N-glycosylation requirements in neuromuscular synaptogenesis

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ABSTRACT
Neural development requires N-glycosylation regulation of intercellular signaling, but the requirements in synaptogenesis have not been well tested. All complex and hybrid N-glycosylation requires MGAT1 (UDP-GlcNAc:α-3-D-mannoside-β1,2-N-acetylglucosaminyltransferase I) function, and Mgt1 nulls are the most compromised N-glycosylation condition that survive long enough to permit synaptogenesis studies. At the Drosophila neuromuscular junction (NMJ), Mgt1 mutants display selective loss of lectin-defined carbohydrates in the extracellular synaptomatrix, and an accompanying accumulation of the secreted endogenous Mind the gap (MTG) lectin, a key synaptogenesis regulator. Null Mgt1 mutants exhibit strongly overelaborated synaptic structural development, consistent with inhibitory roles for complex/hybrid N-glycans in morphological synaptogenesis, and strengthened functional synapse differentiation, consistent with synaptogenic MTG functions. Synapase molecular composition is surprisingly selectively altered, with decreases in presynaptic active zone Bruchpilot (BRP) and postsynaptic Glutamate receptor subtype B (GLURIIIB), but no detectable change in a wide range of other synaptic components. Synaptogenesis is driven by bidirectional trans-synaptic signals that traverse the glycan-rich synaptomatrix, and Mgt1 mutation disruptions both anterograde and retrograde signals, consistent with MTG regulation of trans-synaptic signaling. Downstream of intercellular signaling, pre- and postsynaptic scaffolds are recruited to drive synaptogenesis, and Mgt1 mutants exhibit loss of both classic Discs large 1 (DLG1) and newly defined Lethal (2) giant larvae [L(2)GL] scaffolds. We conclude that MGAT1-dependent N-glycosylation shapes the synaptomatrix carbohydrate repertoire and endogenous lectin localization within this domain, to modulate retention of trans-synaptic signaling ligands driving synaptic scaffold recruitment during synaptogenesis.

KEY WORDS: Synaptomatrix, Trans-synaptic signaling, Synaptic scaffold, Active zone, Glutamate receptor, Neuromuscular junction, Drosophila

INTRODUCTION
N-glycosylation is the most common post-translational modification, involving linkage of diverse carbohydrate trees onto asparagine, targeting primarily cell surface and secreted proteins. Mutation of >20 human N-glycosylation genes result in heritable congenital disorders of glycosylation (CDGs), many of which impair nervous system development (Freeze, 2006; Hennet, 2012; Hewitt, 2009). The Mgt1 gene encoding GlicNAcT1 adds GlicNAc to high-mannose sites (Schachter, 2010); an essential early step in producing all complex and hybrid N-glycans (Pownall et al., 1992; Ye and Marth, 2004). Thus, MGAT1 generates the entire repertoire of polymeric branched N-glycans destined for secretion or presentation on the cell surface (Puthalakath et al., 1996); and Mgt1 null mutants, containing high mannose in place of complex/hybrid N-glycans, are the earliest N-glycan pathway block available to study N-glycosylation requirements in neural development (Schachter and Boulianne, 2011). Mouse Mgt1 knockouts are lethal at embryonic day (E) 9.5, but conditional mutants show movement defects, tremors, paralysis and early death characteristic of neurodevelopmental impairments (Campbell et al., 1995; Grasa et al., 2012; Shi et al., 2004; Ye and Marth, 2004). Drosophila Mgt1 is functionally conserved, and null mutants show the same range of crippling neurological defects, but have the enormous benefit for analysis of being viable.

Drosophila Mgt1 null mutants exhibit severely impaired coordinated movement, and the few adults that eclose usually survive only a few days (Sarkar et al., 2006). Importantly, lifespan shortening is due entirely to neuron-specific requirements, and Mgt1 neuronal overexpression increases lifespan (Sarkar et al., 2010; Schachter and Boulianne, 2011). In the central brain mushroom body learning/memory center, Mgt1 nulls show fused lobes similar to fused lobes (fdl) mutants in β-N-acetylglucosaminidase, which removes the MGAT1-added GlicNAC (Léonard et al., 2006; Sarkar et al., 2006). MGAT1 is also required for a3 fucose addition, a neuron-specific modification routinely labeled with anti-horseradish peroxidase (HRP) (Desai et al., 1994; Paschinger et al., 2009). Overexpression of the fucose transferase generating HRP glycans increases peripheral sensory neuron clustering, ventral nerve cord growth and glial migration (Rendić et al., 2010). MGAT1-dependent N-glycosylation occurs on many neural proteins, including neurotransmitter receptors, SNAREs, fasciclinis, Neuroglian, neurexins and Dystroglycan (Koles et al., 2007; Muntoni et al., 2008; Sun and Xie, 2012).

