litter-specific differences were observed, most likely due to the presence of outliers and small sample size (data not shown).


A
Brain UDP-galNAc
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
UDP-galNAc (pmol/mg brain)
40
30
20
10
0
p=0.013
n=10
n=10
M3/M3
WT/WT
Genotype


B
Brain UDP-glcNAc
M3/M3 F
M3/M3 M
WT/WT F
WT/WT M
UDP-glcNAc (pmol/mg brain)
60
40
20
0
p=0.014
n=10
n=10
M3/M3
WT/WT
Genotype

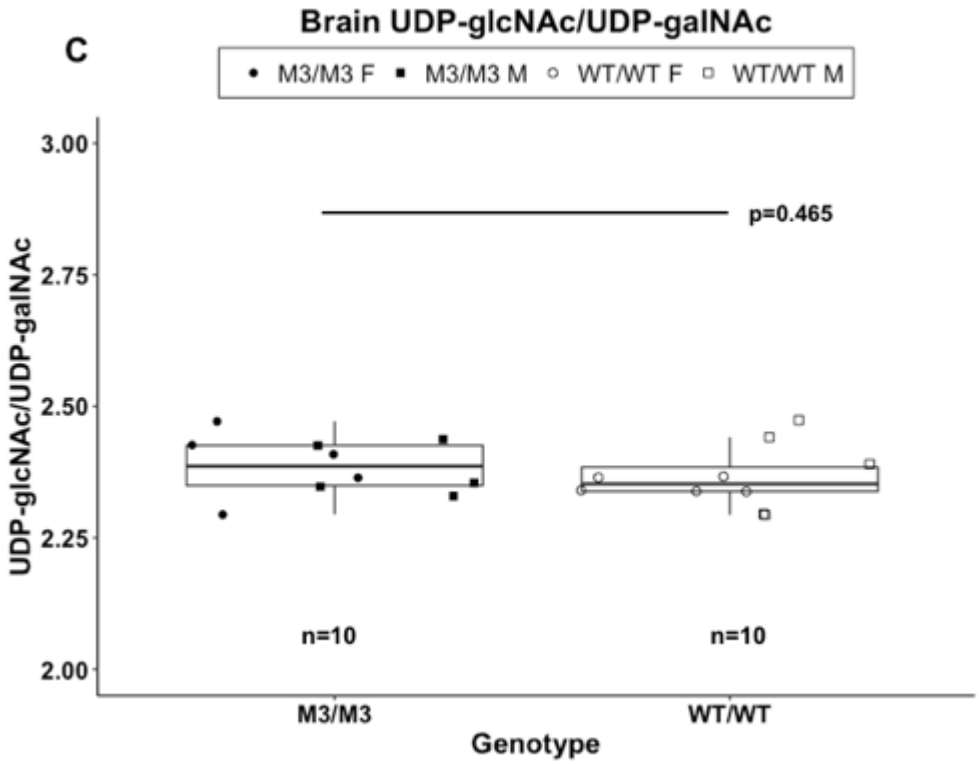


Figure 10. Differences in rat brain homogenate UDP-N-acetylgalactosamine levels, UDP-N-acetylglucosamine levels, and UDP-glcNAc/UDP-galNAc ratio. Two-sample t-tests were conducted for all three comparisons, α =0.05. A, Brain UDP-N-galactosamine. B, Brain UDP-N-glucosamine. C, Brain UDP-glcNAc/UDP-galNAc, x-axis scaled between 2 and 3 to allow for best visualization of sample distributions.

Brain homogenate levels of UDP-galNAc decreased significantly from a mean of 19.45 pmol/mg brain in wildtypes to 15.92 pmol/mg brain in mutants, an 18.1% decrease (Figure 10). A significant decrease was also observed in mutant brain homogenate levels of UDP-glcNAc, who had 37.9 pmol/mg brain as compared to 45.7 pmol/mg brain in wildtypes, representing a decrease of 17.2% (Table 4, Figure 10). Because UDP-galNAc and UDP-glcNAc levels decreased to approximately the same degree, there was no difference in their ratio, with values of 2.39 and 2.36 for mutant and wildtype rats, respectively (Figure 10). Measures of spread were consistent between genotypes for each metabolite and no litter-specific differences were identified.

