

Mediators of a long-term movement abnormality in a *Drosophila melanogaster* model of classic galactosemia

Emily L. Ryan¹, Brian DuBoff², Mel B. Feany² and Judith L. Fridovich-Keil^{3,*}

SUMMARY

Despite neonatal diagnosis and life-long dietary restriction of galactose, many patients with classic galactosemia grow to experience significant long-term complications. Among the more common are speech, cognitive, behavioral, ovarian and neurological/movement difficulties. Despite decades of research, the pathophysiology of these long-term complications remains obscure, hindering prognosis and attempts at improved intervention. As a first step to overcome this roadblock we have begun to explore long-term outcomes in our previously reported *GALT*-null *Drosophila melanogaster* model of classic galactosemia. Here we describe the first of these studies. Using a countercurrent device, a simple climbing assay, and a startle response test to characterize and quantify an apparent movement abnormality, we explored the impact of cryptic *GALT* expression on phenotype, tested the role of sublethal galactose exposure and galactose-1-phosphate (gal-1P) accumulation, tested the impact of age, and searched for potential anatomical defects in brain and muscle. We found that about 2.5% residual *GALT* activity was sufficient to reduce outcome severity. Surprisingly, sublethal galactose exposure and gal-1P accumulation during development showed no effect on the adult phenotype. Finally, despite the apparent neurological or neuromuscular nature of the complication we found no clear morphological differences between mutants and controls in brain or muscle, suggesting that the defect is subtle and/or is physiologic rather than structural. Combined, our results confirm that, like human patients, *GALT*-null *Drosophila* experience significant long-term complications that occur independently of galactose exposure, and serve as a proof of principle demonstrating utility of the *GALT*-null *Drosophila* model as a tool for exploring genetic and environmental modifiers of long-term outcome in *GALT* deficiency.

INTRODUCTION

Classic galactosemia (OMIM 230400) is an autosomal recessive disorder that results from profound impairment of galactose-1-phosphate uridylyltransferase (*GALT*, EC 2.7.7.12), the middle enzyme in the Leloir pathway of galactose metabolism (see review by Fridovich-Keil and Walter, 2008). In most western populations, classic galactosemia occurs with a frequency of at least 1/60,000 live births; the rate is substantially higher in some groups. Infants with classic galactosemia generally appear normal at birth but present with escalating symptoms within days of exposure to dietary galactose, which, as a constituent monosaccharide of lactose, is abundant in breast milk and milk-based formulae. Acute symptoms range from cataracts, failure to thrive, vomiting and diarrhea to hepatomegaly, bleeding abnormalities and *Escherichia coli* sepsis, which can be lethal. Absent intervention, infants with classic galactosemia often succumb in the neonatal period (see review by Fridovich-Keil and Walter, 2008).

The advent of population newborn screening for *GALT* deficiency in the 1960s (e.g. Beutler et al., 1965; Mellman and Tedesco, 1965) and its rapid implementation in the decades that followed enabled early, sometimes even pre-symptomatic, identification of affected infants. Switched to a galactose-restricted diet (generally a soy or elemental formula) these infants appeared to thrive, leading to early predictions that neonatal diagnosis coupled with rigorous life-long dietary restriction of galactose could enable patients with classic galactosemia to escape the negative consequences of their disease (see review by Fridovich-Keil and Walter, 2008).

Unfortunately, the escape was short-lived. By the 1970s and 1980s, first anecdotal reports, and then large retrospective studies, demonstrated that despite early diagnosis and rigorous dietary intervention, patients with classic galactosemia remained at strikingly increased risk for an unusual constellation of long-term complications, including a speech disorder, cognitive disability and neurological or neuromuscular complications in close to half of all patients, and primary or premature ovarian insufficiency in more than 80% of girls and women (e.g. Gitzelmann and Steinmann, 1984; Kaufman et al., 1988; Komrower, 1983; Segal, 1989; Waggoner et al., 1990; Waisbren et al., 2012). Other complications were also noted. Attempts to pinpoint the underlying causes of these disparate complications have been disappointing, hindered in part by the fact that classic galactosemia is a rare disorder so that most studies have been conducted with relatively small numbers of patients, and in part by the failure of a knockout mouse model to mimic patient outcomes (Leslie et al., 1996).

Recently, we reported the development and initial characterization of a *Drosophila melanogaster* model of classic galactosemia (Kushner et al., 2010). These *GALT*-null animals recapitulate the fundamental

¹Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University, Atlanta, GA 30322, USA

²Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

³Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA

*Author for correspondence (jfridov@emory.edu)

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acute patient phenotype in that they die in development if exposed to food containing substantial galactose (in addition to glucose and other nutrients), but live if maintained on a galactose-restricted diet. These animals are also rescued by expression of a wild-type human *GALT* transgene early in development. Finally, *GALT*-null *Drosophila* demonstrate a movement disorder that is evident when they attempt to traverse a countercurrent device; as in patients, this movement abnormality occurs despite life-long dietary restriction of galactose (Kushner et al., 2010).

