Coordinated movement, neuromuscular synaptogenesis and trans-synaptic signaling defects in Drosophila galactosemia models

Patricia P. Jumbo-Lucioni¹, William M. Parkinson¹, Danielle L. Kopke¹ and Kendal Broadie¹,²,*

¹Department of Biological Sciences and ²Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, TN, USA

*To whom correspondence should be addressed at: Kendal Broadie, VU Station B, Box 35-1634, Nashville, Tennessee 37235-1634, TN, USA. Tel: +615-936-3937; Fax: +615-936-0129; Email: kendal.broadie@vanderbilt.edu

Abstract

The multiple galactosemia disease states manifest long-term neurological symptoms. Galactosemia I results from loss of galactose-1-phosphate uridylytransferase (GALT), which converts galactose-1-phosphate + UDP-glucose to glucose-1-phosphate + UDP-galactose. Galactosemia II results from loss of galactokinase (GALK), phosphorylating galactose to galactose-1-phosphate. Galactosemia III results from the loss of UDP-galactose 4’-epimerase (GALE), which interconverts UDP-galactose and UDP-glucose, as well as UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. UDP-glucose pyrophosphorylase (UGP) alternatively makes UDP-galactose from uridine triphosphate and galactose-1-phosphate. All four UDP-sugars are essential donors for glycoprotein biosynthesis with critical roles at the developing neuromuscular synapse. Drosophila galactosemia I (dGALT) and II (dGALK) disease models genetically interact; manifesting deficits in coordinated movement, neuromuscular junction (NMJ) development, synaptic glycosylation, and Wnt trans-synaptic signalling. Similarly, dGALE and dUGP mutants display striking locomotor and NMJ formation defects, including expanded synaptic arbours, glycosylation losses, and differential changes in Wnt trans-synaptic signalling. In combination with dGALT loss, both dGALE and dUGP mutants compromise the synaptomatrix glycan environment that regulates Wnt trans-synaptic signalling that drives 1) presynaptic Futsch/MAP1b microtubule dynamics and 2) postsynaptic Frizzled nuclear import (FNI). Taken together, these findings indicate UDP-sugar balance is a key modifier of neurological outcomes in all three interacting galactosemia disease models, suggest that Futsch homolog MAP1B and the Wnt Frizzled receptor may be disease-relevant targets in epimerase and transferase galactosemias, and identify UGP as promising new potential therapeutic target for galactosemia neuropathology.

Introduction

Galactose is metabolized in a series of reactions catalyzed by the three consecutive enzymes that comprise the Leloir pathway: galactokinase (GALK1), galactose-1-phosphate uridylytransferase (GALT) and UDP-galactose 4’ epimerase (GALE). Human deficits in the activity of any of these enzymes result in galactosemia disease states. Classic galactosemia (CG, OMIM 230400) results from loss of GALT activity (1) the second pathway enzyme (Figure 1A). After GALK1 phosphorylation, GALT converts galactose-1-phosphate and uridine diphosphate-glucose (UDP-glucose) into glucose-1-phosphate and UDP-galactose. GALE catalyzes the final step converting UDP-galactose to UDP-glucose, as well as their N-acetylated forms:
UDP N-acetylglucosamine (UDP-GlcNAc) to UDP N-acetylgalactosamine (UDP-GalNAc; Figure 1A) (2). These four UDP sugars are critical for the biosynthesis of glycoproteins and proteoglycans (3,4), which heavily populate the cell surface and secreted surroundings, including the extracellular synaptomatrix of the synaptic cleft and perisynaptic space (5). UDP-glucose pyrophosphorylase (UGP2) synthesizes UDP-glucose from glucose-1-phosphate and UTP, and alternatively forms UDP-galactose from galactose-1-phosphate and UTP (6), independent of GALT activity (Figure 1A). UGP2 is present in a wide array of organisms (7,8) since UDP-glucose, the active form of glucose, is a central player in almost all living systems. The dual roles of UGP2 in both glucose and galactose metabolic pathways underscore its central importance as an alternative enzymatic route in galactosemia patients (9).

Classic galactosemia (aka galactosemia I) is the second most common inherited metabolic disorder among US children (10), and the most frequent and clinically severe galactosemia disease state (11). Galactosemia II (OMIM 230200) results from loss of GALK1, which we have previously established as a genetically interacting condition (12). Galactosemia III (OMIM 230250) results from loss of GALE, and is the rarest and least well understood form of galactosemia (13). Galactosemia I is often detected pre-symptomatically in newborn screening, and lifelong dietary galactose withdrawal, the current standard of care, resolves at least the acute life-threatening symptoms. Dietary intervention also resolves early onset cataracts in galactosemia II, the main disease symptom (14,15). However, long-term neurological symptoms arise in maturing galactosemia patients, including movement defects, speech delay and cognitive

Figure 1. dGALE phenocopies dGALT and co-removal worsens coordinated movement. (A) Schematic diagram of glucose/galactose pathways showing targeted dGALT, dGALE and dUGP gene products. (B) The dGALT interaction network generated with the Search Tool for Retrieval of Interacting Genes (STRING). Line thickness represents the strength of predicted interactions. (C) Representative NMJs co-labelled with anti-horseradish peroxidase (HRP; green) and anti-Discs Large (DLG; red) in background control (w1118) and homozygous dGALE mutant (dGALE); precise excision dGALT control (dGALT), homozygous dGALT null mutant (dGALTnull) and double mutant (dGALTnull; dGALE) combinations. Movement time for coordinated roll-over behaviour. (D) NMJ bouton. (E) and branch. (F) number in the same genotypes, normalized to respective genetic controls. Sample size: 8 animals per genotype. Error bars show SEM with significance indicated as *P<0.05, **P<0.01 and ***P<0.001.
disability (11), which are not prevented by dietary galactose control. Moreover, galactosemia III is a continuum disorder that can present life-threatening symptoms and long-term neurodevelopmental sequelae (11, 16), and galactose restriction is particularly problematic as GALE plays a pivotal role maintaining UDP sugar balance during the synthesis of glycoproteins. Thus, the current treatment for GALE-associated galactosemia requires a balance between restricting galactose and providing enough for UDP sugar synthesis (17).

A large body of research documents glycosylation defects in galactosemia patients (18–20). Previous studies reveal conserved glycosylation defects in the Drosophila galactosemia I model (dGALT null mutant), and show the correction of glycosylation defects with co-removal of dGALK, in the Drosophila galactosemia II model (12). dGALT nulls exhibit severe coordinated locomotor movement deficits, as well as underlying strikingly overelaborated neuromuscular junction (NMJ) synaptic architecture. Importantly, the disease state is restricted to morphological defects without changes in neurotransmission strength. Assaying the heavily-glycosylated NMJ synapomatrix with a series of lectin probes shows profound synaptogenesis defects without changes in neurotransmission strength. In this assay, wandering L3 larvae (wL3) are placed in an inverted position and the time to righting is measured. Compared to genetic background controls (12). This work strongly suggests NMJ glycan-dependent Wnt signalling defects as a basis for movement deficits in galactosemia I.

