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 Mgat1 nulls are the most compromised N-glycosylation condition that survive long enough to permit synaptogenesis studies. At the Drosophila neuromuscular junction (NMJ), Mgat1 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 Mgat1 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. Synapse molecular composition is surprisingly selectively altered, with decreases in presynaptic active zone Bruchpilot (BRP) and postsynaptic Glutamate receptor subtype B (GLURIIB), but no detectable change in a wide range of other synaptic components. Synaptogenesis is driven by bidirectional trans-synaptic signals that traverse the glycans-rich synaptomatrix, and Mgat1 mutation disrupts 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 Mgat1 mutants exhibit loss of both classic Discs large 1 (DLG1) and newly defined Lethal (2) giant larvae [L(2)GL]. We conclude that MGAT1-dependent N-glycosylation shapes the synaptomatrix carbohydrate environment 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 Mgat1 gene encoding GlcNAcT1 adds GlcNAc 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 Mgat1 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 Mgat1 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 Mgat1 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 Mgat1 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 Mgat1 neuronal overexpression increases lifespan (Sarkar et al., 2010; Schachter and Boulianne, 2011). In the central brain mushroom body learning/memory center, Mgat1 nulls show fused lobes similar to fused lobes (fdl) mutants in β-N-acetylgalcosaminidase, which removes the MGAT1-generated GlcNAc (Léonard et al., 2006; Sarkar et al., 2006). MGAT1 is also required for α3 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 neuronal proteins, including neurotransmitter receptors, SNAREs, fasciculins, 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 (Henríquez and Salinas, 2012; Patton, 2003). Three well-characterized trans-synaptic signals at the Drosophila NMJ are the Wnt protein Wingless (WG), the bone morphogenetic 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) and anti-Fasciclin 2 (Fas2, red) in genetic control w1118, Mgat1Df(2R)BSC430 and UH1-GAL4 driven UAS-Mgat1 in Mgat1 null background. HRP labeling is undetectable in the Mgat1 null, and fully restored by the genetic rescue. (B) Representative NMJ images of Vicia villosa (VVA, red) lectin labeling with Fas2 co-labeling (green) in the same genotypes. VVA labeling is undetectable in Mgat1 nulls, and fully restored by genetic rescue. (C) Quantification of HRP and VVA intensity normalized to w1118. ***P≤0.001 (ANOVA). Sample size is ≥10 NMJs from at least five animals of each genotype. (D) Representative anti-HRP western blot from w1118, Mgat1 null and UH1-Mgat1 rescue conditions. All HRP glycans are undetectable in Mgat1 null, and restored by Mgat1 rescue. The single band (asterisk) represents bleed-through from α-tubulin loading control. (E) Representative NMJ images of Mind the gap (MTG-GFP, green) lectin co-labeled with anti-Fas2 (red), and shown alone (MTG, white), in control (Mgat1+ precise excision) and Mgat1 null. MTG is greatly increased in mutants (P<0.0009; sample size: at least eight NMJs, at least four animals/genotype).
Mgat1 within FAS2-labeled synaptic domains shows a ~70% increase in fluorescent protein (GFP) (Fig. 1E). Quantification of MTG-GFP Mgat1A,C). By contrast, the endogenous MTG lectin is highly elevated at nulls. (A) Representative NMJ images of anti-Fasciclin 2 (Fas2, red) double-labeled with anti-Dystroglycan (DG, green) in w1118 control and Mgat1/Df mutant. (B) Representative western blots double-labeled for Fas2 and HRP (left) and DG and VVA lectin (right) in w1118 and Mgat1/Df. Alpha tubulin is the loading control. (C) Confocal fluorescence intensity quantification shows no significant (n.s.) change in Fas2 or DG at the NMJ in either homozygous Mgat1 or Mgat1/Df conditions compared with w1118. ANOVA statistical analyses were carried out on a sample size of n=16 NMJs for each genotype. P<0.001; Fig. 1C). Anti-HRP recognizes at least 18 protein bands in western blots (Fig. 1D) (Desai et al., 1994). All these N-glycans are lost in Mgat1 nulls and restored by UH1-Gal4 driven UAS-Mgat1 (Fig. 1D). The VVA lectin signal is similarly lost in Mgat1 mutants and rescued with UH1-Gal4 driven UAS-Mgat1 (w1118: 1.0±0.06; Mgat1: 0.02±0.004, P<0.001; Mgat1/Df: 0.04±0.01, P<0.001; Fig. 1A,C). By contrast, the endogenous MTG lectin is highly elevated at null NMJs, as revealed by transgenic MTG tagged with green fluorescent protein (GFP) (Fig. 1E). Quantification of MTG-GFP within FAS2-labeled synaptic domains shows a ~70% increase in nulls compared with controls (Mgat1+/+: 1.0±0.03; Mgat1+/+: 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 (w1118: 1.0±0.03; Mgat1+/+: 1.14±0.09; Mgat1+/+: 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.