In the work described here, we first characterized and then used this movement abnormality to test four fundamental questions about long-term outcome in *GALT*-deficient animals: (1) Does cryptic *GALT* activity impact outcome severity? (2) Does dietary exposure to sublethal galactose in development exacerbate the phenotype and is there a relationship between galactose-1-phosphate (gal-1P) level and outcome severity? (3) Does age impact severity of the phenotype? (4) Are there any structural defects evident in brain or muscle of adult *GALT*-null *Drosophila* that might account for the movement abnormality? Our results demonstrate that cryptic residual *GALT* activity does make a difference: about 2.5% and 6% normal *GALT* activity were each sufficient to significantly improve outcome in our flies. Strikingly, exposure of *GALT*-null larvae to sublethal levels of dietary galactose, which markedly increased their gal-1P levels, did not exacerbate the adult phenotype, although increasing age did have this effect. Finally, microscopic inspection of brain and muscle structures in *GALT*-null and control flies revealed no evident defects, implying that the abnormality is either subtle or physiologic rather than anatomic. These results further our understanding of the etiology of long-term outcome in *GALT*-null *Drosophila*, and by extension, might have implications for our understanding of long-term complications in classic galactosemia.

RESULTS

***GALT*-deficient flies demonstrate a movement abnormality despite lifelong dietary restriction of galactose**

We have reported previously that *GALT*-deficient flies demonstrate a movement abnormality despite lifelong dietary restriction of galactose (Kushner et al., 2010). This phenotype was previously quantified using a classic six-chambered countercurrent device first introduced more than 40 years ago (Akai, 1979; Benzer, 1967). To confirm and expand upon this observation, we repeated the analysis using a custom-made ten-chambered countercurrent device (see Methods). Experiments were conducted using cohorts of approximately 60 male flies between the ages of 36–72 hours post-eclosion.

Using this expanded countercurrent device we confirmed our previous result, demonstrating that *GALT*-null animals, homozygous for the *dGALT*^{ΔAP2} deletion allele (Kushner et al., 2010), were less likely than controls [*dGALT*^{C2} homozygotes (Kushner et al., 2010)] to reach the final chamber of the apparatus in a fixed period of time (Fig. 1). Specifically, under the conditions of the assay the average proportion of *dGALT*^{ΔAP2} homozygotes to reach the final chamber was only 0.21±0.01, whereas the corresponding proportion for control animals was 0.75±0.01. This difference was highly significant ($P<0.0001$).

As a further control, we tested flies homozygous for the P-element insertion allele, *dGALT*^{KG00049}; this is the ancestral allele

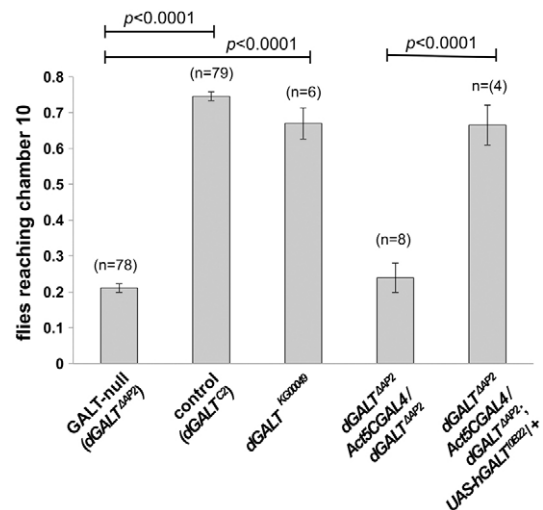


Fig. 1. Movement abnormality in *GALT*-null *D. melanogaster*. The proportion of flies from each indicated cohort to reach the tenth (final) chamber of a countercurrent device. All values were modeled in a one-way ANOVA with a variable to control for day-to-day variations in testing. *dGALT*^{KG00049} carries a P-element insertion just upstream of the *Drosophila GALT* gene that does not interfere with *GALT* expression or activity. *dGALT*^{C2} is a nearly full deletion of the *Drosophila GALT* gene achieved by imprecise excision of the P-element from *dGALT*^{KG00049} (Kushner et al., 2010). *dGALT*^{C2} is a wild-type allele generated by precise excision of the same P-element (Kushner et al., 2010). In flies with endogenous *GALT* enzyme activity (*dGALT*^{C2} and *dGALT*^{KG00049}) more than 60% of each cohort reached the final chamber. Flies lacking *GALT* activity (*dGALT*^{ΔAP2} and *dGALT*^{ΔAP2} with *Actin5c-GAL4*) had difficulty with this behavior and on average only about 20% reached the final chamber. Expression of a human *GALT* transgene rescued the phenotype. Significant differences are indicated. All values represent mean ± s.e.m., n, number of cohorts tested.

from which both *dGALT*^{ΔAP2} and *dGALT*^{C2} were generated by imprecise and precise excision, respectively, of the P-insertion (Kushner et al., 2010). Animals homozygous for the *dGALT*^{KG00049} allele demonstrate essentially normal *GALT* activity (Kushner et al., 2010) and, as expected, progressed to the final chamber of the countercurrent device in proportions comparable with those of the positive control (0.67±0.04 versus 0.75±0.01, respectively).

We also tested flies that were compound heterozygotes for *dGALT*^{ΔAP2} and *Df(2L)Exel7027*, a deficiency that removes the entire *Drosophila GALT* gene (Kushner et al., 2010). As expected, these animals progressed through the countercurrent device indistinguishably from *dGALT*^{ΔAP2} homozygotes (data not shown). Finally, we crossed both the *dGALT*^{ΔAP2} and *dGALT*^{C2} alleles into an Oregon-R background, crossed the resulting animals again to achieve homozygosity at the *dGALT* locus, and then tested the homozygotes using the countercurrent device. As in the *w*¹¹¹⁸ background, the *dGALT*^{ΔAP2} Oregon-R homozygotes were significantly less proficient at progressing to the final chamber than were their *dGALT*^{C2} Oregon-R counterparts (data not shown).