Chapter 3: Discussion, Limitations, and Future Directions

Discussion

In summary, we observed significantly different UDP-gal, UDP-galNAc, and UDP-glcNAc levels as well as the ratio between UDP-glc/UDP-gal between wildtypes and mutants in rat liver tissues. In brain homogenate tissue, however, there were only genotype-specific differences in UDP-galNAc and UDP-glcNAc overall levels. By analyzing the data between litters as well as overall genotype within each tissue, we were able to determine that the trends observed were litter-independent within a given genotype in all but one UDP-sugar comparison (liver UDP-glucose levels).

Overall, absolute levels in all sugars analyzed were higher in liver than brain tissue, regardless of genotype. The greatest difference was observed in mutant Glc-1-p levels, which increased in the liver to approximately 3.5 times the value measured in the brain. Likewise, the spread of each metabolite distribution tended to be wider in liver tissue, regardless of genotype.

As expected, Gal-1-p, galactitol, and galactose levels were all significantly elevated in mutant rat samples, regardless of tissue type, confirming our previous observations[28]. However, glc-1-p values were elevated in mutant brains, which is unlikely a direct result of GALT deficiency since it is a product not a substrate of the deficient GALT enzyme. Perhaps gal-1-p's suspected role as a competitive inhibitor of UDP-glucose pyrophosphorylase (UGP), which is primarily responsible for the conversion of glc-1-p to UDP-glc, contributed to elevated levels of Glc-1-p[17]. This is unlikely, due to the fact that UGP's affinity for Glc-1-p is much greater than its

affinity for gal-1-p[17]; however it is still possible because overall gal-1-p levels were greater than glc-1-p levels. It is important to note that glc-1-p is used as a substrate by enzymes outside of the Leloir pathway as well, such as phosphoglucomutase. It is possible that the competition for glc-1-p by these enzymes may be contributing to the elevated levels observed in this study. In addition, the increase was only observed in brain tissue. This interesting result proposes that metabolite levels can be altered in opposite directions across different tissue types, similar to a previous observation made by our lab that galactose metabolite levels vary between different tissues of our rat model of Classic Galactosemia[28].

Observed trends were consistent across both brain and liver tissue in UDP-galNAc and UDP-glcNAc levels, as well as the ratio between the two sugars. UDP-galNAc and UDP-glcNAc concentrations decreased by approximately the same amount in mutants of both brain (18% and 17%, respectively) and liver (36% and 44%, respectively) tissues (Tables 3 and 4). It has been reported that galactose-1-phosphate serves as an inhibitor of UDP-N-acetylglucosamine pyrophosphorylase[17], which may be a potential explanation for decreased levels of UDP-galNAc and UDP-glcNAc in mutant rats, as they are characterized by marked increases in galactose-1-phosphate levels. Accordingly, neither tissue showed significant differences in the UDP-glcNAc/UDP-galNAc ratio between wildtypes and mutants, with ratios in both tissues centering around 2:1.

However, the observed ratio between UDP-glc and UDP-gal in mutant livers of approximately 7:1 differed substantially from the 2.5:1 ratio observed in wildtype livers, as well as the ratios observed in both wildtype and mutant brains. Two important observations can be made from this result. First, it is interesting that the UDP-glcNAc/UDP-galNAc ratio remained

the same while the UDP-glc/UDP-gal ratio did not due to the fact that GALE is responsible for the interconversion between both sets of UDP-sugars. Second, the tissue-specific differences in ratio posit that some biological process is powerful enough to significantly alter the ratio between UDP-gal and UDP-glc in the liver but not in brain tissue, despite tight regulation by GALE throughout the body. This may be indicative of something that is directly or indirectly altering the way GALE interacts with UDP-gal and UDP-glc only but not UDP-galNAc and UDP-glcNAc. A potential explanation for this is that GALE may have a higher affinity for UDP-galNAc and UDP-glcNAc, making it more difficult to maintain proper ratios of the UDP-gal and UDP-glc metabolites when tissues are under metabolic stress, which may, in turn alter steady state relationships. As previously mentioned, UDP-sugar levels are also maintained by enzymes outside of the Leloir pathway, which may serve as a potential explanation for the observations regarding the absolute levels as well as the ratios. Finally, it is also possible that UDP-gal and UDP-glc are more directly impacted by the GALT enzyme in the Leloir pathway, which may make them more susceptible than UDP-galNAc and UDP-glcNAc to significant alterations.