The heavily glycosylated synaptomatrix is composed of secreted and membrane molecules residing at the interface between presynaptic active zones and postsynaptic receptors. Drosophila genetic analyses show synaptomatrix glycan modification/binding has core roles in structural and functional development of the neuromuscular junction (NMJ) synapse (Broadie et al., 2011; Dani and Broadie, 2012). Glycan-dependent synaptogenic events include presynaptic active zone (AZ) differentiation, postsynaptic glutamate receptor (GluR) localization and extracellular matrix (ECM) organization within the synaptic cleft, driven by the secreted endogenous Mind the gap (MTG) lectin, for example (Dani et al., 2012; Long et al., 2008; Rushton et al., 2012). Synaptogenic events are regulated by bidirectional trans-synaptic signals that traverse the synaptomatrix, and glycosylation of both ligands and receptors alters localization and binding (Henriquez and Salinas, 2012; Patton, 2003). Three well-characterized trans-synaptic signals at the Drosophila NMJ are the Wnt protein Wingless (WG), the bone morphogenic protein Glass bottom boat (GBB), and Jelly belly (JEB) (Del Grosso et al., 2011; Kamimura et al., 2013; Rohrbough and Broadie, 2010; Tanaka et al., 2002).
The goal here is to test N-glycosylation requirements during NMJ synaptogenesis using Mgat1 mutants. We found large alterations in synaptomatrix glycan composition, including complete lack of paucimannose glycans, fucosylated HRP epitopes and Vicia villosa (VV A) lectin reactivity, coupled to strongly elevated MTG expression. Null Mgat1 mutants display increased NMJ growth (increased synapse area, branching and bouton number) and function (increased transmission and FM1-43 dye cycling), showing that MGAT1-dependent N-glycosylation plays inhibitory roles in synaptogenesis. Consistent with the hypothesis that a modified synaptomatrix would alter trans-synaptic signaling, WG, GBB and JEB signaling ligands are all disrupted in the absence of Mgat1 function, together with loss in synaptic recruitment of Discs large 1 (DLG1) and Lethal (2) giant larvae [L(2)GL] membrane scaffolds that modulate NMJ synaptogenesis (Humbert et al., 2008; Staples and Broadie, 2013; Wang et al., 2011). Together, these results show requirements for MGAT1-dependent N-glycosylation in trans-synaptic signaling and synaptic localization of intracellular scaffolds driving neuromuscular synaptogenesis.

RESULTS
MGAT1 shapes the glycosylated synaptomatrix of the NMJ

Lectins have been used to define the specialized carbohydrate environment of pre/postsynaptic membranes and perisynaptic extracellular space (Broadie et al., 2011; Dani and Broadie, 2012). For example, the widely employed anti-HRP antibody binds fucosylated N-glycans in the presynaptic membrane, which require Mgat1 for fucose modification (Sarkar et al., 2006). Likewise, VVA lectin is a synaptic glycan marker at the Drosophila NMJ, which reportedly recognizes primarily postsynaptic Dystroglycan (Haines et al., 2007; Rushton et al., 2012). The endogenous MTG lectin patterns the extracellular glycosylated synaptomatrix (Rohrbough et al., 2007), modulates trans-synaptic signaling and is essential for functional synaptogenesis (Rushton et al., 2009; Rohrbough and Broadie, 2010). To begin to define synaptogenic roles of MGAT1, we labeled the NMJ with each of these lectins in genetic control, Mgat1 null mutants and Mgat1 rescue conditions (Fig. 1).

Wandering third instar NMJs were first probed with anti-horseradish peroxidase (HRP, green) compared to anti-Fasciclin 2 (Fas2, red) in genetic control w1118 and Mgat1 null (Mgat1/Df(2R)BSC430) compared with w1118 genetic control (Fig. 1A). The HRP epitope robustly revealed the presynaptic terminal in control, but was undetectable in mutants. Phenotype rescue was assessed with ubiquitous UH1-Gal4 driven UAS-Mgat1 in Mgat1 null background, showing complete recovery of the HRP signal (Fig. 1A, right). Quantification of mutants normalized to control shows significant loss of HRP signal (w1118: 1.0±0.06; Mgat1: 0.12±0.02, P<0.001; Mgat1/Df: 0.16±0.03.
nulls compared with controls (Fig. 2A). Western blot analyses likewise show no detectable changes in FAS2 or DG protein stability or expression (Fig. 2B). Quantification of NMJ fluorescence intensities did not show significant changes in Mgat1 nulls compared with controls for FAS2 (normalized \( w^{118} \): 1.0±0.04; \( Mgat1^{+9} \): 1.03±0.05; \( Mgat1^{+/Df} \): 0.86±0.08; n.s., not significant; Fig. 2C) or DG (\( w^{118} \): 1.0±0.02; \( Mgat1^{+9} \): 1.06±0.01, \( Mgat1^{+/Df} \): 1.04±0.04; Fig. 2C). Numerous other lectins used as probes, including WGA, PNA, SBA, ECL and WFA (see Materials and methods), did not show detectable changes in \( Mgat1 \) nulls compared with \( w^{118} \) controls. Quantification of fluorescence intensities revealed no significant differences (data not shown). Together, these results show specific lectin changes at \( Mgat1 \) null NMJs, with loss of HRP and VVA labeling but not other lectin labels, but no changes in the abundance or synaptic localization of prominent HRP- (FAS2) and VVA- (DG) labeled proteins.