To further test the connection between loss of *GALT* enzyme activity and the movement abnormality in *dGALT*^{ΔAP2} homozygotes, we restored *GALT* activity in these animals using a human *GALT* transgene driven by the *GAL4/UAS* system. To achieve high level, nearly ubiquitous transgene expression we used

the *Actin5cGAL4* driver. *Drosophila* homozygous for the *dGALT^{ΔAP2}* allele and carrying only the *Actin5cGAL4* driver without the *UAS-hGALT^{10B22}* human *GALT* transgene demonstrated a countercurrent result similar to that described above for *dGALT^{ΔAP2}* homozygotes (Fig. 1). By contrast, *dGALT^{ΔAP2}* homozygotes carrying both the *Actin5cGAL4* driver and the *UAS-hGALT^{10B22}* human *GALT* transgene showed a dramatically improved outcome (Fig. 1); this change was highly significant ($P < 0.0001$).

***GALT*-null *Drosophila* demonstrate a defect in climbing but not startle response**

Progression through a ten-tubed countercurrent device requires repeated climbing and also repeated startle response in the form of rapid recovery from being tapped to the bottom of a tube. The ‘countercurrent defect’ evident in *GALT*-null *Drosophila* as compared with controls might therefore have reflected a defect in one ability, or the other, or both. To distinguish between these possibilities, we subjected *GALT*-null and control flies to a ‘simple climbing’ assay and also to a ‘tap recovery’ assay. All animals tested in these assays were males aged 36–48 hours post-eclosion. To test climbing, cohorts of 9–11 flies were tapped to the bottom of a clear graduated cylinder and then allowed to climb; the number of flies that reached above a predetermined height within 20 seconds were counted and compared with the total number of flies in that cohort.

As with the countercurrent device, *GALT*-null animals had difficulty with the climbing assay. The average proportion of *dGALT^{ΔAP2}* homozygotes that climbed above the predetermined height in 20 seconds was 0.256 ± 0.068 whereas the corresponding number for *dGALT^{C2}* homozygotes was 0.673 ± 0.062 (Fig. 2A). Animals carrying the *Actin5cGAL4* driver without a *UAS-hGALT^{10B22}* transgene in the *dGALT^{ΔAP2}* background performed similarly to *dGALT^{ΔAP2}* homozygotes, with 0.263 ± 0.058 of each cohort climbing above the mark in 20 seconds (Fig. 2A). Finally, expression of human *GALT* in the *dGALT^{ΔAP2}* background rescued this phenotype, with 0.519 ± 0.049 of each rescued cohort climbing above the mark in 20 seconds (Fig. 2A).

To test startle response we modified a previously published assay (Ganetzky and Wu, 1982). In brief, cohorts of three to five animals were subjected to vortex agitation in flat-bottom 25-mm diameter plastic vials at a fixed speed for 10 seconds and then observed. Under these conditions, flies with a normal startle response take less than 15 seconds to stand upright (Ganetzky and Wu, 1982). Repeated cohorts of both *GALT*-null and control animals subjected to this assay were able to right themselves within 1–3 seconds (Fig. 2B); we saw no apparent startle response defect in any of the cohorts tested.

Relationship between *GALT* activity level and severity of the movement abnormality

To test whether trace *GALT* activity might modify long-term outcome severity, we explored the relationship between *GALT* activity and the movement abnormality revealed by the countercurrent device. The lowest levels of *GALT* activity were achieved using a ‘leaky’ human *GALT* transgene, *UAS-hGALT^{10A11}*, that expressed low levels of *GALT* despite the absence of a *GAL4* driver. In a *dGALT^{ΔAP2}* (endogenous *GALT*-null) background, animals carrying one allele of *UAS-hGALT^{10A11}* demonstrated about 2.5% of wild-type *GALT* activity; animals carrying two alleles

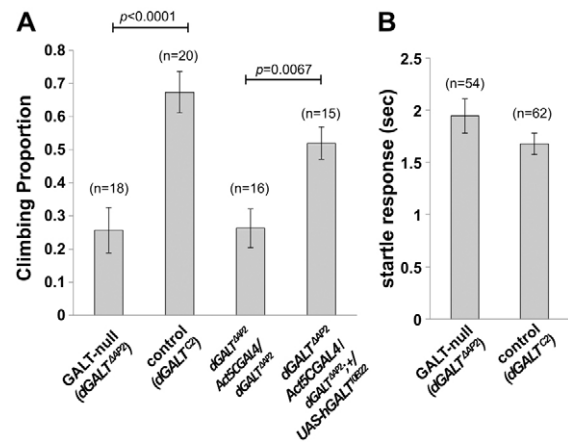


Fig. 2. *GALT*-null *D. melanogaster* are defective in climbing but not startle response. (A) The proportion of flies in each indicated cohort that reached above a designated mark in 20 seconds. (B) The average number of seconds it took all of the flies in each cohort to regain a standing posture after vigorous agitation for 10 seconds. All values were modeled in a one-way ANOVA and mean \pm s.e.m. are plotted. *n*, number of cohorts tested.

demonstrated just over 6% (Table 1). Intermediate *GALT* activity was achieved using animals heterozygous for one allele of the *GALT* deletion, *dGALT^{ΔAP2}*, and one control allele, *dGALT^{C2}*; these animals demonstrated about 82% of wild-type *GALT* activity (Table 1). Finally, *GALT* overexpression was achieved using a *UAS*-human *GALT* transgene coupled with a strong, ubiquitous driver (*Act5cGAL4*) in either an endogenous *GALT*-null (*dGALT^{ΔAP2}*) or a control (*dGALT^{C2}*) background; both genotypes exhibited a dramatic excess of *GALT* activity (Table 1).