These results may have been at least partially affected by the study's small sample size. Despite the lack of statistically significant differences in UDP-glucose levels within either tissue, there was a substantial increase in its level within mutant livers (Table 3). As was indicated in Figure 6B, however, these liver UDP-glc concentrations were significantly different between the two mutant litters, with one displaying a significant increase in comparison to the wildtype group, while the other had no statistically significant changes when compared to wildtypes. The extreme variation in UDP-glc concentration between the two mutant litters is what ultimately led to such a noticeable increase in the mutant genotype compared to wildtypes. Increased

UDP-glc levels in the livers, in turn, undoubtedly contributed to the extremely high UDP-glc/UDP-gal ratio.

However, it is important to note that even if there had been no difference between the UDP-glucose levels in mutant and wildtype liver tissues, there still would have been an overall increase in the UDP-glc/UDP-gal ratio due to the fact that UDP-gal significantly decreased in mutant liver tissue (Figure 5). Therefore, the only way for the ratio to have remained constant despite differences in the individual sugars was if the two sugars differed in the same direction and to the same degree.

Trends in many of the UDP-sugar levels, however, varied according to tissue type. For example, UDP-gal levels decreased significantly by 35% in mutant liver tissue while remaining the same between mutant and wildtype pups in the brain (Tables 3 and 4). This result is not surprising, however, as it reflects the general lack of consensus in reports on UDP-sugar levels, depending upon the tissue or cell-type being analyzed[22,23]. So, in conclusion, it can be stated that the different trends observed between liver and brain tissue confirm that UDP sugar levels and ratios can vary across different tissue types as well as genotypes in ten-day old rat pups.

Limitations

Despite the presence of strong tissue-specific differences in both UDP-sugar levels and ratios between mutant and wild-type rats, this study did have limitations. First and most prominent, this study was restricted by its small sample size. Sample sizes of ten for each genotype posed some challenges during the data analysis stage. Measures of spread were naturally fairly wide and some were not normally distributed. Additionally, it is hard to tell if

observed differences were a product of an actual biological change or just a product of having small numbers. Fortunately, the robustness of the results observed in this study can be improved in the future simply by expanding the size of each genotype sample.

This study required significant optimization of protocol for best quantification of UDP-sugars. Optimization included concentrating samples during metabolite extraction as well as altering HPLC programs that would allow for optimal elution of metabolites, with minimal interference of peaks. Despite the substantial improvements made, we may want to continue to enhance our current method of quantification. These enhancements include confirming peak identity with mass spectrometry and altering HPLC programs based on the results.

Future Directions

The results reported here are indicative of tissue-specific perturbation in both UDP-sugar absolute levels as well as ratios in the rat model of classic galactosemia. This study is the first of its kind, in that we were able to quantify these metabolites in those tissues most relevant to the disease's long-term complications. Our results can contribute to our understanding of UDP-sugar levels and subsequent glycosylation within the brain and liver of human CG patients and might ultimately lead to improved interventions.

In the future, we intend to expand on the current study to deepen our understanding about UDP-sugar levels and their role in classic galactosemia. Previous studies have conflicting reports as to whether differences in UDP-sugar levels and ratios are age-dependent[22,23]. In order to accurately assess this question in our GALT-null rat model, we plan to expand the study

sample so that it includes ten-day old, nursing pups, 4-5 month old adults consuming a low-galactose diet, and prenatal data.

In addition, we also intend to include rat red blood cells in our analysis due to its clinical use a potential biomarker in diagnosis of classic galactosemia in humans[22]. Previous studies analyzing UDP-sugar levels and ratios have been conducted in human red bloods[22,23], therefore, it is critical that we also look at these cells in order to determine if the same trends are recapitulated in rats. It is expected, however, that UDP-sugar levels and ratios in red blood cells may be independent of those observed in liver and brain tissue, due to the fact that RBC levels of galactose-1-phosphate, galactose, and galactitol are poor predictors of those same metabolite levels within other relevant tissues of our rat model[28].

Finally, we hope to include a more thorough analysis of relationships between UDP-sugar levels and other metabolites, as well as their ultimate impact on glycosylation. Through the use of linear model regression analysis, we will be able to elucidate the predictive value of UDP-sugar levels either on other metabolites or between different tissue types. For example, it may be helpful to learn whether or not galactose metabolite levels within mutants are predictive of UDP-sugar level. Analysis of this relationship may be valuable in determining whether or not slight variations in galactose concentrations of CG patients are enough to produce noticeable differences in UDP-sugar levels and thus glycosylation. Once the UDP-sugar levels and ratio trends have been confirmed, we can begin to analyze the impact of metabolite level changes on the process of glycosylation by quantifying levels of N-glycans within each relevant tissue type.

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