**Development of NMJ structural overgrowth in the \( Mgat1 \) null condition**

Our recent genomic survey of glycosylation genes suggested that glycan mechanisms largely function to restrict morphological growth during *Drosophila* NMJ synaptogenesis (Dani et al., 2012). Synaptic architecture is determined by axonal growth properties, branch formation and the differentiation of synaptic boutons as sites of synaptic vesicle storage for neurotransmitter release (Broadie et al., 2011; Nahm et al., 2013). To assay these structural parameters in \( Mgat1 \) mutants, wandering third instar 6/7 NMJs were labeled with anti-FAS2 and measurements made of NMJ length, branch number (process with at least two boutons) and bouton number (≥1 μm in diameter) in six genotypes; \( w^{118} \) background control, \( Mgat1^{+9} \) precise excision control, \( Mgat1^{+/Df} \) homozygous and \( Mgat1^{+/Df} \) null mutants, and muscle 24B-Gal4 and ubiquitous UH1-Gal4 driven UAS-\( Mgat1 \) rescue conditions. Neuronally driven UAS-\( Mgat1 \) resulted in early developmental lethality, and is therefore not included. A summary of these data is shown in Fig. 3.

Null \( Mgat1 \) mutants show a clear increase in NMJ size and structural complexity (Fig. 3A). Quantification of type I bouton number shows a highly significant increase in mutants normalized to control, rescued with ubiquitous but not muscle-targeted \( Mgat1 \), suggesting a neuronal requirement (\( w^{118} \): 1.0±0.03; \( Mgat1^{+9} \): 1.02±0.08; \( Mgat1^{+/Df} \): 1.50±0.06, \( P<0.001 \); Fig. 3B, left). Similarly, quantification of synaptic branch number shows a highly significant increase in \( Mgat1 \) nulls, rescued only with ubiquitous \( Mgat1 \); \( w^{118} \): 1.0±0.03; \( Mgat1^{+9} \): 0.92±0.08; \( Mgat1^{+/Df} \): 1.36±0.06, \( P<0.001 \); \( Mgat1^{+/Df} \): 1.26±0.03, \( P<0.001 \); UH1-Gal4 driven UAS-\( Mgat1 \); 0.89±0.06; Fig. 3B, middle). Finally, synaptic growth, quantified as normalized NMJ length, shows a highly significant increase in \( Mgat1 \) mutants, rescued only with ubiquitous \( Mgat1 \); \( w^{118} \): 1.0±0.03; \( Mgat1^{+9} \): 1.14±0.09; \( Mgat1^{+/Df} \): 1.81±0.08, \( P<0.001 \); \( Mgat1^{+/Df} \): 1.53±0.05, \( P<0.001 \); 24B-Gal4 driven UAS-\( Mgat1 \); 1.73±0.11, \( P<0.001 \); UH1-Gal4 driven UAS-\( Mgat1 \); 0.98±0.05; Fig. 3B, right). Muscle 24B-Gal4 expression of UAS-\( Mgat1 \) did not rescue any structural parameters, suggesting that ubiquitous or at least neuronal \( Mgat1 \) function is required to restore NMJ developmental growth and normal structural synaptogenesis.

**Pre- and postsynaptic \( Mgat1 \) roles restrict synaptic functional differentiation**

Regulation of *Drosophila* NMJ structural and functional synaptogenesis is often genetically separable, but N-glycans are
causally implicated in both developmental processes (Broadie et al., 2011; Dani et al., 2012). However, there have been no studies to assess overall N-glycan contributions to functional synaptic differentiation. We therefore next tested functional properties of Mgat1 null NMJs by measuring synaptic currents using two-electrode voltage-clamp (TEVC) recording. The motor nerve was stimulated with a glass suction electrode at suprathreshold levels to recruit both motoneuron inputs on muscle 6, and the excitatory junction current (EJC) recorded at 0.5 Hz to measure neurotransmission strength. In total, 12 genotypes were assayed: in addition to those described above, including Mgat1 nulls lacking maternal contribution; ubiquitous, neuron-targeted and muscle-targeted Mgat1 RNAi; and appropriate controls for transgenic conditions. A summary of these data is shown in Fig. 4.

NMJ functional strength was clearly and consistently increased in all Mgat1 loss-of-function conditions (Fig. 4A). Null zygotic mutants were comparable to animals lacking both maternal and zygotic expression, showing that a maternal contribution does not mask additional requirements. Mean EJC amplitudes were very significantly elevated in Mgat1 mutants normalized to genetic control (w1118: 1.0±0.02; Mgat1+/+: 1.45±0.10, P<0.001; Mgat1+/+ without maternal contribution: 1.38±0.06, P<0.001; Mgat1+/+Df: 1.40±0.05, P<0.001; Fig. 4A,B). Ubiquitous RNAi Mgat1 knockdown with UH1-Gal4 replicated this phenotype, and the elevated transmission was rescued by ubiquitous expression of UAS-Mgat1 in Mgat1+/+Df (w1118: 1.0±0.02; 24B-Gal4/+ control: 1.04±0.05; 24B-Gal4 UAS-RNAi-Mgat1: 1.29±0.06, P<0.01; Fig. 4E,D). Finally, presynaptic Mgat1 RNAi also elevated transmission strength (w1118: 1.0±0.02; elav-Gal4/+ control: 0.96±0.05; elav-Gal4, UAS-RNAi-Mgat1: 1.51±0.11, P<0.001; Fig. 4G,H). These results reveal separable pre- and postsynaptic Mgat1 roles limiting NMJ functional differentiation.