In the current study, we set forth to characterize a Drosophila galactosemia III model (dGALE mutant), as well as test the bypass pathway (dUGP mutant) for galactose metabolism. We find both dGALE and dUGP phenocopy coordinated movement deficits and impaired NMJ synaptogenesis characterizing dGALT. Loss of dGALT elevates neurotransmission strength, which is further heightened by co-removal of dGALK. Defects in all three mutants include synapomatrix glycosylation losses, elevated synaptic bouton formation and differences in the core Wg/Fz2/Dlp trans-synaptic signalling pathway components. Both dGALT and dUGP display increased Futsch/MAP1b microtubule remodeling in the presynaptic neuron, whereas dUGP exhibits elevated Frizzled nuclear import (FNI) in the postsynaptic muscle. These new findings reveal 1) key glycosylation pathway modifiers of Wnt trans-synaptic signalling, and 2) identify two new diseases-relevant targets, Futsch/MAP1b and Wnt Frizzled receptor, for intervention in galactosemia-associated neuropathology. Both dGALT and dUGP interact with dGALT, modifying behavioural deficits and changes in synaptic architecture in the Drosophila galactosemia I model. Synaptic glycosylation losses, particularly of N-acetylgalactosamine residues, are exacerbated. These findings indicate that differential glycosylation at the NMJ synapomatrix modulates neurological movement outcomes in interacting galactosemia disease states, and further suggest UGP as a promising new therapeutic target for galactosemia treatment.

**Results**

**Neuronal dGALE loss worsens behavioural outcomes in dGALT null mutants**

Movement defects are common among GALT-deficient galactosemia patients (24–28). Similarly, dGALT is necessary for proper coordinated movement in the Drosophila disease model, and dGALK co-removal corrects this behavioural deficit (12). In the galactose pathway, dGALK intersects with dGALT downstream (Figure 1A), and the Search Tool for Retrieval of Interacting Genes (STRING) (29) identified dGALK as a promising candidate interactor (Figure 1B), consistent with previous evidence of an interactive relationship (30). GALE-deficient galactosemia patients also exhibit deficits in coordinated motor skills that appear early in development (31, 32). However, most of the severe patient cases arise from consanguineous parents, which raises the possibility that homozygosity of autosomal recessive alleles other than GALE may underlie some of the severe movement symptoms reported in galactosemia III (31). Animal models have shown GALE activity is essential for viability (33) and normal development (34, 35), but movement phenotypes have not been investigated despite evidence of GALE expression in neurons and muscle in C. elegans (35). We therefore set out to assay coordinated movement in our Drosophila model. Null dGALE mutants manifest early lethality, confirming that dGALE is essential for viability, and therefore viable dGALE hypomorphic mutants with residual ~8% dGALE activity (34) were tested.

A well-established test for Drosophila coordinated movement is the larval rollover assay, which requires a complex set of integrated behaviours to be properly executed, as previously described (12). In this assay, wandering L3 larvae (wL3) are placed in an inverted position and the time to righting is measured. Compared to genetic background controls (12). We hypothesized dGALT would exacerbate dGALT movement impairments since dGALE synthesizes UDP-sugar precursors when limited by dGALT deficiency (12). Consistently, the double mutants (homozygous dGALT<sub>AP2</sub>; dGALE<sub>AP2</sub>) manifest 2- and 4-fold slower responses (17.7 ± 6.0 s, n = 23, P < 0.01; Figure 1D). We hypothesized dGALK would exacerbate dGALE movement impairments since dGALK synthesizes UDP-sugar precursors when limited by dGALK deficiency (23). Similarly, as previously reported (12), dGALT null (dGALT<sub>AP2</sub>) animals are significantly movement impaired, also manifesting ~2-fold slower righting time compared to precise-excision genetic background (dGALT<sub>AP2</sub>) matched controls (n = 36,38, P < 0.001; Figure 1D). We found that dGALT and dGALE contribute equally to the control of coordinated movement, and that loss of the two genes has an additive effect on coordinated movement deficits.
(9.0 ± 0.94 s, n = 14, P = 0.0002; Figure 2B). Similarly, targeted muscle dGALE RNAi (24B-Gal4→dGALE-RNAi) causes ≥2-fold slower rollover (10.4 ± 1.64 s, n = 24) compared to genetic controls (24B-Gal4/+ , 4.7 ± 0.46 s, n = 28, P = 0.002). These findings identify a neuronal and muscle dGALE requirement for properly controlled coordinated movement. Similar to the above dGALT; dGALE double mutant condition, targeted dGALE neuronal knockdown in the dGALT null background (homozygous dGALT<sup>ΔAQP</sup>; elav-Gal4/+), causes further slowing of rollover time (34.6 ± 11.46 s, n = 25) compared to dGALT with driver alone (dGALT<sup>ΔAQP</sup>; elav-Gal4/+) animals (15.7 ± 1.61 s, n = 29), as well as precise excision control (dGALT<sup>ΔC8</sup>) animals (8.8 ± 0.77 s, n = 51, P < 0.001; Figure 2B). Conversely, postsynaptic targeted dGALE knockdown in the dGALT null background (homozygous dGALT<sup>ΔAQP</sup>; 24B-Gal4→dGALE-RNAi) does not further compromise locomotor coordination (20.8 ± 2.14 s, n = 22) compared to dGALT alone (dGALT<sup>ΔAQP</sup>; elav-Gal4/+ : 18.4 ± 2.67 s, n = 9). Taken together, these results demonstrate that neuronal and muscle dGALE both contribute to properly controlled coordinated movement, and that presynaptic, but not postsynaptic, dGALE is a strong genetic modifier of dGALT disease model behavioural deficits in an additive manner. As these movement defects have been closely associated with changes in NMJ morphological development (12), we next examined the synaptic architecture in dGALE single mutants and in dGALE; dGALT double mutant animals.

**Neuronal dGALE regulates neuromuscular synaptogenesis and transmission strength**

We conducted a Drosophila glycosene screen using transgenic RNAi knockdown of a wide range of N/O-linked glycans, glycosaminoglycans, glycosyltransferases and glycan-binding lectins to test effects on NMJ structure and function (21). This screen identified dGALT as a strong negative regulator of NMJ structural synaptogenesis, but not functional differentiation, and we subsequently confirmed that dGALT acts to restrict terminal arbour branching and synaptic bouton formation (12). Since defects in NMJ morphogenesis have been shown to underlie locomotor deficits (12,36,37), we hypothesized that similar defects could account for the movement limitations occurring in the absence of neuronal dGALE, as well as for the worsening of movement deficits in dGALT; dGALT double mutant conditions. To assay synapse architecture, muscle 4 NMJs from wL3 animals were co-labelled with presynaptic anti-horseradish-peroxidase (HRP, green) and postsynaptic anti-Discs Large (DLG, red) in single and double mutant animals. Synaptic boutons (>1μm in minimum diameter) and branches (axonal processes with >2 boutons) were counted. Representative images and quantification for mutant and control genotypes are shown in Figures 1 and 2.

