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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-Keil³,*

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 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
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 introduced more than 40 years ago (Akai, 1979; Benzer, 1967). To confirm and expand upon this observation, we repeated the analysis introduced more than 40 years ago (Akai, 1979; Benzer, 1967).

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\text{C2}\text{-null} carries a P-element insertion just upstream of the Drosophila GALT gene that does not interfere with GALT expression or activity. dGALT\text{AP2} is a nearly full deletion of the Drosophila GALT gene achieved by imprecise excision of the P-element from dGALT\text{C2}\text{-null} (Kushner et al., 2010). dGALT\text{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\text{AP2} and dGALT\text{C2}) more than 60% of each cohort reached the final chamber. Flies lacking GALT activity (dGALT\text{AP2} and dGALT\text{C2} with ActinSC-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.

Using this expanded countercurrent device we confirmed our previous result, demonstrating that GALT-null animals, homozygous for the dGALT\text{AP2} deletion allele (Kushner et al., 2010), were less likely than controls [dGALT\text{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\text{AP2} 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\text{C2}\text{-null}, this is the ancestral allele from which both dGALT\text{AP2} and dGALT\text{C2} were generated by imprecise and precise excision, respectively, of the P-insertion (Kushner et al., 2010). Animals homozygous for the dGALT\text{C2}\text{-null} 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\text{AP2} 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\text{AP2} homozygotes (data not shown). Finally, we crossed both the dGALT\text{AP2} and dGALT\text{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\text{1118} background, the dGALT\text{AP2} Oregon-R homozygotes were significantly less proficient at progressing to the final chamber than were their dGALT\text{C2} Oregon-R counterparts (data not shown).

To further test the connection between loss of GALT enzyme activity and the movement abnormality in dGALT\text{AP2} 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*<sup>AP2</sup> allele and carrying only the *Actin5cGAL4* driver without the *UAS-hGALT<sup>10B22</sup>* human GALT transgene demonstrated a countercurrent result similar to that described above for *dGALT*<sup>AP2</sup> homozygotes (Fig. 1). By contrast, *dGALT*<sup>AP2</sup> homozygotes carrying both the *Actin5cGAL4* driver and the *UAS-hGALT<sup>10B22</sup>* 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*<sup>AP2</sup> homozygotes that climbed above the predetermined height in 20 seconds was 0.256±0.068 whereas the corresponding number for *dGALT<sup>C2</sup>* homozygotes was 0.673±0.062 (Fig. 2A). Animals carrying the *Actin5cGAL4* driver without a *UAS-hGALT<sup>10B22</sup>* transgene in the *dGALT*<sup>AP2</sup> background performed similarly to *dGALT*<sup>AP2</sup> 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*<sup>AP2</sup> 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<sup>10A11</sup>*, that expressed low levels of GALT despite the absence of a GAL4 driver. In a *dGALT*<sup>AP2</sup> (endogenous GALT-null) background, animals carrying one allele of *UAS-hGALT<sup>10A11</sup>* demonstrated about 2.5% of wild-type GALT activity; animals carrying two alleles demonstrated just over 6% (Table 1). Intermediate GALT activity was achieved using animals heterozygous for one allele of the GALT deletion, *dGALT<sup>AP2</sup>* and one control allele, *dGALT<sup>C2</sup>*; 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 (*Actin5cGAL4*) in either an endogenous GALT-null (*dGALT*<sup>AP2</sup>) or a control (*dGALT<sup>C2</sup>*) 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...
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-ΔC2 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

<table>
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<th>Relevant genotype</th>
<th>GALT activity ± s.e.m. (n)</th>
<th>GALT activity (% of wild type)</th>
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<tr>
<td>dGALTΔC2 homozygote</td>
<td>30.77±2.29 (12)</td>
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<tr>
<td>dGALTΔAP2 homozygote</td>
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<td>0</td>
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<tr>
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<tr>
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<td>1.995±0.429 (4)</td>
<td>6.5</td>
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<tr>
<td>dGALTΔAP2 / dGALTΔC2</td>
<td>25.39±3.88 (3)</td>
<td>82.5</td>
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<tr>
<td>dGALTΔAP2 Act5cGAL4 / dGALTΔC2 / UAS-hGALT10B22/+</td>
<td>395.7±85.5 (4)</td>
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</tr>
<tr>
<td>dGALTΔC2 / Act5cGAL4; UAS-hGALT10B22/+</td>
<td>618.97±259.57 (5)</td>
<td>2017</td>
</tr>
</tbody>
</table>

Activities were measured in lysates prepared from adult flies of the indicated genotypes as described in Methods.
Movement disorder in GAL T-null flies

**RESEARCH ARTICLE**

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 GAL T-null fly brain or muscle

Considering the nature of the movement abnormality we hypothesized that GAL T-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 normal configuration of major brain structures (Fig. 6A, top). The cortex, which contains cell bodies of neurons and glia, was well preserved in GAL T-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).

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 GAL T-null Drosophila adults exhibit a movement defect despite being raised on food with no added...
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<sup>AP2</sup> and dGALT<sup>C2</sup>, and the human UAS-hGALT transgenes UAS-hGALT<sup>10A11</sup> and UAS-hGALT<sup>40B22</sup> used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010). All other alleles or stocks, including the P-element insertion stock y<sup>1w</sup>d<sup>67C2</sup>, P[SupPor-P]GALT<sup>hG0049</sup> and P[UAS-hGALT<sup>40B22</sup>] used here have been described previously (Kushner et al., 2010).
recombined onto the same second chromosome. Therefore, in experiments with human GALT transgene rescue, the following genotypes were used: dGALTΔAP2 ActinSc-GALA/ dGALTΔAP2; +/+ and dGALTΔAP2 ActinSc-GALA/ dGALTΔAP2; UAS-hGALTΔOB22/+.

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 dGALTΔAP2 and dGALTΔC2 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 dGALTΔAP2 and dGALTΔC2 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 dGALTΔC2 or dGALTΔAP2 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).

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

TRANSLATIONAL IMPACT

Clinical issue

Classic galactosemia is an autosomal recessive disorder caused by galactose-1-phosphate uridyltransferase (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: 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 melanogaster model currently available for outcome studies: 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.

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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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