**MGAT1 regulates development of synaptic vesicle cycling properties**

As loss of Mgat1 function increases both synaptic morphogenesis and functional differentiation, the next step was to determine whether the overgrown structure simply mediates more transmission, or if structural and functional defects are due to separable Mgat1 requirements. Synaptic vesicle (SV) cycling with FM1-43 dye measures synaptic function within single boutons, thus allowing a clear separation of structure and function (Long et al., 2010; Nahm et al., 2013). To study SV endocytosis, FM1-43 dye was loaded under endogenous activity conditions over a prolonged period, and in response to acute depolarization with 90 mM K+ saline. To study SV exocytosis, NMJ terminals were depolarized a second time in the absence of FM1-43 to drive dye release. The ratio of loading to unloading provides a measure of SV cycling rate within individual synaptic boutons. A summary of these data is shown in Fig. 5.

Representative images of endogenous activity loading is shown in control and Mgat1+/+Df NMJs at 1, 10 and 30 minutes in Fig. 5A. Faint dye incorporation was present in boutons (arrows) after 1 minute loading in both genotypes; however, loading occurred significantly faster in control compared with mutant (Fig. 5A,B).
Comparing intensities over time points revealed a significant decrease in \(Mgat1\) loading [1 minute: \(23.1\pm1.9\) (\(w^{1118}\)) versus \(5.0\pm0.3\) (\(Mgat1\)), \(P<0.0001\); 10 minutes: \(79.5\pm3.5\) (\(w^{1118}\)) versus \(37.3\pm2.2\) (\(Mgat1\)), \(P<0.0001\); 30 minutes: \(124.8\pm12.6\) (\(w^{1118}\)) versus \(74.7\pm6.6\) (\(Mgat1\)), \(P<0.006\); Fig. 5B]. This difference could represent reduced central activity in locomotor pattern generation, reduced SV cycling in the NMJ or elevated dye release compared with uptake. To distinguish these possibilities, FM1-43 dye was loaded (5 minutes) and then partially unloaded (2 minutes) with acute high [K+] depolarization (Fig. 5C). Representative images control and \(Mgat1^{1/1}\) NMJs are shown on the left, with higher magnification images of individual boutons shown on the right. Two defects are qualitatively apparent: \(Mgat1\) nulls incorporate less dye, but release dye faster (Fig. 5C). Quantification of mean fluorescence intensities shows decreased loading in \(Mgat1\) nulls normalized to control (\(w^{1118}\): 1.0±0.03; \(Mgat1^{1}\): 0.83±0.04, \(P<0.05\); \(Mgat1^{1/Df}\): 0.76±0.04, \(P<0.001\); Fig. 5D, left). More strikingly, SV cycling rate (unloaded/downloaded fluorescence intensity) is increased in \(Mgat1\) nulls compared with control (\(w^{1118}\): 1.0±0.03; \(Mgat1^{1}\): 0.46±0.05, \(P<0.001\); \(Mgat1^{1/Df}\): 0.37±0.03, \(P<0.001\); Fig. 5D, right). Thus, \(Mgat1\) mutants exhibit altered SV cycling within individual boutons, independent of the increased bouton number, with a strong increase in cycling rate in response to acute depolarization.

**Selective loss of pre-and postsynaptic components in \(Mgat1\) null mutants**

Functional differentiation of the NMJ requires recruitment and organization of presynaptic SV cycle proteins and AZ release sites, and postsynaptic glutamate receptors (GluRs; Featherstone et al., 2005; Long et al., 2008; Richmond and Broadie, 2002). N-glycosylation may be important for localization and maintenance of these key proteins during synaptogenesis, hypothesized to be dependent on MGAT1 function (Dani et al., 2012; Kwon and Chapman, 2012). We therefore next conducted a thorough confocal microscopy expression survey of synaptic proteins in the \(Mgat1\) null condition to test for changes in presynaptic and postsynaptic composition. Most proteins were unchanged in \(Mgat1\) mutants, and only a few molecular changes were identified. A summary of these results is shown in Fig. 6.

Postsynaptic GluRs are believed to form tetramers composed of three essential subunits (GLURIIIC-E) and a single variable subunit (GLURIIA or B) generating two distinct GluR functional classes.
obvious decrease in GLURIIB-class receptors in Mgat1 null condition (data not shown). By contrast, there was a clear and essential GLURIIC) or the GLURIIA-class receptors in the change in the overall expression of total GluRs (labeled with the specific for GLURIIA, B and C subunits. There was no detectable elevation of these negative data, we show vesicular glutamate transporter (VGLUT) and V-SNARE Synaptobrevin (Fig. 6D). The selective loss of just BRP shows a focused presynaptic requirement for MGAT1 function.

Multiple trans-synaptic signaling pathways altered in Mgat1 null mutants

Active zone (i.e. BRP) and class-specific GluR (i.e. GLURIIB) recruitment are both dependent on tightly regulated trans-synaptic signaling between pre- and postsynaptic cells during synaptogenesis (Dani et al., 2012; Marqués, 2005; Rohrbough et al., 2013). We therefore hypothesized that Mgat1 mutants would manifest defects in bidirectional trans-synaptic signals. To test this idea, we assayed signaling ligands for three well-characterized trans-synaptic pathways: (1) anterograde Wnt Wingless (WG); (2) retrograde BMP Glass bottom boat (GBB); and (3) newly defined ligand Jelly belly (JEB). Representative NMJ images and the compiled quantification for these studies are shown in Fig. 7.
right). By contrast, the GBB signal was clearly reduced and poorly localized to the synaptic domain in mutants (Fig. 7B, middle), and likewise significantly decreased in quantified intensity (w1118: 1.0±0.08; Mgat11/Df: 0.55±0.08, P<0.001; Fig. 7B, right). The third signal JEB was similarly decreased in Mgat11/Df compared with control (w1118: 1.0±0.07; Mgat11/Df: 0.63±0.07 P<0.01; Fig. 7C). These results reveal a differential MGAT1 role in modulating trans-synaptic signaling, with increased abundance of WG ligand and decreases in both GBB and JEB ligands. A primary role of trans-synaptic signaling is to recruit synaptic scaffolds, which in turn bind synaptic proteins to seed the process of synaptogenesis.