The synaptic bouton number is increased by >50% in dGALE mutants (30.3 ± 2.0, n = 8 animals) compared to w<sup>1118</sup> genetic background controls (19.7 ± 1.7, n = 10, P = 0.002; Figure 1E).
Targeted neuronal dGALE knockdown (elav-Gal4 → dGALE-RNAi) animals similarly show a significant, although smaller (~17%), increase in bouton number compared to driver alone (elav-Gal4/+ ) genetic controls (n = 13,16; P = 0.01; Figure 2C). While both dGALE mutants and neuron-targeted dGALE-RNAi animals display obvious NMJ over elaboration (Figures 1C, 2A), muscle-targeted dGALE-RNAi does not cause a detectable change in synaptic architecture or a significant increase in synaptic bouton number compared to genetic controls (25.1 ± 1.1 boutons, n = 35 vs. 26.9 ± 1.2 boutons, n = 35, respectively). These findings show a specific requirement for dGALE in the neuron, in this case to restrict NMJ synaptogenesis. In double mutant conditions, the NMJ architectural complexity characterizing dGALE nulls is exacerbated by simultaneously reducing dGALE activity (Figure 1C). Compared with the significantly greater number of boutons in dGALT single mutants (34.5 ± 1.6, n = 33), double mutant synapses (homozygous dGALT<sup>−/−</sup>; dGALT<sup>−/−</sup>) develop ~50% and ~20% more boutons (39.8 ± 2, n = 40), compared to genetic control (dGALT<sup>−/+</sup>) (26.4 ± 1.3, n = 26, P < 0.001; Figure 1E) and dGALE single mutants (Figure 1E), respectively. Neuronal dGALT knockdown in the dGALT null background did not further increase NMJ structural complexity (Figure 2C,D). Single dGALT<sup>−/−</sup> and dGALE-RNAi also did not increase synaptic branching (Figures 1F,2D), in contrast to dGALE mutants (3.8 ± 0.2, n = 33) which increased synaptic branching compared to controls (2.6 ± 0.1, n = 24, P < 0.001; Figure 1F). There is a tendency for branching to increase further in dGALE; dGALE double mutants (4 ± 2, n = 40), but the effect is not significant (Figure 1F).

To test NMJ functional differentiation, we performed two-electrode voltage clamp (TEVC) electrophysiological recordings (12). The innervating motor nerve was stimulated with a glass suction electrode while recording from the voltage-clamped muscle 6. Excitative junctional current (EJC) records were made at 0.2 Hz frequency with 0.5 ms duration stimuli at a suprathreshold voltage. ≥25 NMJs from ≥10 different w13 animals were recorded from each of three genotypes: genetic background control (w<sup>1118</sup>), dGALT alone (dGALT<sup>−/−</sup>) and a double mutant in combination with dGALT (dGALT<sup>−/−</sup>; dGALT<sup>−/−</sup>). Neurotransmission strength is very obviously elevated with loss of dGALE activity (Figure 3A). Mean EJC amplitudes significantly increased in mutants compared to control (313.2 ± 15.6 nA vs. 241.4 ± 8.6 nA; P < 0.001; Figure 3B). Strikingly, the double mutant combination exhibits further heightened neurotransmission (Figure 3A). Mean EJC amplitudes significantly increased in double mutants (dGALT<sup>−/−</sup>; dGALT<sup>−/−</sup>; dGALE<sup>−/−</sup>; dGALE<sup>−/−</sup>) compared to both w<sup>1118</sup> control (P < 0.001, Figure 3B) and single dGALE mutants (P < 0.001, Figure 3B). In summary, these results show that dGALE is a strong genetic modulator of both neuromuscular structural synaptogenesis and functional differentiation alone and in combination with dGALT loss at the NMJ synapse.

**dGALT genetic modifier dUGP regulates coordinated movement and NMJ architecture**

STRING analyses (29) further identified dUGP as a second highly-associated dGALT interactor (Figure 1A,B). In Drosophila, CG4347 encodes UDP-glucose pyrophosphorylase (EC 2.7.7.9) as the only enzyme capable of producing UDP-glucose from glucose-1-P and UTP (38-41) (Figure 1A). Like dGALT, dUGP is essential for viability and, consistently, no human patients with UGP loss-of-function mutations have been reported. We therefore characterized two dUGP hypomorphic mutations (dUGP<sup>−/−</sup>, dUGP<sup>−/−</sup>) generated by transposable element insertion (42). Neither mutant is homozygous viable, but viability is restored with significantly reduced survival in heteroallelic combination (dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup>). We first tested dUGP mutants for coordinated movement behaviour using the same larval roll-over assay as above. Compared to w<sup>1118</sup> genetic background controls (7.6 ± 0.71 s, n = 29), UGP<sup>−/−</sup> (11.6 ± 1.33 s, n = 15, P < 0.05) and UGP<sup>−/−</sup> (16.0 ± 1.82 s, n = 15, P < 0.001) animals are both significantly slower and obviously less coordinated (Figure 4B). The coordinated movement time for UGP<sup>−/−</sup> is not significantly different from UGP<sup>−/−</sup> but displays a significant further reduction in coordinated movement (18.6 ± 1.73 s, n = 15) compared to single dUGP heterozygotes (P < 0.05) and w<sup>1118</sup> controls (P < 0.001; Figure 4B). Ubiquitous dUGP knockdown (UH1-Gal4 > dUGP-RNAi) caused the strongest effect, with ≥25-fold longer time to upright position (25.2 ± 2.78 s, n = 10) compared to control (UH1-Gal4: 9.3 ± 1.47 s, n = 9, P = 0.004). Tissue-targeted neural (elav-Gal4 > dUGP-RNAi: 13.6 ± 1.82 s, n = 29, P < 0.05) and muscle (24B-Gal4 > dUGP-RNAi: 19.7 ± 4.40 s, n = 12, P < 0.05) knockdown both significantly impaired coordinated movement compared to the driver alone controls (elav-Gal4/+; 8.8 ± 0.70 s, n = 23; 24B-Gal4/+; 8.2 ± 1.0 s, n = 14).

UGP2 loss causes a dramatic reduction in UDP-glucose, an essential precursor for the biosynthesis of proteoglycans (43), which are key components of the NMJ synaptomatrix and established regulators of NMJ development (5). Although UGP2 has a higher affinity for glucose-1-P, it also catalyzes UDP-galactose from galactose-1-P and UTP (6,9), particularly at the high galactose-1-P levels in GALT-deficient galectosia patients (11). Thus, UGP2 represents an alternate GALT-independent pathway for galactose metabolism (9) (Figure 1A). We therefore hypothesized that dUGP loss would enhance behavioural impairments in dGALT mutants and produce an exacerbated phenotype. Since dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup> is the most severely impaired condition, coordinated movement was next assayed in the homozygous dGALT<sup>−/−</sup>; dUGP<sup>−/−</sup> dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup> combination (Figure 4B). As predicted, these double mutant animals are obviously less coordinated than either of the single mutant conditions, with a 2-fold slower roll-over time (44.1 ± 10.94 s, n = 27) compared to dGALT nulls alone (22.4 ± 2.53 s, n = 23, P < 0.05) and ≥4-fold change compared to genetic background controls (10.3 ± 0.90 s, n = 50, P < 0.001; Figure 4B). These results show that dUGP plays a particularly important role enabling coordinated movement, and that co-deletion of dUGP and dGALT combinatorially further impairs the ability to move in a coordinated manner.