Phenotypic analyses of animals representing these different genotypes revealed a very steep relationship between *GALT* activity and outcome at the lowest levels of *GALT* activity. Animals expressing as little as about 2.5% or 6.5% of wild-type *GALT* activity demonstrated significant phenotypic rescue ($P < 0.0001$, Fig. 3). This relationship leveled off asymptotically as *GALT* activity approached or exceeded the carrier level so that there was no marked difference in outcome between *GALT* heterozygotes, wild-type animals, and animals expressing more than tenfold excess *GALT* activity (Fig. 3). Indeed, flies expressing a human *GALT* transgene on top of an intact endogenous *Drosophila GALT* gene demonstrated close to 20-fold excess *GALT* activity (Table 1), and yet even these animals exhibited ‘normal’ progression through the countercurrent device (0.79 ± 0.05 reached the tenth chamber, data not illustrated in Fig. 3). These results demonstrate the importance of even trace *GALT* activity on long-term outcome, and also confirm that in flies, as in humans, long-term outcome in galactosemia, like acute outcome, is recessive.

Impact of sublethal dietary galactose exposure in development and gal-1P accumulation on severity of the movement abnormality

Previously, we have demonstrated that *GALT*-null *Drosophila* raised in the absence of dietary galactose exhibit a movement abnormality as adults (Kushner et al., 2010), but that observation left open the question of whether dietary exposure to low, sublethal

Table 1. GALT enzyme activity levels detected in adult flies

Relevant genotype	GALT activity \pm s.e.m. (n) (pmol/ μ g protein/minute)	GALT activity (% of wild type)
dGALT ^{C2} homozygote	30.77 \pm 2.29 (12)	100
dGALT ^{ΔAP2} homozygote	Not detected (10)	0
dGALT ^{ΔAP2} ; UAS-hGALT ^{10A11} /+	0.7037 \pm 0.2950 (5)	2.3
dGALT ^{ΔAP2} ; UAS-hGALT ^{10A11} homozygote	1.995 \pm 0.429 (4)	6.5
dGALT ^{ΔAP2} / dGALT ^{C2}	25.39 \pm 3.88 (3)	82.5
dGALT ^{ΔAP2} Act5cGAL4 / dGALT ^{ΔAP2} ; UAS-hGALT ^{10B22} / +	395.7 \pm 85.5 (4)	1286
dGALT ^{C2} / Act5cGAL4; UAS-hGALT ^{10B22} / +	618.97 \pm 259.57 (5)	2017

Activities were measured in lysates prepared from adult flies of the indicated genotypes as described in Methods.

levels of galactose during development might exacerbate the phenotype. This is an important question considering the clinical parallels. To address this question, we tested the outcome severity of both *GALT*-null (*dGALT*^{ΔAP2} homozygotes) and control (*dGALT*^{C2} homozygotes) flies reared on food containing either glucose as the sole monosaccharide or both glucose and a small amount of galactose. The level of galactose used in these experiments (50 mM) represents <10% of the monosaccharide in the fly food, and we have previously demonstrated that this level of galactose causes no survival loss in *GALT*-null larvae (data not shown). All animals to be tested in the countercurrent device were switched to glucose-only food upon eclosion so that dietary galactose exposure was limited to the larval period.

Countercurrent analyses of all four categories of flies (those with and without *GALT*, and with and without larval exposure to dietary galactose) reconfirmed that *GALT*-null flies have a

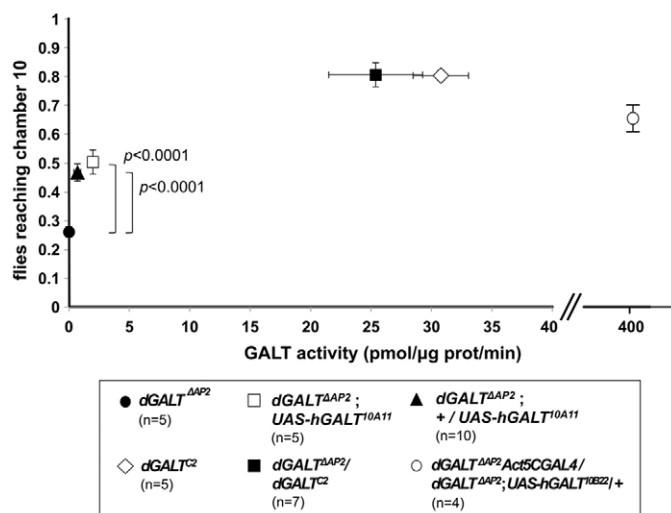
movement abnormality revealed by the countercurrent device, but also that early galactose exposure has no apparent impact on that phenotype (Fig. 4A).

To test whether exposure to galactose at this low level has any impact on the *GALT*-null *Drosophila*, we characterized the gal-1P levels in late stage larvae, both *GALT*-null and control, each harvested after 7 days of life on food containing either the standard level of glucose (555 mM), or that level of glucose supplemented with 50 mM galactose. Our results (Fig. 4B) confirmed that *GALT*-null larvae exposed to 50 mM galactose accumulate very high levels of gal-1P, whereas control larvae do not.