**Loss of key synaptic scaffolds driving synaptogenesis in Mgat1 null mutants**

Discs large 1 (DLG1) is a particularly well-characterized synaptic scaffold at the Drosophila NMJ, which is modulated downstream of WG, GBB and JEB trans-synaptic signaling and, in turn, drives the appropriate recruitment of synaptic proteins including cell adhesion molecules, ion channels and GLURIB-containing receptors (Chen and Featherstone, 2005; Marqués, 2005; Marurus et al., 2004). In addition, we have just recently defined Lethal (2) giant larvae [L(2)GL] as another key synaptic scaffold, which presynaptically facilitates the assembly of BRP-containing active zones to regulate SV cycling, and postsynaptically regulates GluR subunit composition (Staples and Broadie, 2013). We hypothesized that, downstream of MGAT1-dependent changes in trans-synaptic signaling, defects in recruiting these synaptic scaffolds could explain changes in pre/postsynaptic molecular composition. To test this idea, we imaged DLG1 and L(2)GL scaffolds at the wandering third instar NMJ, comparing Mgat1 nulls to genetic controls. A summary of these data is shown in Fig. 8.

The DLG1 scaffold is expressed in both pre- and postsynaptic compartments, but is most apparent in the subsynaptic reticulum (SSR) overlapping with postsynaptic glutamate receptors (Fig. 8A). DLG1 levels are clearly and strongly decreased in Mgat1 null mutants compared with controls. When intensity levels were quantified, there was a very significant decrease in mutants normalized to genetic control (w1118: 1.0±0.08; Mgat11/Df: 0.66±0.06, P<0.01; Fig. 8C, top). Similarly, the L(2)GL scaffold is present in the FAS2-labeled NMJ terminals in both presynaptic boutons and the postsynaptic domain, with clearly higher levels of expression in genetic control compared with the Mgat1 null condition (Fig. 8B). Quantification of fluorescence intensity shows a highly significant decrease in L(2)GL (w1118: 1.0±0.07; Mgat11: 0.50±0.06, P<0.001; Fig. 8C, bottom). L(2)GL and DLG1 scaffolds are both known to regulate active zone and glutamate receptor composition, so changes in their abundance and localization are likely to be causal in MGAT1-dependent changes in NMJ synaptogenesis.

**DISCUSSION**

We began with the hypothesis that disruption of synaptomatrix N-glycosylation would alter trans-synaptic signaling underlying NMJ synaptogenesis (Dani and Broadie, 2012). MGAT1 loss transforms the synaptomatrix glycan environment. Complete absence of the HRP epitope, α1-3-fucosylated N-glycans, is expected to require MGAT1 activity: key HRP epitope synaptic proteins include fasciclin, Neurotactin and Neuroglian, among others (Desai et al., 1994; Paschinger et al., 2009). We show that HRP epitope modification of the key synaptogenic regulator Fasciclin 2 is not required for stabilization or localization, suggesting a role in protein function. However, complete loss of VVA lectin synaptomatrix labeling is surprising because the epitope is a terminal β-GalNAc
Drosophila complex formation in Dystroglycan glycosylation blocks extracellular ligand binding and synaptic context. Importantly, VVA labels Dystroglycan and loss of glycans/glycosphingolipids may be present on N-glycans in this enriched at the NMJ, and that the terminal GalNAc expected on O-(Martin, 2003). This result suggests that the N-glycan LacdiNAc is enriched at the NMJ, and that the terminal GalNAc expected on O-glycans/glycosphingolipids may be present on N-glycans in this synaptic context. Importantly, VVA labels Dystroglycan and loss of Dystroglycan glycosylation blocks extracellular ligand and complex formation in Drosophila (Haines et al., 2007; Nakamura et al., 2010), and causes muscular dystrophies in humans (Ervasti et al., 1997; Muntoni et al., 2008; Tran et al., 2012). This study shows that VVA-recognized Dystroglycan glycosylation is not required for protein stabilization or synaptic localization, but did not test functionality or complex formation, which probably requires MGAT1-dependent modification. Conversely, the secreted endogenous lectin MTG is highly elevated in Mgat1 null synaptomatrix, probably owing to attempted compensation for complex and hybrid N-glycan losses that serve as MTG binding sites. MTG binds GlcNAc in a calcium-dependent manner and pulls down a number of HRP-epitope proteins by immunoprecipitation (Rushton et al., 2012), although the specific proteins have not been identified. It will be of interest to perform immunoprecipitation on Mgat1 samples to identify changes in HRP bands. Importantly, MTG is crucial for synaptomatrix glycan patterning and functional synaptic development (Rohrbough et al., 2007). MTG regulates VVA synaptomatrix labeling (Rushton et al., 2009), suggesting a mechanistic link between the VVA and MTG changes in Mgat1 mutants. The MTG elevation observed in Mgat1 nulls provides a plausible causative mechanism for strengthened functional differentiation (Rohrbough and Broadie, 2010; Rushton et al., 2012).