We next assayed NMJ synaptogenesis in dUGP mutants (Figure 4A). Like both dGALT and dGALT mutants, loss of dUGP causes NMJ overgrowth and structural over elaboration in UGP<sup>−/−</sup>, UGP<sup>−/−</sup>- and UGP<sup>−/−</sup>/UGP<sup>−/−</sup> mutants. Compared to the w<sup>1118</sup> genetic background control (21.2 ± 1.0 boutons, n = 22), both UGP<sup>−/−</sup> (+28.3 ± 2.1, n = 12, P < 0.05) and UGP<sup>−/−</sup> (+30.7 ± 1.4, n = 17, P < 0.01), as well as dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup> (28.8 ± 2.7, n = 6, P < 0.001), developed significantly more synaptic boutons (Figure 4B). Synaptic branching tends to increase in all dUGP mutants, but is only significantly greater in UGP<sup>−/−</sup> (+2.8 ± 0.2, n = 12) compared to w<sup>1118</sup> genetic controls (2.3 ± 0.2, n = 22, P < 0.05; Figure 4D). Single dGALT nulls (dGALT<sup>−/+</sup>; dGALT<sup>−/+</sup>; 32.9 ± 1.9, P < 0.05, n = 19) and particularly, the double mutant combination (dGALT<sup>−/−</sup>; dGALT<sup>−/−</sup> dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup>; dUGP<sup>−/−</sup>; 35.7 ± 1.6, P < 0.001, n = 29) developed significantly more synaptic boutons compared to the dGALT<sup>−/−</sup> precise excision controls (24.9 ± 1, n = 22; Figure 4C). Synaptic branching increases in dGALT<sup>−/−</sup> mutants (3.3 ± 0.3, n = 19)
compared to dGALT D C2 controls (2.7 ± 0.1, n = 22), but there is a further significant increase in branching in double mutants (3.9 ± 0.2, n = 29, P < 0.001; Figure 4D). Consistently, ubiquitous dUGP knockdown (UH1-Gal4 > dUGP-RNAi) significantly increases synaptic bouton (35.5 ± 1.56, n = 11, P < 0.0003) and branch (3.36 ± 0.20, n = 11, P < 0.0017) numbers compared to driver alone controls (UH1-Gal4/+; 25.2 ± 1.56, n = 10 and 2.25 ± 0.23, n = 10, respectively). However, structure is over-elaborated in pan-neuronal dUGP-knockdown animals (26.3 ± 1.0 boutons, n = 10, P = 0.007), although unaffected in muscle-targeted dUGP-RNAi larvae (25.4 ± 2.4 boutons, n = 11) compared to their appropriate controls (22 ± 1.1, n = 12 and 20.2 ± 1.4, n = 15, respectively).

As above, we next tested NMJ functional differentiation with TEVC electrophysiological recordings (Figure 3). >12 NMJs from ≥6 different w1118 animals were recorded from each of three genotypes: genetic background control (w1118), dUGP single mutant (dUGP w1118) and double mutant animals (dGALT^A^P2 dUGP w1118). (B) EJC peak amplitude quantification for all three genotypes. Sample size: ≥25 NMJs for each genotype. Error bars show SEM with significance indicated: ***P < 0.001. (C) Representative EJC traces from control (w1118), single dUGP mutant (dUGP/dUGP) and double mutant animals (dGALT^A^P2 dUGP/dUGP). (D) EJC peak amplitude quantification for all three genotypes. Sample size: ≥12 NMJs for each genotype. There is no significant differences.

compared to dGALT^A^P2 controls (2.7 ± 0.1, n = 22), but there is a further significant increase in branching in double mutants (3.9 ± 0.2, n = 29, P < 0.001; Figure 4D). Consistently, ubiquitous dUGP knockdown (UH1-Gal4 > dUGP-RNAi) significantly increases synaptic bouton (35.5 ± 1.56, n = 11, P < 0.0003) and branch (3.36 ± 0.20, n = 11, P < 0.0017) numbers compared to driver alone controls (UH1-Gal4/+; 25.2 ± 1.56, n = 10 and 2.25 ± 0.23, n = 10, respectively). However, structure is over-elaborated in pan-neuronal dUGP-knockdown animals (26.3 ± 1.0 boutons, n = 10, P = 0.007), although unaffected in muscle-targeted dUGP-RNAi larvae (25.4 ± 2.4 boutons, n = 11) compared to their appropriate controls (22 ± 1.1, n = 12 and 20.2 ± 1.4, n = 15, respectively).

As above, we next tested NMJ functional differentiation with TEVC electrophysiological recordings (Figure 3). >12 NMJs from ≥6 different w1118 animals were recorded from each of three genotypes: genetic background control (w1118), dUGP transheterozygotes (dUGP/dUGP) and double mutant animals (dGALT^A^P2 dUGP/dUGP). Unlike dGALT, neurotransmission strength is closely comparable between all three genotypes (Figure 3C). Mean EJC amplitudes from single mutants (219.35 ± 12.60 nA) are not significantly different from w1118 control (230.36 ± 19.81 nA) or the double mutant combination (242.58 ± 15.09 nA, P > 0.05, Figure 3D). Taken together, these results show that dUGP is important for coordinated movement and neuromuscular structural synaptogenesis, but is not detectably required for synapse function. These findings show that co-removal of dUGP and dGALT interact to modify the severity of behavioural and structural mutant phenotypes. Since there is extensive evidence showing synaptic glycosylation restricts NMJ morphogenesis (12,21,22), we next proceeded to examine the synaptic glycan environment in single and double mutant combinations.

dGALT and dUGP both shape NMJ synaptomatrix glycan composition

GALT plays a crucial role maintaining UDP-sugar balance for glycosylation (2), with a primarily role in UDP-galNAc biosynthesis. Unlike UDP-glc and UDP-glcNAc, UDP-galNAc cannot be synthesized by a GALT-independent pathway (Figure 1A). Indeed, recent studies reveal that GALT loss-of-function in C. elegans causes a strong reduction of UDP-galNAc levels
accompanied by developmental defects unique to this UDP-sugar (35). We previously found similar glycan losses at the NMJ synaptomatrix in the absence of dGALT (12). Since this is the only glycan defect corrected by transgenic hGALT expression in the dGALT null background (12), it provides a potential mechanistic basis for the coordinated movement and morphological synaptogenesis defects in this disease model. We hypothesized that dGALE deficiency, like loss of dGALT, would compromise galNAc abundance in the NMJ synaptomatrix in the new Drosophila epimerase galactosemia model, driving the synaptogenesis and movement deficits in dGALE mutants. To test this hypothesis, we first probed with Wisteria floribunda lectin (WFA, green) to label terminal galNAc residues in NMJs marked with Fasciclin-II (FasII, red). Representative images and data summary are shown in Figure 5.

The synaptic marker FasII does not vary significantly between tested genotypes, comparing w¹¹¹⁸ genetic background control and dGALE⁰ mutant NMJs (Figure 5A). In sharp contrast, while w¹¹¹⁸ control NMJs (n = 38) are very highly enriched with WFA-labelled galNAc residues, dGALE⁰ synapses (n = 10) show a dramatic ~60% loss of WFA label (P < 0.001; Figure 5A,C). To test whether dGALT co-removal would worsen the phenotype, dGALT; dGALE double mutants were next examined. The dGALT⁰/dGALE⁰ single mutant decreases WFA labelling by ~25% compared to precise controls (n = 42, 46; P < 0.001; Figure 5A,C), and the double mutants (dGALE⁰/dGALT⁰; dGALE⁰/dGALT⁰; dGALT⁰/dGALE⁰; dGALT⁰/dGALE⁰) exhibit a further ~35% reduction, significantly different from the single mutant condition (dGALT⁰/dGALE⁰, P < 0.05; Figure 5C). Fucosylation defects are also detected in galactosemia (44), and the Drosophila dGALT disease model manifests a loss of anti-HRP labelling at the NMJ (12), a commonly employed synaptic marker recognizing α1,3-fucosylation. We therefore hypothesized dGALE mutants would manifest a similar glycosylation defect that would be exacerbated by co-removal of dGALT. Consistently, dGALE⁰ NMJs show significantly reduced HRP glycan levels compared to controls (n = 6, 6; P = 0.006), similar to dGALT⁰/dGALT⁰ NMJs compared to controls (n = 16, 17; P = 0.05), with the dGALT; dGALE double mutants exhibiting a stronger ~30% reduction in HRP glycan levels (n = 16, P < 0.001 compared to dGALT⁰/dGALT⁰; Figure 5D). These results show that dGALT and dGALE both shape the glycan composition of the developing NMJ synaptomatrix.