Finally, to ask whether the movement abnormality observed in *GALT*-null adult flies might reflect a continued presence of high gal-1P in these animals we also measured gal-1P in newly eclosed adults and in adults transferred to glucose-only food for 48 hours before analysis. As illustrated in Fig. 4B, gal-1P levels remained marginally elevated in newly eclosed *GALT*-null flies exposed to galactose during development, but after 48 hours on food lacking galactose this gal-1P had fallen essentially to baseline. These data confirm that by the time adult *GALT*-null flies were tested in the countercurrent device they no longer harbored high levels of gal-1P, regardless of whether or not they had been exposed to galactose as larvae.

Impact of age on the movement abnormality in *GALT*-null *Drosophila*

Climbing behavior in normal adult *D. melanogaster* slows as a function of age, largely due to decreased climbing speed (Rhodenizera et al., 2008). To test the impact of age on the movement phenotype of *GALT*-null *Drosophila*, we collected newly eclosed mutant and control males and allowed them to age, maintained at 25°C under non-overcrowding conditions, for an additional 7 or 14 days before subjecting the different cohorts to the countercurrent assay. The results were striking (Fig. 5). As expected, the control animals demonstrated a slow, progressive loss of ability to navigate the countercurrent device, such that at 2 days of age >70% of the animals reached the final chamber, at 9 days of age only about 50% reached the final chamber, and at 16 days of age fewer than 30% reached the final chamber. By contrast, the *GALT*-null animals demonstrated a loss of ability that was both accelerated and profound, such that at 2 days of age about 20% reached the final chamber, and at 9 or 16 days of age <5% reached the final chamber. In terms of raw numbers of flies that lost the ability to reach the final chamber between 2 and 9 days of age, the


Fig. 3. Relationship between GALT activity and a movement abnormality in *D. melanogaster*.

The ability of animals of each indicated genotype to reach the tenth chamber of a countercurrent device is plotted as a function of their adult GALT enzymatic activity. As GALT activity increased from a null toward wild-type levels, an animal's ability to reach the tenth chamber improved. There was no apparent impact from GALT overexpression. All values were modeled in a one-way ANOVA with a variable to control for day-to-day variations in testing. All values represent mean \pm s.e.m. *n*, number of cohorts tested.

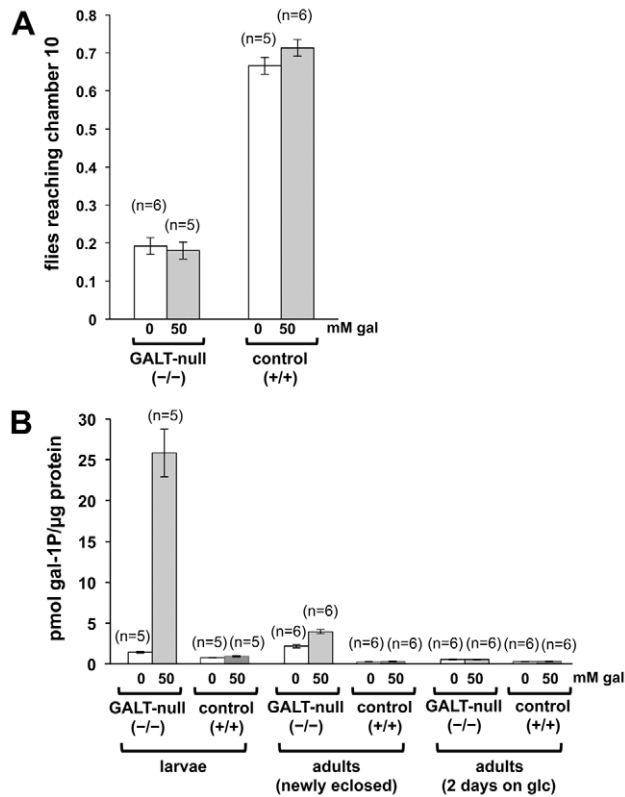


Fig. 4. Low-level galactose exposure during development has no impact on a movement abnormality in adult *GALT*-null *D. melanogaster*. (A) *GALT*-null and control animals were tested in the countercurrent device after being raised on a diet of food containing 555 mM glucose as the sole monosaccharide (white bars), or 555 mM glucose supplemented with 50 mM galactose (shaded bars). (B) Gal-1P accumulation in *GALT*-null and control larvae, newly eclosed adults or adults transferred following eclosion to glucose-only food for 2 days prior to analysis. As described in A, some animals were exposed during development to food containing glucose as the sole monosaccharide (white bars), whereas others were exposed to food containing glucose spiked with 50 mM galactose (shaded bars). Late-stage larvae and adult animals were collected and analyzed as described in Methods. Gal-1P levels were standardized to protein concentration. All values represent mean \pm s.e.m. *n*, number of cohorts tested.

GALT-null and control flies showed similar losses. However, in relative terms the losses were markedly different; the control animals suffered less than a 30% loss, whereas the mutants suffered a greater than fourfold loss. In short, aging the animals by 1 or 2 weeks prior to testing greatly widened the outcome gap between mutants and controls.

