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

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Abstract

The multiple galactosemia disease states manifest long-term neurological symptoms. Galactosemia I results from loss of galactose-1-phosphate uridyltransferase (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 (GALK), galactose-1-phosphate uridytransferase (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 (dGALT) and double mutant (dGALT; 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 coordinate locomotor movement defects, as well as underlying strikingly overelaborated neuromuscular junction (NMJ) synaptic architecture. Importantly, the disease state is restricted to morphological defects without changes in neurotransmission. Importantly, coordinated movement, NMJ development, synaptic architecture. Importantly, the disease state is restricted to morphological defects without changes in neurotransmission.

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 defects 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 synaptomatrix 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 re-modeling 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 galactoseemia-associated neuropathology. Both dGALE 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 synaptomatrix 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 defect (12). In the galactose pathway, dGALT intersects with dGALK downstream (Figure 1A), and the Search Tool for Retrieval of Interacting Genes (STRING) (29) identified dGALE 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 homology 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 (w1118) showing rapid and well-coordinated movement (8.2 ± 1.09, n = 24 animals), dGALEh mutants exhibit clearly uncoordinated and >2-fold slower responses (17.7 ± 5.64, n = 23, P = 0.01; Figure 1D). Mutant larvae make several attempts to twist and roll before they successfully right themselves. Similarly, as previously reported (12), dGALT null (dGALT<trans>) animals are significantly movement impaired, also manifesting ~2-fold slower righting time compared to precise-excision genetic background (dGALT<del>) matched controls (n = 36; P < 0.001; Figure 1D). We hypothesized dGALE would exacerbate dGALT movement impairments since dGALE synthesizes UDP-sugar precursors when limited by dGALT deficiency (Figure 1A). Consistently, the double mutants (homozygous dGALT<del>; dGALE<del>) manifest >2- and >4-fold longer period to complete the coordinated movement (56.3 ± 17.77, n = 15) compared to dGALT single mutants (P < 0.01) and genetic controls (P < 0.001, Figure 1D), respectively. Our findings suggest 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.

To test the cellular requirements for dGALE in bilateral coordinated movement control, dGALE was knocked-down with transgenic RNAi (Figure 2). Like dGALE null mutants, ubiquitous (UH1-Gal4) dGALE knockdown results in 100% early lethality, and therefore could not be assayed. Like the dGALE<del> mutants, targeted neuronal dGALE RNAi (elav-Gal4 > dGALE-RNAi) also results in overly uncoordinated animals, which likewise manifest >2-fold slower roll-over behaviour (18.7 ± 1.96, n = 16) compared to their driver alone (elav-Gal4/+); genetic controls
Figure 2. Targeted neuronal dGALE knockdown also phenocopies dGALT mutants. (A) Representative NMJs co-labelled with presynaptic anti-HRP (green) and postsynaptic anti-DLG (red) in driver alone controls (elav-Gal4/+), neuronal-targeted dGALE RNAi (elav-Ga4–dGALE-RNAi); precise excision dGALT controls (dGALT<sup>ΔΔ</sup>), dGALT nulls with driver alone (dGALT<sup>ΔΔ</sup> elav-Gal4/+), and the double mutant animals (dGALE<sup>ΔΔ</sup>; dGALT<sup>ΔΔ</sup> elav-Gal4–dGALE-RNAi). Movement time for coordinated roll-over behaviour. (B) NMJ bouton. (C) and branch. (D) number in the same genotypes, normalized to respective genetic controls. Sample size: ≥10 animals for each genotype. Error bars show SEM with significance; *P<0.05, **P<0.01 and ***P<0.001.