We next tested dUGP single and double mutants with the same WFA and HRP probes (Figure 5A,B). While WFA-labelling in single dUGP mutants (UGP¹/+, n = 8; UGP²/+, n = 9) is not significantly different from matched controls (n = 26), the stronger UGP²/UGP² combination shows a ~40% reduction in WFA-labelled galNAc residues (n = 9, P < 0.01; Figure 5C). The dGALT⁰/dUGP² single mutant shows a ~25% decrease (n = 42) compared to controls (n = 46, P < 0.001; Figure 5C), and the double mutants (dGALT⁰/dUGP²/dGALE⁰; dUGP²/dGALE⁰) exhibit a further decrease in WFA labelling (~30% reduction, n = 26) compared to matched controls (n = 46, P < 0.001; Figure 5C). Anti-HRP labelling for fucosylation defects shows dUGP²/dUGP² exhibits a ~30% reduction (n = 10) compared to w¹¹¹⁸ genetic controls (n = 11, P < 0.05; Figure 5D). Both single dGALE⁰ (n = 29) and double dGALT⁰/dGALE⁰ (n = 18) combinations show a similar ~20% decrease in fucosylated residues at the NMJ, a significant decrease compared to controls (n = 30; P < 0.05 and P < 0.01, respectively; Figure 5D). Taken together, these results show that dGALT and dGALE are strong genetic modifiers of NMJ synaptomatrix glycan composition, partially overlapping with dGALT requirements in synaptic glycosylation. Since we have previously found that these synaptomatrix defects alter NMJ...
synaptogenesis via modulation of the Wg trans-synaptic signalling pathway (12), we next test whether this pathway is compromised in dGALE and dUGP single and double mutant combinations.

**dGALE and dUGP both regulate wnt trans-synaptic signalling at the NMJ**

The heavily-glycosylated cell surface and extracellular space modulates ligand-mediated signalling in normal and disease states (45), and the Drosophila NMJ glycosylated synaptomatrix has been repeatedly shown to fine-tune trans-synaptic signalling driving synaptogenesis (5,12,22). In the core Wnt pathway, UDP-sugar availability could also impact the glycosylation status of the secreted Wingless (Wg) glycoprotein ligand (46) and biosynthesis of Wg co-receptor HSPG Dlp (21), required for the optimal processing, availability and presentation of Wg within the synaptic cleft. We have previously shown that dGALT mutants increase Wg and reduce Dlp levels at the Drosophila NMJ (12). We therefore hypothesized that dGALE and dUGP mutants would likewise impact the Wg...
trans-synaptic signalling pathway to similarly alter NMJ synapticogenesis and impair coordinated movement. We tested this hypothesis by assaying the dual outputs of the Wg pathway; 1) presynaptic Wg activation of Futsch/MAP1b remodeling of the synaptic bouton microtubule cytoskeleton (47) and 2) postsynaptic Wg activation of the Frizzled nuclear import (FNI) pathway involving cleavage of the Frizzled-2 (Fz2) receptor and trafficking of the carboxyl-terminal signalling domain (Fz2-C) to the muscle nuclei (48–50). Representative images and data summaries of these analyses are shown in Figures 6 and 7.
Compared to w1118 genetic background controls, dGALE mutant NMJs display a striking and immediately obvious loss of the Wg signalling ligand (Figure 6A). In quantifying Wg label intensity, dGALEh exhibits a highly significant \(50\%\) decrease in Wg compared to matched controls \((n=10,10; P=0.0007; \text{Figure } 6B)\). This contrasts sharply with dGALT mutants, which show the opposite \(50\%\) increase in Wg levels compared to matched controls \((n=31,36; P<0.001; \text{Figure } 6A,B)\). Perhaps additively, double mutants \((dGALEh/dGALEh; dGALT^AP2/dGALT^AP2)\) show a restoration of Wg ligand levels to the wildtype condition \((n=23; P<0.001 \text{ compared to } dGALT^AP2; \text{Figure } 6A,B)\). In contrast, compared to genetic controls both dGALE and dGALT mutant NMJs display lower levels of the Wg co-receptor Dlp (Figure 6C). Quantification of Dlp labelling intensity shows a similar \(20\%\) loss in dGALEh compared to controls \((n=15,16; P=0.03)\) and dGALT^AP2 compared to controls \((n=56,59; P<0.05; \text{Figure } 6D)\).
Double mutants were not significantly worse than the single mutants alone, but display a very significant loss of Dlp compared to matched controls (n = 26,59; P < 0.01; Figure 6D). These results show that dGALE strongly impacts Wg pathway components, but that dGALE effects on Wg ligand are different compared to dGALT, with a similar effect on the Wg co-receptor.

In presynaptic Fz2 receptor activation, Wg binds the receptor to drive phosphorylation of Futsch/MAP1b, which mediates NMJ growth and bouton formation via regulation of the synaptic microtubule cytoskeleton (48). To assay this pathway, dGALT and dGALT single and double mutants were co-labelled with anti-Futsch (green) compared to anti-HRP (red), with Futsch labelling quantified in synaptic boutons as absent, bundled, diffuse or looped (48) (Figure 6E). In quantifying these different categories, dGALE wild-type single mutants display a significantly greater percentage of looped boutons (0.13 ± 0.03, n = 14) compared to w1118 controls (0.05 ± 0.01, n = 15, P = 0.023; Figure 6F). Similarly, dGALT/dGALE single mutants show a higher level of Futsch loops (0.17 ± 0.02, n = 28, Figure 6G) compared to dGALT/dGALE genetic background controls (0.09 ± 0.01, n = 36, P < 0.001; Figure 6F). The double mutants (dGALT/dGALE, dGALT/dGALE/dGALT) exhibit greatly elevated Futsch loops in synaptic boutons (0.19 ± 0.02, n = 24), a highly significant increase compared to matched controls (P < 0.001; Figure 6F). These results show dGALT and dGALT display Futsch-driven microtubule changes predictive of the above NMJ overelaboration defects (47). In postsynaptic receptor activation, Wg binding causes Fz2-C cleavage and trafficking to muscle nuclei (49,50), which can be quantified by measuring anti-Fz2-C nuclear fluorescence intensity. For all dGALE and dGALT single and double mutants, Fz2-C accumulation in the nuclei is not significantly different from matched controls (control: 1.0 ± 0.05; dGALT single mutants: 0.96 ± 0.08; and double mutant animals: 0.90 ± 0.09). These results indicate that both dGALT and dGALT selectively impact presynaptic Wg signalling.