Consistent with our recent glycosylation gene screen findings (Dani et al., 2012), Mgat1 nulls exhibit increased synaptic growth and structural overelaboration. Therefore, complex and hybrid N-glycans overall provide a brake on synaptic morphogenesis, although individual N-glycans may provide positive regulation. Likely players include MGAT1-dependent HRP-epitope proteins (e.g. fasciclin, Neurotactin, Neuroglan), and position-specific (PS) integrin receptors and their ligands, all of which are heavily glycosylated and have well-characterized roles regulating synaptic architecture (Beumer et al., 1999; Rushton et al., 2009; Beck et al., 2012; Enneking et al., 2013). An alternative hypothesis is that Mgat1 phenotypes may result from the presence of high-mannose glycans on sites normally carrying complex/hybrid structures (Schachter, 2010), suggesting possible gain of function rather than loss of function of specific N-glycan classes. NMJ branch and bouton number play roles in determining functional strength (Thomas and Sigrist, 2012), although active zones and GluRs are also regulated independently (DiAntonio, 2006). Thus, the increased functional strength could be caused by increased structure at Mgat1 null NMJs. However, muscle-targeted UAS-Mgat1 rescues otherwise Mgat1 null function, but has no effect on structural defects, demonstrating that these two roles are separable. Presynaptic Mgat1 RNAi also causes strong functional defects, showing there is additionally a presynaptic requirement in functional differentiation. Neuron-targeted Mgat1 causes lethality, indicating that MGAT1 levels must be tightly regulated, but preventing independent assessment of Mgat1 presynaptic rescue of synaptogenesis defects.

Presynaptic glutamate release and postsynaptic glutamate receptor responses drive synapse function. Using lipophilic dye to visualize SV cycling, we found Mgat1 null mutants endogenously cycle less than controls, but have greater cycling upon depolarizing stimulation. The endogenous cycling defect is consistent with the sluggish locomotion of Mgat1 mutants (Sarkar et al., 2006), whereas the elevated stimulation-evoked cycling is consistent with electrophysiological measures of neurotransmission. Similarly, mutation of dPOMT1, which glycosylates VVA-labeled Dystroglycan, decreases SV release probability (Wairkar et al., 2008), although dPOMT1 adds mannose not GalNAc. Null Mgat1

![Fig. 7. Multiple trans-synaptic signaling pathways altered in Mgat1 null mutant. Assays of three well-characterized NMJ trans-synaptic signals: Wingless (WG), Glass bottom boat (GBB) and Jelly belly (JEB). (A) Representative NMJ images of WG (red) double-labeled with HRP (blue), and shown alone (WG, white). High-magnification bouton comparison of genetic control (w1118) and Mgat1/Df. Right: Quantification of relative fluorescence intensity reveals increased WG in mutant. (B) Representative images of GBB (green) double-labeled with HRP (blue), and shown alone (GBB, white). High-magnification bouton comparison of w1118 and Mgat1/Df. Right: Quantification shows decreased GBB in mutant. (C) Representative NMJ images of JEB (green) double-labeled with HRP (blue), and shown alone (JEB, white). High-magnification bouton comparison of w1118 and Magat1/Df. Right: Quantification shows decreased JEB in mutant. Fluorescence intensities measured within the NMJ domain (white dotted line), normalized to genetic control w1118. **P<0.01 and ***P<0.001 (Student’s t-test) for pairwise comparisons. The sample size is n=18 NMJs for each label and each genotype.](image-url)
mutants display no change in SV cycle components (e.g. Synaptobrevin, Synaptotagmin, Synaptogyrin, etc.), but exhibit reduced expression of the key active zone component Bruchpilot (Wagh et al., 2006; Kittel et al., 2006). Other examples of presynaptic glycosylation requirements include the Drosophila FUSEless (FUSL) glycans transporter, which is critical for Cacophony (CAC) voltage-gated calcium channel activity in active zones (Long et al., 2008), and the mammalian GalNAc transferase (GALGT2), whose overexpression causes decreased active zone assembly (Martin, 2003). Postsynaptically, Mgat1 nulls show specific loss of GLURIIA-containing receptors. Similarly, dPOMT1 mutants exhibit specific GLURIII loss (Wairkar et al., 2008), although dystroglycan nulls display GLURIIA loss (Bogdanik et al., 2008). Selective GLURIIA loss in Mgat1 nulls may drive increased neurotransmission owing to channel kinetics differences in GLURIIA versus GLURIII receptors (DiAntonio et al., 1999).

Bidirectional trans-synaptic signaling regulates NMJ structure, function and pre/postsynaptic composition (Dani et al., 2012; Enneking et al., 2013; Müller and Davis, 2012). This intercellular signaling requires ligand passage through, and containment within, the heavily glycosylated synaptomatrix (Dani and Broadie, 2012; Martin, 2003), which is strongly compromised in Mgat1 mutants. In testing three well-characterized signaling pathways, we found that WG accumulates, whereas both GBB and JEB are reduced in the Mgat1 null synaptomatrix. WG has two N-glycosylation sites, but these do not regulate ligand expression (Tang et al., 2012), whereas both GBB and JEB are reduced in the Mgat1 null synaptomatrix. WG overexpression increases NMJ activity, similarly to the reduced endogenous SV cycling in Mgat1 mutants (Rohrbough and Broadie, 2010). Moreover, the MTG lectin negatively regulates JEB accumulation in NMJ synaptomatrix (Rohrbough and Broadie, 2010), consistent with elevated MTG causing JEB downregulation in Mgat1 nulls.