Microscopy reveals no clear anatomical defects in adult *GALT*-null fly brain or muscle

Considering the nature of the movement abnormality we hypothesized that *GALT*-null flies might have an anatomical defect visible in brain or muscle, and so performed the following histological studies. Sections from paraffin-embedded adult male animals, 24–48 hours post-eclosion, were stained with hematoxylin and eosin to visualize overall anatomy and tissue integrity. Histological examination of the brain demonstrated

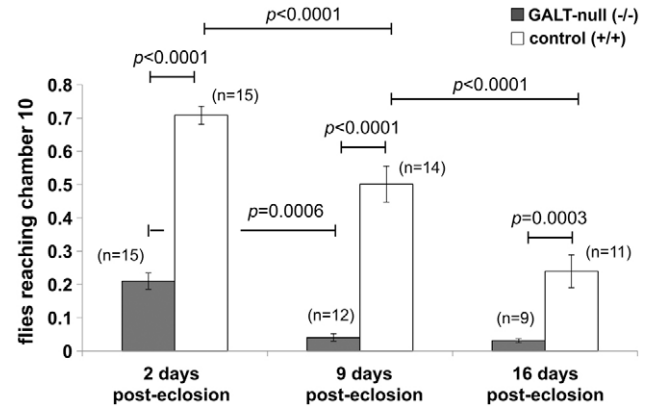


Fig. 5. Impact of age on movement in adult *GALT*-null *D. melanogaster*. *GALT*-null (shaded bars) and control flies (white bars) were tested using the countercurrent device at ages 2, 9 and 16 days post-eclosion. Whereas the control flies demonstrated a gradual, progressive decline with age the *GALT*-null flies demonstrated a decline that was notably accelerated and profound. All values represent mean \pm s.e.m. *n*, number of cohorts tested.

normal configuration of major brain structures (Fig. 6A, top). The cortex, which contains cell bodies of neurons and glia, was well preserved in *GALT*-null animals (Fig. 6A, top, arrows) as was the neuropil (Fig. 6A, top, asterisks). Vacuoles (Fig. 6A, top, arrowheads), which often accompany neurodegeneration in *Drosophila*, were modest in size and number and were present in both mutants and controls with equivalent frequencies. Histological examination of indirect flight muscle similarly revealed overall normal structure with no clear indication of malformation or degeneration (Fig. 6A, bottom).

To probe brain structures further, we performed immunostaining for well-characterized markers of mitochondria (ATP synthase, Fig. 6B), synapses (synapsin and the vesicular glutamate transporter) and axons (futsch) (Fig. 6C). No clear abnormalities were evident in the *GALT*-null animals. Finally, we repeated these studies on control and *GALT*-null flies that had been aged for 1 or 2 weeks following eclosion; again no clear differences were detected (data not shown).

DISCUSSION

Effective newborn screening coupled with prompt and rigorous dietary restriction of galactose prevents or resolves the acute and potentially lethal sequelae of classic galactosemia but does little, if anything, to prevent the long-term complications of the disease. Our goal is to understand the fundamental bases of long-term complications in galactosemia in the hope that this knowledge will lead to improved options for prognosis and intervention. In the work reported here, we have applied a *D. melanogaster* model of classic galactosemia to begin defining the genetic and environmental factors that modify long-term outcome in *GALT* deficiency. Of note, *Drosophila* is the only animal model reported to date that recapitulates aspects of either the acute or long-term complications of classic galactosemia (Kushner et al., 2010).

Previously, we reported that *GALT*-null *Drosophila* adults exhibit a movement defect despite being raised on food with no added

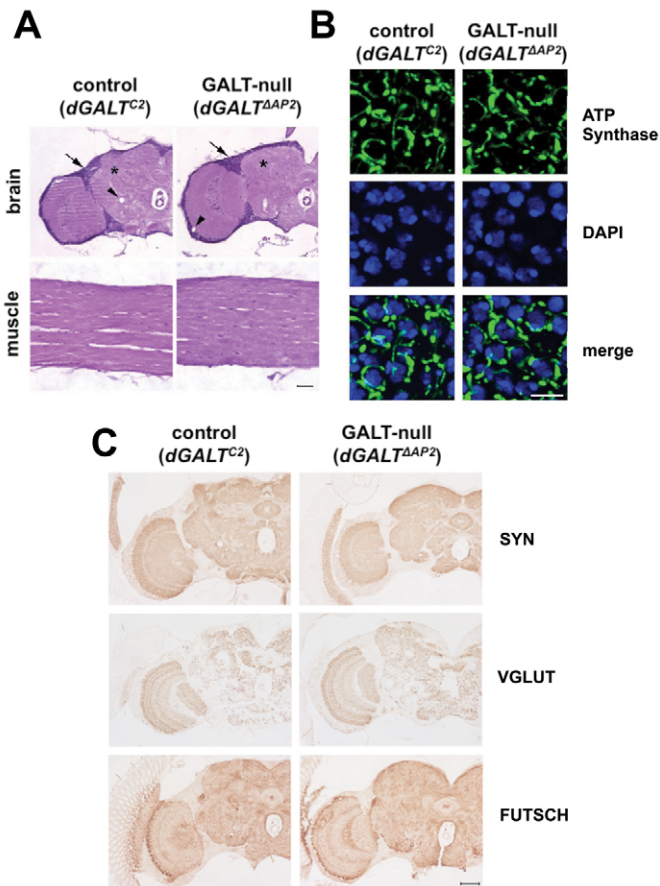


Fig. 6. *GALT*-null *D. melanogaster* show no apparent morphological defects in brain or muscle. (A) Representative images of adult brain (upper panels) and muscle tissue (lower panels) from *GALT*-null and control animals. Arrows in the brain images point to cortex, arrowheads point to vacuoles and the asterisk indicates normal neuropil. There were no apparent differences in gross anatomical structures between the *GALT*-null and control animals in either tissue. Scale bar: 20 μ m. (B) Representative immunofluorescence stains for mitochondria using an antibody directed to ATP synthase reveals no clear abnormalities of mitochondrial structure or number in *GALT*-null neurons. A DAPI counterstain was used to stain nuclei. Scale bar: 5 μ m. (C) Representative immunohistochemical stains for synapsin (SYN), the vesicular glutamate transporter (VGLUT) and the microtubule binding protein futsch revealed no clear defects in *GALT*-null animals. Scale bar: 40 μ m.

galactose (Kushner et al., 2010). Here we have extended from that result in five important ways.