**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; w1118 background control, Mgat1+/+ precise excision control, Mgat1 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 1 bouton number shows a highly significant increase in mutants normalized to control, rescued with ubiquitous but not muscle-targeted Mgat1, suggesting a neuronal requirement (w1118: 1.0±0.03; Mgat1+/+: 1.02±0.08; Mgat1+/+: 1.50±0.06, P<0.001; Mgat1/Df: 1.58±0.05, P<0.001; 24B-Gal4 driven UAS-Mgat1: 1.49±0.06; UH1-Gal4 driven UAS-Mgat1: 0.97±0.08; Fig. 3B, left). Similarly, quantification of synaptic branch number shows a highly significant increase in Mgat1 nulls, rescued only with ubiquitous Mgat1 (w1118: 1.0±0.03; Mgat1+/+: 0.92±0.08; Mgat1+/+: 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 (w1118: 1.0±0.03; Mgat1+/+: 1.14±0.09; Mgat1+/+: 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).

**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
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 null 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±1.9 (\(\text{w}^{1118}\)) versus 5.0±0.3 (\(Mgat1\)), \(P<0.0001\); 10 minutes: 79.5±3.5 (\(\text{w}^{1118}\)) versus 37.3±2.2 (\(Mgat1\)), \(P<0.0001\); 30 minutes: 124.8±12.6 (\(\text{w}^{1118}\)) versus 74.7±6.6 (\(Mgat1\)), \(P<0.006\); 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 \(Mgat1^{11}/\text{Df}\) 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 (\(\text{w}^{1118}\): 1.0±0.03; \(Mgat1^{11}\): 83±0.04, \(P<0.05\); \(Mgat1^{11}/\text{Df}\): 76±0.04, \(P<0.001\); Fig. 5D, left). More strikingly, SV cycling rate (unloaded/loaded fluorescence intensity) is increased in \(Mgat1\) nulls compared with control (\(\text{w}^{1118}\): 1.0±0.03; \(Mgat1^{11}\): 0.46±0.05, \(P<0.001\); \(Mgat1^{11}/\text{Df}\): 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.
obvious decrease in GLURIIB-class receptors in Mgat1 null condition (data not shown). By contrast, there was a clear and essential GLURIC-class or the GLURIIA-class receptors in the change in the overall expression of total GluRs (labeled with the specificity for GLURIIA, B and C subunits. There was no detectable

As exemplars of these negative data, we show vesicular glutamate (Gyr)] were not detectably altered in Synaptotagmin, Cysteine string protein (CSP), Synaptogyrin (Gyr)] were not detectably altered in Mgat1 nulls (data not shown).

The presynaptic apparatus includes key AZ protein Bruchpilot (BRP), V/T-SNARES of the core SNARE complex, and a host of SV associated and integral proteins regulating the SV cycle (Chapman, 2002; Hallermann et al., 2010). We assayed a wide range of these presynaptic proteins for changes in Mgat1 null mutants, both at low level magnification of the entire NMJ and in higher resolution images of individual synaptic boutons (Fig. 6A). Quantitative measures of fluorescence intensity show a highly significant decrease in GLURIIB labeling in Mgat1/Df normalized to the genetic control (w^{1118}: 1.0±0.09; Mgat1/Df: 0.73±0.04, P<0.01; Fig. 6D, left). This selective loss of GLURIIB shows that this one receptor class alone appears dependent on MGAT1 function.

The synaptotagmin family provides a ready example of proteins that traverse the synaptomatrix and are highly glycosylated (Dani et al., 2012; Marqués, 2005; Rohrbough et al., 2013). We therefore hypothesized that Mgat1 mutants would manifest defects in bidirectional trans-synaptic signaling between pre- and postsynaptic cells during synaptogenesis (Dani et al., 2012; Marqués, 2005; Rohrbough et al., 2013). Moreover, both trans-synaptic signaling pathway components and the synaptomatrix they traverse are highly glycosylated, and it is known that glycan mechanisms play key roles in the regulation of this developmental communication (Rohrbough and Broadie, 2010; 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.

Well-characterized antibodies for all three signaling ligands were used to label wandering third instar NMJs, comparing fluorescence intensities within the synaptic domain (dotted white outlines) in Mgat1/Df null normalized to the w^{1118} genetic control (Fig. 7). The WG signal was obviously increased in mutants (Fig. 7A, middle), and quantification of fluorescence intensity confirmed a significant elevation (w^{1118}: 1.0±0.10; Mgat1/Df: 1.56±0.16, P<0.001; Fig. 7A,
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; Mgat1/Df: 0.55±0.08, P<0.001; Fig. 7B, right). The third signal JEB was similarly decreased in Mgat1/Df compared with control (w1118: 1.0±0.07; Mgat1/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; Marrus 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 presynaptic 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; Mgat1/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 compared to the Mgat1 null condition (Fig. 8B).