Trans-synaptic signaling drives recruitment of scaffolds that, in turn, recruit pre- and postsynaptic molecular components (Ataman et al., 2006; Koles et al., 2012). Specifically, DLG1 and L(2)GL scaffolds regulate the distribution and density of both active zone components (e.g. BRP) and postsynaptic GluRs (Chen and Featherstone, 2005; Staples and Broadie, 2013), and both of these scaffolds are reduced at Mgat1 null NMJs. Importantly, dlg1 mutants display selective loss of GLURIIA, with GLURIIA unchanged, similar to Mgat1 nulls (Chen and Featherstone, 2005), suggesting a causal mechanism. Moreover, l(2)gl mutants display both a selective GLURIIA impairment as well as reduction of BRP aggregation in active zones, similarly to Mgat1 nulls (Staples and Broadie, 2013), suggesting a separable involvement for this synaptic scaffold. DLG1 and L(2)GL are known to interact in other developmental contexts (Humbert et al., 2008), indicating a likely interaction at the developing synapse. Although synaptic ultrastructure has not been examined in l(2)gl mutants, dlg1 mutants exhibit impaired NMJ development, including a deformed SSR (Lahey et al., 1994). These synaptogenesis requirements predict similar ultrastructural defects in Mgat1 mutants, albeit presumably due to the combined loss of both DLG1 and L(2)GL scaffolds. Our future work will focus on electron microscopy analyses to probe N-glycosylation mechanisms of synaptic development.

**MATERIALS AND METHODS**

**Drosophila genetics**

All genotypes were made in the w+ background, with w1118 used as genetic control. Df(2R)BSC430 removing Mgat1 was obtained from the Bloomington Drosophila Stock Center (Indiana University). Imprecise excision Mgat1+ and precise excision Mgat1+2 lines have been characterized (Sarkar et al., 2006). Transgenic studies were done with the pan-neural elav-Gal4, muscle 248-Gal4 and ubiquitous UH1-Gal4 driver lines (Brand and Perrimon, 1993; Lin and Goodman, 1994; Rohrbough et al., 2007) crossed to UAS-Mgat1 (Sarkar et al., 2010) or UAS-RNAi-Mgat1 lines obtained from the Vienna Drosophila RNAi Center (VDRC). The Mind the gap (MTG) cDNA fused to GFP coding sequence (UAS-MTG:GFP; Rushton et al., 2006) was used as the control for FACS analyses.
al., 2009) was placed into w; Mgtat^{1}/CyO-GFP to express MTG::GFP in the null background.

**Immunocytochemistry**

Studies were performed as described previously (Rushion et al., 2012; Dani et al., 2012). Briefly, wandering third instars were dissected in physiological saline consisting of 128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 0.2 mM CaCl₂, 70 mM sucrose, 5 mM trehalose and 5 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 7.1). Preparations were fixed in ice-cold methanol for 5 minutes (GLURIIA) or 4% paraformaldehyde for 10 minutes at room temperature (RT; all other labels). Preparations were then either processed with detergent [phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) + 0.2% Triton X-100] for cell-permeabilized studies, or detergent-free (PBS with 1% BSA) conditions for non-permeabilized studies. Primary antibodies used included: rabbit anti-HRP (1:200, Sigma); mouse anti-Fasciclin 2 [FA52, 1:10, 1D4, Developmental Studies Hybridoma Bank (DHSB), University of Iowa]; rabbit anti-dg (1:1000, gift from Wu Deng, Department of Biological Sciences, The Florida State University); mouse anti-glutamate receptor II A (GLURIIA, 1:100, 84BD2[MHZB), DHSB); rabbit anti-GLURIIB (1:1000) (Marrus et al., 2004), and rabbit anti-GLURIC (1:500) (Marrus et al., 2004); mouse anti-BRP (1:100, NC82, DHSB); rabbit anti-vesicular glutamate transporter (VGLUT, 1:10,000) (Daniels et al., 2004); rabbit anti-SYB (1:500) (Littleton et al., 1993); mouse anti-Cysteine string protein (CSP, 1:250) (Zinsmaier et al., 1990); rabbit anti-Syaptotagrypin (GYR, 1:500) (Stevens et al., 2012); mouse anti-Wingless (WG, 1:2, 4D4, DHSB); rabbit anti-GaBb (1:100) (Dani et al., 2012), guinea pig anti-JEB (1:2000) (Lee et al., 2003); mouse anti-DLG (1:200, DLG1, DHSB); rabbit anti-L2 (GL2) (1:300) (Ohshiro et al., 2000). Lectins used included: Vicia villosa agglutinin (VVA-Trifc, 1:200, R-4061, E.Y. Laboratories); and wheat germ agglutinin (WGA, 1:200, B-1025, Vector Laboratories), peanut agglutinin (PNA, 1:250, B-1075, Vector Laboratories), soybean agglutinin (SBA, 1:200, B-1015, Vector Laboratories), Erythrina cristagalli lectin (ECL, 1:250, B-1145, Vector Laboratories) and Wisteria floribunda lectin (WFA, 1:250, B-1355, Vector Laboratories), all from Vector Laboratories. Secondary Alexa fluorophore antibodies (Invitrogen) used included: goat anti-mouse 488 and 568 (1:250), goat anti-rabbit 488/568 (1:250), goat anti-guinea pig 488/568 (1:250) and streptavidin 488 (1:250). Primary antibodies and lectins were incubated at 4°C overnight; secondary antibodies were incubated at RT for 2 hours. Dissections were mounted in Fluoromount-G (Electron Microscopy Sciences). Z-stacks were taken with a Zeiss LSM 510 META laser-scanning confocal microscope using 40x or 63x water immersion objectives. Optical sections were done starting immediately above and ending immediately below the NMJ. Stacks were projected on the z-axis, with NMJ signals highlighted and average intensity for each recorded. Intensities were quantified using ImageJ (Abramoff et al., 2004).