First, we tested whether the ‘countercurrent’ abnormality reported earlier reflects a defect in climbing or in startle response. This is an important question because of implications for mechanism. The answer was that the abnormality reflects a defect in climbing, not in startle response.

Second, we asked whether trace levels of *GALT* activity might impact the severity of the movement defect. The answer was positive in that about 2.5% of wild-type *GALT* activity was sufficient to rescue most of the movement defect. This is an important result that parallels the clinical experience.

Third, we tested whether low-level galactose exposure in development, and the elevated gal-1P values that result, would

impact the severity of the movement defect in *GALT*-null adult flies. In our earlier report (Kushner et al., 2010), we demonstrated that the countercurrent defect occurred despite complete dietary galactose restriction, but we did not test whether cryptic galactose exposure might make the phenotype more severe. Here we demonstrate that exposure to 50 mM galactose, which causes no significant increase in mortality despite a greater than 20-fold increase in gal-1P accumulation, does not exacerbate the movement defect. This result challenges the idea that accumulated gal-1P leads to long-term complications in *GALT* deficiency.

Fourth, we tested the impact of age on the movement phenotype and noted a marked difference between controls and *GALT*-null animals. The decline with age for controls was gradual and progressive, but for *GALT*-null flies it was rapid and profound. This result suggests that physiological changes associated with aging overlap with the pathways that underlie the climbing defect in *GALT*-null flies.

Finally, careful light microscopic studies of brain and muscle in *GALT*-null and control flies collected at three different ages revealed no clear morphological differences. Although this result cannot rule out the existence of morphological defects that are subtle or tissue-specific, or evident only at a specific developmental stage, at face value it strengthens the argument that the defect in the mutants might be physiological rather than anatomical. Simply put, our current understanding is that a primary defect in biochemistry (*GALT* deficiency) leads to physiological changes that are either localized or systemic, and that these physiological changes ultimately lead to impaired neuronal or neuromuscular function and a movement abnormality in the mutant flies.

Combined, these data add substantially to our knowledge of long-term outcomes in *GALT*-null *Drosophila*, better characterizing the nature of the movement defect and also using it as a tool to begin exploring mechanism. These results further establish the utility of the fly model system for studies of long-term outcome in galactosemia, setting the stage for future work to define other outcomes and the genetic and/or environmental factors that underlie and modify those outcomes.

METHODS

Fly stocks and maintenance

All stocks were maintained at 25°C on molasses-based food that contained 44.4 g/l corn meal, 19.2 g/l yeast extract, 6 g/l agar, 52.5 ml/l molasses, 3 ml/l propionic acid and 13.8 ml/l methyl paraben (tegosept, 10% w/v in ethanol). For experiments designed to test the impact of dietary galactose exposure, animals were fed a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] with supplemental galactose added, as indicated. The *Drosophila GALT* alleles *dGALT*^{ΔAP2} and *dGALT*^{C2} and the human *UAS-hGALT* transgenes *UAS-hGALT*^{10A11} and *UAS-hGALT*^{10B22} used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock *y*^{1w^{67c23}}; P{SUPor-P}GALT^{KG00049}cup^{KG00049} (FBst0014339) from which the excisions were made, *w*¹¹¹⁸; Df(2L)Exel7027/Cyo (FBst0007801), and *y*^{1w*}; P{Act5C-GAL4}25FO1/Cyo,*y*⁺ (FBst0004414) were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. To generate animals carrying *Actin5c-GAL4* in the *dGALT*^{ΔAP2} background, the two alleles were

recombined onto the same second chromosome. Therefore, in experiments with human *GALT* transgene rescue, the following genotypes were used: *dGALT*^{ΔAP2} *Actin5c-GAL4*/ *dGALT*^{ΔAP2}; +/+ and *dGALT*^{ΔAP2} *Actin5c-GAL4*/ *dGALT*^{ΔAP2}; *UAS-hGALT*^{10B22}/+.

Countercurrent analysis of a movement abnormality in flies

For each experiment, a cohort of approximately 60 male flies, each less than 24 hours post-eclosion, was collected and aged for 36–48 hours on molasses food. On the day of testing, flies were added to the first tube in the lower rack and given 15 seconds per round to climb into the corresponding inverted tube in the upper rack. After each round, flies in the inverted tubes were shifted into juxtaposition with the next tubes in the lower rack and tapped down. After completing all nine rounds, flies were removed from all tube positions and counted. The proportion of flies in the final chamber (tube 10) was calculated. Every experimental day, cohorts of *dGALT*^{ΔAP2} and *dGALT*^{C2} homozygotes were analyzed in parallel with all the other experimental cohorts. Details of the data analysis are described in the ‘Statistics and regression analysis’ section.

GALT enzyme activity assays

Lysates from 10–20 male flies, each less than 24 hours post-eclosion, were prepared and analyzed as previously described (Sanders et al., 2010). *GALT* activity values less than 0.05 pmol/μg protein/minute were indistinguishable from zero and were reported as not detectable.