(9.0 ± 0.94 s, n = 14, P = 0.0002; Figure 2B). Similarly, targeted muscle dGALE RNAi (24B-Ga4→dGALE-RNAi) causes 2-fold slower rollover (10.4 ± 1.64 s, n = 24) compared to genetic controls (24B-Ga4/+, 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>ΔΔ</sup>; elav-Ga4→dGALE-RNAi) causes further slowing of roll-over time (34.6 ± 11.46 s, n = 25) compared to dGALT with driver alone (dGALT<sup>ΔΔ</sup>; elav-Ga4/+) animals (15.7 ± 1.61 s, n = 29), as well as precise excision control (dGALT<sup>ΔΔ</sup>; elav-Ga4/+). Conversely, postsynaptic targeted dGALE knockdown in the dGALT null background (homozygous dGALT<sup>ΔΔ</sup>; 24B-Ga4→dGALE-RNAi) does not further compromise locomotor coordination (20.8 ± 2.14 s, n = 22) compared to dGALT alone (dGALT<sup>ΔΔ</sup>; elav-Ga4/+: 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 glycogene 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<sup>Δ</sup> 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 overelaboration (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 dGALT 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>AAP2</sup>; dGALE<sup>B</sup>) develop ~50% and ~20% more boutons (39.8 ± 2; n = 40), compared to genetic control (dGALT<sup>W118B</sup>) (26.4 ± 1.3; n = 26, P < 0.001; Figure 1E) and dGALT single mutants (Figure 1E), respectively. Neuronal dGALE knockdown in the dGALT null background did not further increase NMJ structural complexity (Figure 2C,D). Single dGALE<sup>B</sup> and dGALE-RNAi also did not increase synaptic branching (Figures 1F,2D), in contrast to dGALT 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; dGALT double mutants (4 ± 0.2, 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. Excitatory 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>118B</sup>, dGALE alone (dGALE<sup>B</sup>) and a double mutant in combination with dGALT (dGALT<sup>AAP2</sup>; dGALE<sup>B</sup>). Neurotransmission strength is very obviously elevated with loss of dGALE activity (Figure 3A). Mean EJC amplitudes significantly increased in mutants compared to control (313.3 ± 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>AAP2</sup>; dGALE<sup>B</sup>; 413.2 ± 12.4 nA) compared to both w<sup>118B</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 modifier 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 dGALE, 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>1</sup>, dUGP<sup>2</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>1</sup>/dUGP<sup>2</sup>). We first tested dUGP mutants for coordinated movement behaviour using the same larval roll-over assay as above. Compared to w<sup>118B</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>, although UGP<sup>+/+</sup> appears qualitatively more behaviourally compromised. Combined dUGP<sup>+/dUGP<sup>2</sup> mutants display 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>118B</sup> controls (P < 0.001; Figure 4B). Ubiquitous dUGP knockdown (UH1-Gal4 > dUGP-RNAi) caused the strongest effect, with >2.5-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 gluc-6-P levels in dUGP<sup>-/-</sup> mutants, 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 gluc-6-P levels in dUGP<sup>-/-</sup> mutants, but is only sig-
compared to dGALT \textsuperscript{D}C\textsubscript{2} 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 (\(\text{UH1-Gal4} > \text{dUGP-RNAi}\)) significantly increases synaptic bouton (35.5 ± 1.56, \(n = 11\), \(P < 0.0003\)) and branch (3.3 ± 0.23, \(n = 10\), respectively). However, structure is over-elaborated in pan-neuronal dUGP-knockdown animals (26.3 ± 1.0 boutons, \(n = 10\), \(P = 0.007\)) 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). \(\geq 12\) NMJs from \(\geq 6\) different \(w\textsubscript{1118}\) animals were recorded from each of three genotypes: genetic background control (\(w\textsubscript{1118}\)), dUGP single mutant (\(d\text{UGP}\textsuperscript{C}\)) and double mutant animals (\(d\text{GALT}\textsuperscript{AP2}; d\text{UGP}\textsuperscript{C}\)). Unlike \(d\text{GALT}\), 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 \(w\textsubscript{1118}\) 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 \(d\text{UGP}\) is important for coordinated movement and neuromuscular structural synaptogenesis, but is not detectably required for synapse function. These findings show that co-removal of \(d\text{UGP}\) and \(d\text{GALT}\) 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.

dGAL\text{E} and dUGP both shape NMJ synaptomatrix glycan composition

GAL\text{E} 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 GAL\text{E}-independent pathway (Figure 1A). Indeed, recent studies reveal that GAL\text{E} loss-of-function in \(C.\text{elegans}\) 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 deficit 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 S5.