**Western blotting**

Dissected wandering third instar ventral nerve cords (6) were homogenized in buffer [67 mM NaCl, 2 M urea, 1.3% sodium dodecyl sulfate (SDS), 1 mM EDTA, Tris pH 8] and centrifuged for 30 minutes at 16,000 g. Soluble fractions with 1× NuPage sample buffer (Invitrogen) and 5% 2-mercaptoethanol were boiled for 10 minutes. Samples were loaded onto 4-12% Bis-Tris SDS gels (Invitrogen), electrophoresed at 200 V for 90 minutes in 1× MES buffer and transferred to nitrocellulose membranes (Biorad) in 1× NuPage transfer buffer with 300 mA for 1 hour at 4°C. Membranes were blocked in 2% BSA (Sigma) in Tris-buffered saline + Tween 20 (TBST; 10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 hour at RT. Rabbit anti-HRP (Sigma (1:1000)) or biotinylated VVA (EY labs) (1:1000) were diluted in blocking buffer and incubated for 1 hour at RT and washed for 5 minutes in TBST (six times). Mouse anti-tubulin (Sigma) (1:5000), rabbit anti-dg (1:1000) or mouse anti-FAS2 (34B3C2) (1:100) were diluted in blocking buffer and incubated overnight at 4°C. Preparations were washed for 5 minutes with TBST (six times), and streptavidin-800 (Rockland) (1:10,000), goat anti-mouse 680 (Invitrogen) (1:10,000) or goat anti-rabbit 800 (Rockland) (1:10,000) were incubated for 1 hour at RT. Blots were washed for 5 minutes in TBST (six times) and then imaged using an Odyssey Infrared Imaging System.

**Electrophysiology**

TEVC electrophysiology was performed as previously reported (Rohrbough and Broadie, 2002). Briefly, staged larvae were glued with 3M Vetbond tissue adhesive (World Precision Instruments) to sylgard-coated glass coverslips, cut longitudinally along the dorsal midline, internal organs removed and sides glued down for neuromusculature access. Peripheral nerves were cut near the ventral nerve cord (VNC). Dissections and recordings were performed at 18°C in saline consisting of 128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 1 mM CaCl₂, 70 mM sucrose, 5 mM trehalose and 5 mM HEPES (pH 7.1). Preparations were imaged using a Zeiss Axioskop microscope with 40x water immersion objective. A fire-polished glass suction electrode was used for evoked nerve stimulation with a 0.5-second supraphasalpolar stimulus at 0.2 Hz from a Grass S88 stimulator (Rohrbough and Broadie, 2010). Muscle 6 in abdominal segments 2/3 was impaled with two microelectrodes of ~15 MΩ resistance filled with 3 M KCl. The muscle was clamped at ~60 mV using an Axoclamp-2B amplifier. EJC records were filtered at 2 kHz. To quantify EJC amplitudes, ten consecutive traces were averaged and the peak of the averaged trace recorded. Clampex software was used for all data acquisition, Clampfit software was used for all data analysis, and GraphPad InStat 3 software was used for statistical tests.

**FM1-43 dye imaging**

Synaptic vesicle cycling was imaged using lipophilic dye FM1-43, as previously reported (Long et al., 2010; Nahm et al., 2013). Briefly, for endogenous labeling, dissected preparations were incubated in physiological saline (1.0 mM Ca^{2+} plus 10 μM FM1-43 (Invitrogen). To stop labeling at staged intervals, the saline was replaced several times in quick succession with Ca^{2+}-free saline lacking FM1-43. For evoked depolarization dye loading, preparations were stimulated with 90 mM K⁺ plus 10 μM FM1-43 for 5 minutes. After imaging, preparations were unloaded with the same depolarizing stimulation without FM1-43 for 2 minutes. Z-stacks were taken with a Zeiss LSM 510 META laser-scanning confocal microscope using a 63x water immersion objective. Fifteen slices were acquired and stacks projected in ImageJ. To quantify loaded/unloaded fluorescence intensities, five individual boutons per NMJ were outlined and average intensity measured in ImageJ, with muscle background intensities subtracted.

**Statistics**

All statistics were performed using GraphPad InStat3 software. ANOVA tests were used for all data sets of at least three comparisons. Student’s t-test was used for pairwise comparisons.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

W.P. performed and analyzed most of the experiments. M.L.D. ran all western blots and related quantification in Figs 1 and 2. E.R. imaged MTG in Fig. 1E. W.P. and K.B. designed experiments and wrote the manuscript.

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