We next turned to testing dGALT roles in Wg signalling, either in the presence and absence of dGALT (Figure 7). Like dGALE, dUGP1/dUGP2 mutants exhibit significantly lower Wg ligand levels at the NMJ compared to w1118 controls (n = 28,26; P = 0.0038; Figure 7A,B). However, unlike dGALE, the double mutants (dGALT/dGALE, dUGP1/dUGP2) did not revert the elevated Wg ligand levels characterizing dGALT/dGALT alone (n = 34,46; P < 0.01 compared to dGALT/dGALT control), but rather display a modest further increase (~15%) in Wg ligand abundance (n = 33, P < 0.001; Figure 7A,B). Like dGALT, dUGP mutants (dUGP1/dUGP2) show a ~20% decrease in the Wg co-receptor Dlp (n = 18) compared to w1118 controls (n = 19, P = 0.002; Figure 7C,D). Similar to dGALT nulls (n = 28), double mutants also display a significant Dlp depletion at the NMJ (n = 20), which is significantly lower than matched controls (n = 31, P < 0.01; Figure 7D). Pre- and postsynaptic Wg signalling was next explored. In the presynaptic pathway, the number of Futsch-positive bouton loops in dGALT single mutants (Futsch-positive bouton loops/total number of boutons: 0.09 ± 0.02, n = 6) is indistinguishable from appropriate controls (Futsch-positive bouton loops/total number of boutons: 0.05 ± 0.01, n = 15). Our findings confirm the elevated Futsch loops that characterized the single dGALT mutant condition (Futsch-positive bouton loops/total number of boutons: 0.20 ± 0.02, n = 11, P = 0.002), compared to the precise excision control group (Futsch-positive bouton loops/total number of boutons: 0.08 ± 0.02, n = 10). This trait remains unaffected by the co-deletion of dUGP (Futsch-positive bouton loops/total number of boutons: 0.20 ± 0.03, n = 10) in the postsynaptic pathway, however, nuclear Fz2-C accumulation is very significantly increased by ~50% in dUGP1/dUGP2 mutants compared to w1118 genetic controls (n = 16, 7; P < 0.01; Figure 7E, F). As reported above, dGALT nulls again show no significant difference in nuclear Fz2-C levels compared to controls (n = 48,40; Figure 7E,F), however the double mutants (dGALT/dGALE, dUGP1/dUGP2, dUGP1/dUGP2, n = 25) exhibit a very striking increase in Fz2-C nuclear localization compared to both controls (P < 0.001) and dGALT alone (P < 0.01; Figure 7E,F). These findings suggest that specifically increased activation of the postsynaptic FNI pathway underlies dUGP NMJ synaptogenesis and coordinated movement deficits.

Discussion

Galactosemias result from deficits in any of the three enzymes of the Leoloi pathway: GALK, GALT and GALE. Through this highly conserved cascade, galactose is converted into precursors for the galactosylation of proteins and lipids (2). This mechanism is thought to be the key to disease state chronic neurological symptoms (51,52), particularly in the transferase and epimerase-associated galactosemias. We previously discovered severe galactose glycogen losses, synaptogenesis defects and changes in Wnt trans-synaptic signalling components at the neuromuscular synapse correlated with coordinated movement impairments in the Drosophila GALT-deficient galactosemia disease model (12). Since the long-term neurodevelopmental and movement symptoms reported for GALT-deficient galactosemia are similar (11), we set forth here to characterize a new Drosophila GALT-deficient neurological disease model. Unlike GALT and GALK, no human patient completely lacking GALE activity has been reported (13), consistent with the essential requirement for GALE in Drosophila and C. elegans (34,35). Thus, while most severe GALT-deficient patients have no detectable enzymatic activity (53), the most severe GALT-deficient patients reported exhibit >5% residual activity (54). Consistently, we report here on dGALT mutants with ~3% enzymatic activity, as well as targeted neuronal dGALE knockdown, and show both display compromised coordinated movement and NMJ synaptogenesis defects as severe as dGALT nulls completely lacking enzymatic activity (55).

Both dGALT and dGALE mutants show striking impairments in coordinated movement. These mutant classes both move in an overtly uncoordinated manner, and roll over slowly after being placed in an inverted position (56). This twist-and-roll response is a complex behaviour requiring the animal to contract multiple muscles on one side in a close sequence, while simultaneously relaxing contralateral muscles. Such bilateral motor control requires tight regulation of neuromuscular connectivity (12,57). Consistently, aberrant NMJ architecture is associated with the striking coordination deficits in both dGALT and dGALE mutant animals. Double dGALT; dGALE mutants show an additive exacerbation of the behavioural phenotypes. Since dGALE is downstream of dGALT in the pathway, one might predict that double mutant animals would behave like dGALT single mutants. The fact that this is not the case indicates that these enzymes must have non-overlapping functions outside of the linear Leoloi pathway (58). What might be the basis of this genetic interaction? Unlike dGALT, dGALE mediates an energetically-reversible enzymatic reaction (Figure 1A). In the absence of dGALT, there is little UDP-galactose present, which has been suggested to shift the reversible dGALE reaction towards the formation of UDP-galactose from UDP-glucose (30). Loss of dGALT activity would not allow for such a responsive shift and would account for the worsening of the dGALT coordinated movement phenotypes reported here.
UDP-glucose pyrophosphorylase represents an alternate GALT-independent route for galactose metabolism (9). UGP catalyzes the formation of UDP-glucose from glucose-1-phosphate and UTP, but can also catalyze the formation of UDP-galactose from galactose-1-P and UTP (6,9). This second function is proposed to occur particularly with high levels of galactose-1-P, such as occurs in GALT-deficient galactosemia (11). Importantly, overexpression of human UGP2 (hUGP2), the ortholog for dUGP, in the yeast system has shown the ability to rescue galactose-dependent survival of GALT-deficient strains (59). It has therefore been hypothesized that UGP2 upregulation could also protect against long-term neurological complications in GALT-deficient galactosemia. Consistently, our studies reveal that dUGP deficiency is indeed a strong negative modulator of neurobehavioral outcomes. Null dGALT coordinated movement deficits are exacerbated in dGALT; dUGP double mutant combinations. Similar to dGALE, dUGP mediates a reversible enzymatic reaction (Figure 1A), and can alternately use galactose as the enzymatic substrate (6,9). Under dGALT null conditions where there are increasing amounts of galactose-1-P, the dUGP reaction would shift towards the formation of UDP-galactose from galactose-1-P, in order to restore the UDP-sugar balance for proper glycosylation. Based on this interaction, co-removal of dUGP would be predicted to aggravate coordinated movement deficits in the double mutants, as reported here.

NMJ synaptogenesis is regulated by distinct mechanisms controlling axonal branching and synaptic bouton maturation (60,61). Indeed, while new bouton formation is plastic, activity-dependent and occurs at variable rates throughout postembryonic development, the number of synaptic branches is established early and remains relatively stable thereafter (60,61). Like dGALT, dUGP mutants strongly restrict synaptic bouton development but, unlike dGALT, do not affect synaptic branching. Loss of dGALT augments only the number of synaptic boutons, whereas double dGALT; dUGP mutant animals display an increase in both branching and supernumerary bouton formation. Likewise, loss of dUGP significantly increases new synaptic bouton formation in both heterozygous and transheterozygous conditions, demonstrating the need for >50% dUGP activity for proper NMJ synaptic morphogenesis. Like dGALT, dUGP mutants have only a minimal impact on NMJ branching, although co-removal of dGALT augments the axonal branching defect in double mutant conditions. These findings show a clear need for dGALT, dUGP and dGALT in limiting synaptic bouton development, whereas dGALT and to a lesser extent dUGP are also required earlier to establish a proper synaptic branching architecture. This dUGP involvement is not surprising, since it is the only enzyme in Drosophila capable of synthesizing UDP-glucose, which is well-known to have a central role in anabolic and catabolic pathways regulating cell growth and development (38-40).