The synaptic marker FasII does not vary significantly between tested genotypes, comparing w1118 genetic background control and dGALEh mutant NMJs (Figure 5A). In sharp contrast, while w1118 control NMJs (n = 38) are very highly enriched with WFA-labelled galNAc residues, dGALEh 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 dGALTAP2 single mutant decreases WFA labelling by ~25% compared to precise controls (n = 42, 46; P < 0.001; Figure 5A,C), and the double mutants (dGALEh/dGALTAP2, dGALEh/dGALTAP2P) exhibit a further ~35% reduction, significantly different from the single mutant condition (dGALTAP2/dGALTAP2P, P < 0.05; Figure 5C). Fucosylation defects are also reported 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, dGALEh NMJs show significantly reduced HRP glycan levels compared to controls (n = 6, 6; P = 0.006), similar to dGALTAP2 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 dGALTAP2; 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 (UGP+/+, n = 8; UGP+/+, n = 9) is not significantly different from matched controls (n = 26), the stronger UGP1/UGP2 combination shows a ~40% reduction in WFA-labelled galNAc residues (n = 9, P < 0.01; Figure 5C). The dGALTAP2 single mutant shows a ~25% decrease (n = 42) compared to controls (n = 46, P < 0.001; Figure 5C), and the double mutants (dGALTAP2/dGALTAP2, dGALTAP2/dUGP1) 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 dUGP1/dUGP2 exhibits a ~30% reduction (n = 10) compared to w1118 genetic controls (n = 11, P < 0.05; Figure 5D). Both single dGALTAP2 (n = 29) and double dGALTAP2/dUGP1 combination shows 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 dUGP 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 synaptomatix 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 synaptogenesis 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.

Figure 6. Presynaptic Wnt signalling upregulated in dGALE and dGALT double mutant. (A) Representative NMJ boutons co-labelled with anti-HRP (green) and anti-Wingless (Wg, red) in genetic control (w^{1118}) and dGALE mutant (dGALE^t); precise excision controls (dGALT^D-C), dGALT mutant (dGALT^D), and double mutant (dGALT^DAP2; dGALE^t). (B) Wg intensity in single and double mutants normalized to appropriate genetic controls. (C) NMJ boutons imaged with anti-HRP (green) and anti-Dally-like Protein (Dlp, red) in the same 5 genotypes. (D) Dlp intensity in single and double mutants normalized to genetic controls. (E) Representative NMJs probed with anti-HRP (red) and anti-Futsch (green) in the same 5 genotypes. (F) Percentage of Futsch-positive loop boutons in single and double mutants. (G) Higher magnification image of a microtubule loop within a single synaptic bouton probed with membrane anti-HRP (red), anti-Futsch (green) and the merged channels. Sample size: ≥10 NMJs/animals per genotype. Error bars show SEM with significance indicated as *P<0.05, **P<0.01 and ***P<0.001.
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, dGALE₉₅ exhibits a highly significant ~50% decrease in Wg compared to matched controls (n = 10,10; P = 0.0007; 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; Figure 6A,B). Perhaps additively, double mutants (dGALE₉₅/dGALE₉₅; dGALT⁵⁵⁵/dGALT⁵⁵⁵) show a restoration of Wg ligand levels to the wildtype condition (n = 23; P < 0.001 compared to dGALT⁵⁵⁵; 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 dGALE₉₅ compared to controls (n = 15,16; P = 0.03) and dGALT⁵⁵⁵ compared to controls (n = 56,59; P < 0.05; Figure 6D).