Quantification of fluorescence intensity shows a highly significant decrease in L(2)GL (w1118: 1.0±0.07; Mgat1/Df: 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 2, 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...
(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 binding 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 and Broadie, 2010; Rushton et al., 2012). Neuron-targeted Mgat1 RNAi also causes strong functional 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 capacity 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
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) glycan transporter, which is critical for Cacophony (CAC) voltage-gated calcium channel recruitment to active zones (Long et al., 2008), and the mammalian GaINAc transferase (GALGT2), whose overexpression causes decreased active zone assembly (Martin, 2003). Postsynaptically, Mga1 nulls show specific loss of GLURIIA-containing receptors. Similarly, dPOMT1 mutants exhibit specific GLURIIA loss (Wairkar et al., 2008), although dystroglycan nulls display GLURIIA loss (Bogdanik et al., 2008). Selective GLURIIA loss in Mga1 nulls may drive increased neurotransmission owing to channel kinetics differences in GLURIIA versus GLURIIB 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 Mga1 mutants. In testing three well-characterized signaling pathways, we found that WG accumulates, whereas both GBB and JEB are reduced in the Mga1 null synaptomatrix. WG has two N-glycosylation sites, but these do not regulate ligand expression (Tang et al., 2012), with high-magnification boutons in side panels. (A) Representative images of L(2)GL (red) co-labeled with Fas2 (green), with similar ultrastructural defects in Mga1 null NMJs. Importantly, GBB loss impairs presynaptic active zone development similarly to the phenotype of Mga1 nulls (Chen and Featherstone, 2005), suggesting a separable 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 Mga1 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 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 Mga1 null NMJs. Importantly, dlg1 mutants display selective loss of GLURIIA, with GLURIIA unchanged, similar to Mga1 nulls (Chen and Featherstone, 2005), suggesting a causal mechanism. Moreover, l(2)gl mutants display impaired NMJ development, including a deformed SSR 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^{1118} background, with w^{1118} used as genetic control. Df(2R)BSC430 removing Mga1 was obtained from the Bloomington Drosophila Stock Center (Indiana University). Imprecise excision Mga1^1 and precise excision Mga1^1*E* lines have been characterized (Sarkar et al., 2006). Transgenic studies were done with the pan-neural elav-Gal4, muscle 24B-Gal4 and ubiquitous UH1-Gal4 driver lines (Brand and Perrimon, 1993; Lin and Goodman, 1994; Rohrbough et al., 2007) crossed to UAS-Mga1 (Sarkar et al., 2010) or UAS-RNAi-Mga1 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

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**Fig. 8. Synaptic scaffolds DLG1 and L(2)GL reduced in the Mga1 null mutants.** Imaging of DLG1 and L(2)GL synaptic scaffolds at the wandering third instar NMJ. (A) Representative images of DLG1 (red) co-labeled with glutamate receptor type IIC (GLURIIIC, green), with high-magnification boutons in side panels. (B) Representative images of L(2)GL (red) co-labeled with Fas2 (green), with high-magnification boutons in side panels. (C) Quantification of DLG1 fluorescence intensity (top: Mga1^1, n=15; w^{1118}, n=22) and L(2)GL fluorescence intensity (bottom: Mga1^1, n=12; w^{1118}, n=12)**, **P<0.001 (Student’s t-test) for pairwise comparisons.
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, ID4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa]; rabbit anti-DG (1:1000, gift from Wu Deng, Department of Biological Sciences, The Florida State University); mouse anti-glutamate receptor IIA [GLU/RIA, 1:100, 8B4D2(MHZB), DSHB], rabbit anti-GLUR1B (1:1000) (Marrus et al., 2004), and rabbit anti-GLUR1C (1:500) (Marrus et al., 2004); mouse anti-BRP (1:100, NC82, DSHB); 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-Synaptogyrin (GYR, 1:500) (Stevens et al., 2012); mouse anti-Wingless (WG, 1:2, 4D4, DSHB); rabbit anti-GGB (1:100) (Dani et al., 2012), guinea pig anti-JEB (1:2000) (Lee et al., 2003); mouse anti-DLG (1:200, DLG1, DSHB); rabbit anti-L2 (G2L) (1:300) (Ohshiro et al., 2000). Lectins used included: *Vicia villosa* agglutinin (VVA-Tritc, 1:200, R-4601-2, E.Y. Laboratories); and wheat germ agglutinin (WGA, 1:200, B-1015, 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 40× or 63× 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 (3B3C2) (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 Brodie, 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 Axiostar microscope with 40× water immersion objective. A fire-polished glass suction electrode was used for evoked nerve stimulation with a 0.5-second suprathreshold stimulus at 0.2 Hz from a Grass S88 stimulator (Rohrbough and Brodie, 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 Ca2+) plus 10 μM FM1-43 (Invitrogen). To stop loading at staged intervals, the saline was replaced several times in quick succession with Ca2+-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 63× 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 for genetic lines and antibodies, respectively. We are most grateful to Gabrielle Boulianne (University of Toronto, Canada) for Mgat1 alleles; and to Wu-Min Deng (Florida State University) for anti-DG, Aaron DiAntonio (Washington University School of Medicine) for anti-GLURIB/C, Ruth Palmer (Umea Institute, Sweden) for anti-JEB, and Daniela Zarnescu (University of Arizona) for anti-L(2)GL antibodies.

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