We have previously shown that dGALT activity did not significantly alter functional differentiation driving neurotransmission strength at the NMJ, but rather appears to specifically modulate synaptic architecture (12). This is possible since NMJ structure and function are well established to be independently regulated, and coordinated movement defects can occur with NMJ structural defects in the absence of overt synaptic transmission defects (56). The result is surprising, however, since N-glycans have been causally implicated in both Drosophila NMJ structural and functional synaptogenesis (21,22), Consistent with the essential role of dGAL in the biosynthesis of glycoconjugates (62-64), we find here that dGAL is a strong determinant of neurotransmission strength, with significantly elevated synaptic function under conditions of reduced dGAL activity. Moreover, neurotransmission is further elevated with co-removal of dGALT, showing a significant genetic interaction upstream of synaptic function. However, like dGALT, viable levels of dUGP loss do not have a similar impact on neurotransmission strength. There is no significant change in NMJ function either in the single dUGP mutant condition, or with co-removal of dGALT. Unlike dGAL, dUGP is not at the crossroads of UDP sugar balance and this perhaps explains the differential requirement of the two enzymes in synaptic functional differentiation.

NMJ synaptogenesis is highly dependent on extracellular glycan mechanisms (5). Indeed, we previously demonstrated dGALT null NMJ overelaboration is driven by a striking depletion of galactosyl, N-acetylgalactosamine and fucosylated HRP moieties within the synaptomatrix (12). However, only loss of N-acetylgalactosamine is rescued by hGALT expression, and is reduced in wildtype animals after galactose overfeeding, suggesting this is the primary causative synaptic glycan change (12). Similar to dGALT, C. elegans GALE loss-of-function causes strong reduction of UDP-galNAc levels, accompanied by developmental defects associated with UDP-galNAc deficits (35). Importantly, GALE is primarily responsible for UDP-galNAc biosynthesis, since UDP-galNAc cannot be synthesized by GALE-independent pathways (Figure 1A). Consistently, our Drosophila GALE-deficient galactosemia model exhibits a dramatic loss of GalNAc in the NMJ synaptomatrix. Furthermore, co-removal of dGALE and dGALT strikingly exacerbates GalNAc losses compared to the single dGALT null condition. dUGP-deficient animals also manifest loss of GalNAc in the NMJ synaptomatrix. Moreover, both dGALT and dUGP mutants similarly display synaptic loss of HRP epitope, revealing bifucosylated N-glycans. This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) suggests it is the primary causative synaptic glycan change (12). This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) further suggests it is the primary causative synaptic glycan change (12). This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) further suggests it is the primary causative synaptic glycan change (12). This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) further suggests it is the primary causative synaptic glycan change (12). This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) further suggests it is the primary causative synaptic glycan change (12). This apparent cross-talk between Leloir and GDP-mannose metabolic pathways regulating cell growth and development (12) further suggests it is the primary causative synaptic glycan change (12).
the new dGALT-deficient galactosemia model shows specific hyper-activation of presynaptic Futsch-mediated microtubule rearrangements modulating NMJ morphogenesis. Thus, both galactosemia disease models similarly activate the presynaptic Wnt pathway.

In contrast to dGALT and dGALe requirements, dUGP mutants specifically activate the postsynaptic FNI pathway. Presynaptic Wg activation requires the inhibition of the Glycogen Synthase Kinase 3β (GSK3β) homolog Shaggy (Sgg) (70), a protein kinase with the ability to phosphorylate glycogen synthase. Previous experiments have demonstrated that UGP loss is associated with increased phosphorylation of glycogen synthase and subsequent reduction of its catalytic activity (38). This predicts that GSK3β is hyper-activated in dUGP mutants, which would prevent Wg activation of the presynaptic pathway. As we have shown many times previously (21–23), predicting the directionality of Wg signalling changes based on Wg ligand, Fz2 receptor levels and Dlp co-receptor is profoundly difficult owing to the interplay between the 3 players, as described in the ‘Exchange Factor Mechanism’ (21, 23, 71). Wg may be actively signalling or sequestered extracellularly in a non-signalling state, and Dlp plays both positive and negative roles in signalling, dependent on the relative abundance of Wg and Fz2 (71), as well as other factors. In the galactosemia disease models, extracellular Wg abundance is oppositely misregulated in dGALT vs. dGALe mutants, although the presynaptic signalling activation is similar. Likewise, dGALT and dUGP mutants exhibit an opposite effect on Wg ligand levels, but only dUGP mutants show a significant activation of the postsynaptic FNI pathway. We hypothesize that the fundamental defect in Leorio pathway mutants is the altered levels of the Wg co-receptor HSPG Dlp, which are low in both single and double mutant combinations. Previous studies have associated reduced Dlp levels to locally increased extracellular Wg levels (69, 72), as is the case for dGALT mutants, and activation of Wg signalling, similar to what we report here. The direction of Wg activation, pre- or postsynaptic, appears different in dGALT, dGALe and dUGP mutants due to additional factors that we have yet to resolve. The identity and role of Wnt pathway ligand/receptor/coreceptor changes, and directional signalling defects at the NMJ, will be the focus of our future studies.

In conclusion, the results presented here are the first to reveal coordinated movement deficits, NMJ structure and functional defects, NMJ glycosylation losses and differential Wnt trans-synaptic signalling dysfunction under conditions of GALE-deficient galactosemia and UGP2 deficiency. Unlike GALT-deficient galactosemia, both GALE and UGP2 are indispensable for viability, and substantial residual enzymatic activities are required for survival, but nevertheless partial loss-of-function manifests severe neurobehavioural defects due to a neuronal requirement. GALE-deficient galactosemia shares with classic galactosemia numerous pathogenic factors underlying the long-term neurological impairments characterized by coordinated movement disabilities. Our results suggest that changes in Wnt signalling and Futsch/MAP1B microtubule cytoskeletal organization underlie neuromuscular synapse development defects and impaired coordinated movement in both disease states. Our results also reveal UGP2 activity and GalNAc glycosylation roles in neuromuscular synaptogenesis, raising the intriguing possibilities that targeted UGP2 strategies and/or UDP-galNAc supplementation might relieve neurological complications in the galactosemia disease states, as previously suggested (59, 62).

Materials and Methods

Drosophila genetics

Figure 1A shows in parallel the enzymatic steps of the glucose and galactose metabolic pathways, listing the names and Drosophila CG numbers of all the genes targeted in this study, as well as the biochemical functions of the encoded proteins. All stocks were obtained from the Bloomington Drosophila Stock Center and Vienna Drosophila Resource Center, and reared at 25°C under low-density rearing conditions on standard molasses-based food. We used two dGALT excision alleles generated by mobilizing a P-element insertion in the 5’-untranslated region of CG9232 (KG00049); dGALTΔC2 (precise excision control) and dGALTΔCF (null deficiency), with measured normal and undetectable enzymatic activity, respectively (55). A dGALe (CG12030) allele, dGALe8, was generated by mobilizing P-element insertion P[EPgy2]CG12030EV22205 with H[Pdelta2-3]HoP8,y, y,w+,Dr/TM3, Sb (34). Activity assays show dGALe is a strong hypomorphic allele (measured ~8% enzymatic activity), with unaltered dGALK and dGALT activities (34). The UAS-dGALe-RNAi line w1118, P[GD7464]u74708 was compared to Gal4 driver alone controls. UH1-Gal4 driven UAS-dGALe-RNAi results in 100% lethality, consistent with the essential requirement for the gene (33). We used two dUGP stocks; w1118; P{EPgy2}CG12030EV22205 w1118; P{GP2}CG9232 (null deficiency), with measured normal activity insertion control) and dUGP1, P{XP}UGPd07256/TM6B, Tb1 (dUGP1) and w1118, P{XP}U603756/TM6B, Tb1 (dUGP2) generated by P-element insertion (42); and UAS-dUGP-RNAi line P[C12088]u21832 for tissue-targeted experiments. Pan-neuronal elav-Gal4 and muscle 24B-Gal4 driver were used for tissue-specific studies, compared to driver alone controls. dGALe and dUGP hypomorphic alleles were used to create double mutant stocks with dGALTΔCF, w1118 was used as the genetic background control for dGALe and dUGP, and precise excision controls dGALTΔC2 were used in double mutant combinations of these alleles.