Figure 7. Postsynaptic Wnt signalling upregulated in dUGP and dGALT double mutant. (A) Representative NMJ boutons co-labelled with anti-HRP (green) and anti-Wg (red) in genetic control (w¹¹¹⁸), dUGP mutant (dUGP¹/dUGP²), precise excision control (dGALT⁵⁵⁵), dGALT single (dGALT⁵⁵⁵) and double (dGALT⁵⁵⁵/dUGP¹/dUGP²) mutants. (B) Quantification of Wg intensity normalized to genetic controls. (C) NMJ boutons imaged with anti-HRP (green) and anti-Dlp (red) in the same 5 genotypes. (D) Quantification of Dlp intensity in single and double mutants normalized to genetic controls. (E) NMJs probed with anti-HRP (red) and anti-Fz2-C (Fz2-C, green) in the same 5 genotypes. The bottom row shows nuclear Fz2-C labelling only. The “n” labels postsynaptic muscle nuclei. (F) Quantification of Fz2-C intensity within muscle nuclei. Sample size: ≥7 NMJs/animals per genotype. Error bars show SEM with significance indicated as *P<0.05, **P<0.01 and ***P<0.001.
Double mutants were not significantly worse than the single mutants alone, but displayed 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 assess this pathway, dGALE 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 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, dGALTAKAP mutants show a higher level of Futsch loops (0.17 ± 0.02, n = 28, Figure 6G) compared to dGALT10c genetic background controls (0.09 ± 0.01, n = 36, P < 0.001; Figure 6F). The double mutants (dGALE/dGALE; dGALTAKAP/dGALTAKAP) 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 dGALE 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 ± 0.05; dGALE single mutants: 0.96 ± 0.08; and double mutant animals: 0.90 ± 0.09). These results indicate that both dGALE and dGALT selectively impact presynaptic Wg signalling.

We next turned to testing dUGP 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, 36; P = 0.0038; Figure 7A,B). However, unlike dGALE, the double mutants (dGALTAKAP/dGALTAKAP; dUGP1/dUGP2) did not revert the elevated Wg ligand levels characterizing dGALTAKAP alone (n = 34, 46; P < 0.01 compared to dGALT10c control), but rather display a modest further decrease (~15%) in Wg ligand abundance (n = 33, P < 0.001; Figure 7A,B). Like dGALE, 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 very 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 dUGP 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 characterize 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-removal 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 (dGALTAKAP/dGALTAKAP; 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 < 0.001; 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 Leloir 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 glycan 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 GALE-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 GALE-deficient patients have no detectable enzymatic activity (53), the most severe GALE-deficient patients reported exhibit >5% residual activity (54). Consistently, we report here on dGALE mutants with ~8% 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 overly 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 dGALE; dGALT 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 Leloir 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 dGALE 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 modifier of neuro-behavioural 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 alternate 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 synaptomorphogenesis. 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, dGAL and dUGP 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 GALT-deficient galactosemia model exhibits a dramatic loss of GalNAc in the NMJ synaptomatrix. Furthermore, concomitant removal of dGALT and dGAL strikingly exacerbates GalNAc losses compared to the single dGALT null condition. dGAL-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-Fucose production pathways is intriguing, especially in the absence of a Fucose salvage route in Drosophila. It would be interesting to determine how altered nucleotide sugar metabolism affects protein glycosylation in addition to processing. In eukaryotes, fucosylation occurs primarily in the Golgi, and recent evidence suggests a close connection between UDP-sugar balance and Golgi N-glycan branching (65), which may explain these findings. Furthermore, recent studies from our lab (12,22) provide evidence that HRP glycan loss is accompanied by increased synaptic growth, similar to what we report here for both dGALT and dUGP mutants.