Dietary galactose exposure

Cohorts of newly eclosed *dGALT*^{ΔAP2} and *dGALT*^{C2} adults were allowed to lay embryos for 24–48 hours in vials containing food with either 555 mM glucose as the sole sugar, or 555 mM glucose plus 50 mM galactose. At the end of this time period the adults were removed and the embryos were allowed to develop and eclose. Cohorts of approximately 60 adult male flies, each less than 24 hours post-eclosion, were then collected from among the F1 generation and placed for 36–48 hours in vials containing food with 555 mM glucose as the sole sugar. These cohorts were tested in the countercurrent apparatus in parallel with their counterparts who had developed on food containing molasses. For animals of each genotype there was no statistical difference between the results obtained with any of these different cohorts.

Galactose metabolites in larvae and adults

Cohorts of newly eclosed male and female *dGALT*^{C2} or *dGALT*^{ΔAP2} animals were allowed to lay embryos for 24–48 hours in vials containing food with either 555 mM glucose as the sole sugar, or 555 mM glucose spiked with 50 mM galactose. Cohorts of larvae from these vials (approximately 100 μl packed volume) were collected after 7 days and washed in phosphate-buffered saline (PBS) prior to analysis. Animals remaining in the vials were allowed to pupate and eclose. Some of the newly eclosed males, in cohorts of 10, were collected directly for analysis. Other flies were transferred to vials containing food with 555 mM glucose as the sole sugar, where they were allowed to remain for 2 days prior to harvest for analysis. Finally, each cohort of larvae or adult flies to be analyzed for metabolites was resuspended in 125 μl of ice-cold HPLC-grade water and then homogenized, processed and analyzed as previously described (Kushner et al., 2010).

TRANSLATIONAL IMPACT

Clinical issue

Classic galactosemia is an autosomal recessive disorder caused by galactose-1-phosphate uridylyltransferase (*GALT*) deficiency; acute symptoms in response to dietary galactose exposure can be fatal in neonates if untreated. In populations served by newborn screening, and despite life-long dietary restriction of galactose, long-term complications – often including speech, cognitive, behavioral, ovarian and neurological dysfunction – are one of the most problematic aspects of the disease, and the current lack of accurate prognostic factors adds uncertainty to the burden for patients and families. Identifying the mechanisms underlying long-term complications, as well as potential points of therapeutic intervention, are important challenges that have yet to be addressed.

Results

This work represents the first exploration of candidate prognostic factors of one aspect of long-term outcome severity using the only animal genetic model currently available for outcome studies: *GALT*-null *Drosophila melanogaster*. They authors show that, similar to human patients, *GALT*-null flies show long-term movement abnormalities despite dietary restriction of galactose. In line with clinical data, cryptic residual *GALT* activity significantly lessens the severity of this long-term outcome. Surprisingly, exposure to low-level dietary galactose during development did not worsen severity, despite increasing levels of galactose-1-phosphate (gal-1P) by more than 20-fold. The authors also show that the movement disorder worsened with age, and suggest that the underlying defect might be physiological, because *GALT*-null flies do not show gross malformations in brain or muscle tissue.

Implications and future directions

These results have implications for possible prognostic factors in patients, and serve as proof of principle that *GALT*-null *Drosophila* are a useful model for studies of genetic and environmental modifiers of long-term outcome in *GALT* deficiency. In addition, the data challenge the idea that the accumulation of gal-1P in development causes long-term complications in the disease.

Histological analysis

Male flies aged for 1–14 days post-eclosion were fixed in 4% paraformaldehyde and processed for paraffin embedding. Serial 4-μm sections were taken through the entire head (for analysis of the brain) or thorax (for indirect flight muscle analysis). Slides were processed through xylene and ethanol, and into water. Standard hematoxylin and eosin staining was performed to evaluate overall anatomy and tissue integrity. Antigen retrieval by boiling in sodium citrate, pH 6.0, was used before immunostaining. Slides were blocked in PBS containing 0.3% Triton X-100 and 5% milk. Immunostaining was performed using the following mouse monoclonal primary antibodies: anti-synapsin (Developmental Studies Hybridoma Bank), anti-vGlut (Feany laboratory), anti-futsch (Developmental Studies Hybridoma Bank) and anti-ATP synthase (MitoSciences). For immunofluorescence, an Alexa-Fluor-488-conjugated anti-mouse secondary antibody was used. For immunohistochemistry, biotin-conjugated anti-mouse secondary antibody and avidin-biotin-peroxidase complex (Vectastain) staining was performed. Histochemical detection was performed by developing with diaminobenzidine (DAB).

Statistics and regression analysis

Data were analyzed using JMP SAS software version 8.0. Countercurrent data illustrated in Fig. 1 were modeled using a one-way ANOVA with a variable to control for day-to-day variation and test for differences in experimental conditions. Data illustrated

in Fig. 4A were analyzed using a hierarchically well-formulated linear regression to control for day-to-day variation and to test the interaction term (diet*genotype). In all instances, the data presented in the figures are the averages and standard errors from these analyses. Multiple comparisons were corrected using the Bonferroni correction ($\alpha=0.05/n$). Mean and standard error of the mean for enzyme activity (Table 1 and Fig. 3) and gal-1P levels (Fig. 4B) were calculated from the individual values.

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AUTHOR CONTRIBUTIONS

E. L. Ryan performed all experiments except those illustrated in Fig. 6; M. B. Feany and B. DuBoff performed the microscopy illustrated in Fig. 6. J. L. Fridovich-Keil conceived of and directed the project. All authors contributed to writing and editing the manuscript.

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