Behavioral assays

Movement assays were performed as previously described in isolated wandering third-instar (wi3) male larvae (56). Larvae were placed individually on a fresh, room temperature (RT) 1% agar plate and allowed to move freely for ~2min before the assay. Using forceps, the larva was rolled to an inverted position as defined by the upright ventral midline. Once released, a timer recorded the amount of time the larva took to completely right itself as defined by the upright dorsal midline (56). Three consecutive time measurements were recorded for each larva and then averaged to produce one data point. The maximum amount of time allowed for a given animal to rollover was 5 minutes. Behavioural experiments were done on ≥15 individual animals for each genotype. Data were analyzed by student’s t-test for pairwise comparisons, and ANOVA tests for all data sets of ≥3 comparisons.

Immunocytochemical imaging

Wandering L3 larvae were used for all immunocytochemistry imaging as previously described (21, 73). Reagents were purchased from Sigma-Aldrich unless otherwise specified. Larvae were dissected in physiological saline (128mM NaCl, 2mM KCl, 4mM MgCl2, 0.2mM CaCl2, 70mM sucrose, 5mM trehalose, 5mM HEPES), fixed in 4% paraformaldehyde for 10 minutes at RT, and then either processed with PBTX (PBS + 1% BSA + 0.2% Triton X-100) detergent, for cell permeabilized studies, or detergent-free (PBS with 1% BSA or without BSA when using lectins) conditions, for
non-permeabilized studies. Primary antibodies included: Alexa Fluor-488 goat anti-heroserdish peroxidase (HRP, 1:200, Jackson Labs); Cy3-conjugated goat anti-HRP (1:200, Jackson Labs); mouse anti-Fascin II (FasII, 1:10, Developmental Studies Hybridoma Bank (DSHB), University of Iowa); mouse anti-Discs Large (DLG, 1:250; DSHB); mouse anti-Wingless (Wg, 1:2; DSHB); mouse anti-Dally-like Protein (Dlp, 1:4; DSHB); rabbit anti-dFz-C (1:500) (75); mouse anti-Futsch (1:100; DSHB). Secondary antibodies included: Alexa Fluor-488-conjugated goat anti-mouse IgG (1:200, Invitrogen-Molecular Probes) and Alexa Fluor-488-conjugated goat anti-rabbit IgG (1:250, Invitrogen-Molecular Probes). Wisteria floribunda (WFA-Fitc, 1:250, Vector Labs) was used as fluorophore-conjugated lectin to label N-acetyl galactosamine residues (12). Primary antibodies and lectins were incubated at 4°C overnight; secondary antibodies were incubated at RT for 2 hrs. Dissections were mounted on slides in Fluormount G (Electron Microscopy Sciences).

All mutant and control larvae were dissected, labelled and imaged completely in parallel. Z-stacks were taken with a Zeiss LSM 510 META laser-scanning confocal, using either 40x or 63x Plan Apo oil-immersion objectives. Optical sections were taken starting immediately above and ending immediately below the NMJ. The stacks were projected on the Z-axis, with NMJ signals highlighted and the average intensity for each NMJ recorded. Intensities were quantified using ImageJ software. For structural analyses, preparations were double-labelled with anti-HRP and anti-DLG, with counts made at muscle 4 in segments A2/3 on both right and left sides. Structural data from hemisegments were averaged for each animal to produce one data point. For quantification, a bouton was defined as an axon varicosity >1 μm in minimum diameter, and >2 boutons on an axon defined a NMJ branch. For quantification of Wg trans-synaptic signalling components (i.e. Wg ligand, HSPG Dlp, Fz2-C and Futsch), each NMJ was treated as an independent replicate. For Futsch labelling, NIH ImageJ was used to count bouton numbers, with high magnification imaging used to classify all boutons as empty (no Futsch labelling), diffuse (Futsch-positive, unorganized), bundled (Futsch-positive bundle) and looped (Futsch-positive loop) (48).

Electrophysiology

Two-electrode voltage-clamp (TEVC) electrophysiology was performed as previously described (74). Briefly, w1.3 were secured with 3M Verbond tissue adhesive (World Precision Instruments) to sylgard-coated glass coverslips, cut longitudinally along the dorsal midline, internal organs removed, and the larval cuticle glued down laterally to allow access to the neuromusculature. Peripheral nerves were then cut at the base of the ventral nerve cord (VNC). Dissections and recordings were performed at 18°C in saline solution consisting of 128mM NaCl, 2mM KCl, 4mM MgCl2, 1.0mM CaCl2, 70mM sucrose, 5mM trehalose and 5mM HEPES, with pH adjusted to 7.1 using NaOH. Preparations were imaged using a Zeiss Axiostep microscope with 40X water immersion objective. Muscle 6 in abdominal segments 2/3 was impaled with two microelectrodes of ~15 MΩ resistance filled with 3M KCl. The muscle was clamped at -60 mV using an Axoclamp-2B amplifier. A fire-polished glass suction electrode containing saline was used for evoked nerve stimulation of the severed motor nerve with a 0.5 ms supratreshold stimuli at 0.2 Hz from a Grass S88 stimulator (74). Excitatory junctional current (EJC) records were filtered at 2 kHz. To quantify EJC amplitudes, 10 consecutive traces were averaged and the peak of the averaged trace recorded. Clampex software was used for all data acquisition and Clampfit software was used for all data analyses.

Statistical Analyses

All behavioural and NMJ structural data were averaged per genotype and each replicate value was calculated as the fold-change relative to the average value of the appropriate control. For signal intensity and structural data, control and mutant animals were always dissected, labelled and imaged in parallel. Intensity was calculated as the fold-change relative to the average control value recorded in the same experiment. The proportion of boutons displaying Futsch-positive loops was calculated relative to the total number of boutons at each NMJ. Unpaired t-tests (pairwise comparisons) or Mann-Whitney tests (>3 comparisons) were used to compare differences between mutants and controls as indicated in figure legends. The criterion for statistical significance was P < 0.05, with higher levels of significance classified as P < 0.01, P < 0.001 and P < 0.0001. All statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego California, USA).

Acknowledgements

This work was solely supported by National Institutes of Health grant MH096832 to K.B. We are grateful to Vivian Budnik (University of Massachusetts Medical School, Worchester MA) for antibody reagents. We also particularly thank the Developmental Studies Hybridoma Bank at the University of Iowa for essential antibodies, and the Drosophila Bloomington Stock Center at Indiana University and Vienna Drosophila Resource Center for essential genetic lines.

Conflict of Interest statement

None declared.

Funding

This work was fully funded by NIH grant R01 MH096832 to K.B.

References