NMJ synaptogenesis is modulated by trans-synaptic signalling that occurs via secreted glycoprotein ligands traversing the highly-glycosylated synaptomatrix (5,66,67). In particular, the founding Wnt Wingless (Wg) signalling ligand is a potent driver of synaptic development (50,68). Wg is secreted from presynaptic neuron and glia to bind Fz2 receptors on both neuron and postsynaptic muscle via interaction with the Wg co-receptor HSPG Dlp, a well-known regulator of Wg extracellular distribution and signalling (21,68,69). Postsynaptic Wg activates Futsch/MAP1B phosphorylation and binding to the synaptic microtubule cytoskeleton, driving the budding of new synaptic boutons and NMJ expansion (47,48). Null dGALT NMJs exhibit elevated Wg and reduced Dlp (21), but the impact on Wg signal transduction was not previously investigated. We discover here that postsynaptic FNI signalling is not affected in the dGALT-deficient galactosemia model, but rather there is a strong upregulation of presynaptic Fusch signalling, consistent with the observed elevated synaptic bouton formation (47). Likewise,
the new dGALE-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 dGALE vs. dGALE mutants, although the presynaptic signalling activation is similar. Likewise, dGALE 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 Leloir 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 dGALE mutants, and activation of Wg signalling, similar to what we report here. The direction of Wg activation, pre- or postsynaptic, appears different in dGALE, dGALE and dUGP mutants due to additional factors that we have yet to resolve. The identity and role of Wnt pathway ligand/receptor/co-receptor 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); dGALT<sup>AC2</sup> (precise excision control) and dGALT<sup>MD2</sup> (null deficiency), with measured normal and undetectable enzymatic activity, respectively (55). A dGALE (CG12030) allele, dGALE<sup>u1</sup>, was generated by mobilizing P-element insertion P{EPseg}CG12030<sup>EV22205</sup> with H{P{eq}Delta2-3;HoP8,y,w};Dr/TM3,Sb (34). Activity assays show dGALE<sup>u1</sup> is a strong hypomorphic allele (measured ~8% enzymatic activity), with unaltered dGALK and dGALT activities (34). The UAS-dGALE-RNAi line w<sup>1118</sup>, P{GD7464}u47408 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; w<sup>1118</sup>, Pbac(WHAGAL<sup>107756</sup>/TM6B, Tb<sup>1</sup> (dUGP<sup>1</sup>) and w<sup>1118</sup>, P{XP}uGALE<sup>107756</sup>/TM6B, Tb<sup>1</sup> (dUGP<sup>2</sup>) generated by P-element insertion (42); and UAS-dUGP-RNAi line P{GD11288};w<sup>21832</sup> 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 dGALK<sup>MD2</sup>, w<sup>1118</sup> was used as the genetic background control for dGALE and dUGP, and precise excision controls dGALT<sup>AC2</sup> were used in double mutant combinations of these alleles.

Behavioural assays

Movement assays were performed as previously described in isolated wandering third-instar (wL3) male larvae (56). Larvae were placed individually on a fresh, room temperature (RT) 1% agar plate and allowed to move freely for ~2 min 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 (128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 0.2 mM CaCl₂, 70 mM sucrose, 5 mM trehalose, 5 mM HEPES), fixed in 4% paraformaldehyde for 10 minutes at RT, and then either processed with PBTR (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
quantification, a bouton was defined as an axon varicosity. Intensities were averaged for each animal to produce one data point. For both right and left sides. Structural data from hemisegments analyses, preparations were double-labelled with anti-HRP and 3M Vetbond tissue adhesive (World Precision Instruments). Optical sections were taken starting immediately above and ending immediately below the NMJ. ImageJ was used to 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 high- and ending immediately below the NMJ. Apo oil-immersion objective. Muscle 6 in abdominal segments 2/3 was imaged using 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 Imagent 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, wL3 were secured with 3M Verbond tissue adhesive (World Precision Instruments) to syngiard-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 128 mM 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 Axiostar microscope with 40X water immersion objective. Muscle 6 in abdominal segments 2/3 was impaled with two micropipettes 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 suprathreshold 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 and P ≤ 0.001. All statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego California, USA).

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Conflict of Interest statement. None declared.

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