Two Protein N-Acetylgalactosaminyl Transferases Regulate Synaptic Plasticity by Activity-Dependent Regulation of Integrin Signaling

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Using a *Drosophila* whole-genome transgenic RNAi screen for glycogenes regulating synapse function, we have identified two protein α -N-acetylgalactosaminyltransferases (*pgant3* and *pgant35A*) that regulate synaptic O-linked glycosylation (GalNAc α 1-O-S/T). Loss of either *pgant* alone elevates presynaptic/postsynaptic molecular assembly and evoked neurotransmission strength, but synapses appear restored to normal in double mutants. Likewise, activity-dependent facilitation, augmentation, and posttetanic potentiation are all suppressively impaired in *pgant* mutants. In non-neuronal contexts, *pgant* function regulates integrin signaling, and we show here that the synaptic Position Specific 2 (α PS2) integrin receptor and transmembrane tenascin ligand are both suppressively downregulated in *pgant* mutants. Channelrhodopsin-driven activity rapidly (<1 min) drives integrin signaling in wild-type synapses but is suppressively abolished in *pgant* mutants. Optogenetic stimulation in *pgant* mutants alters presynaptic vesicle trafficking and postsynaptic pocket size during the perturbed integrin signaling underlying synaptic plasticity defects. Critically, acute blockade of integrin signaling acts synergistically with *pgant* mutants to eliminate all activity-dependent synaptic plasticity.

Key words: activity dependent; *Drosophila*; integrin; neuromuscular junction; O-linked glycosylation; plasticity

Introduction

The heavily glycosylated transmembrane and extracellular synaptomatrix at the synaptic interface plays pivotal roles in synaptogenesis, neurotransmission, and synaptic plasticity (Dityatev and Schachner, 2003; Broadie et al., 2011; Dani and Broadie, 2012). Neurological disease states arising from aberrant glycosylation occur in numerous congenital disorders of glycosylation and dystroglycanopathies (Freeze, 2006). However, the mechanisms by which synaptomatrix glycan modifications regulate normal synapse function and dysfunction in heritable disease states remain poorly understood (Ohtsubo and Marth, 2006). *Drosophila* is a powerful genetic model to pursue these synaptic glycan mechanisms, given the conservation of glycan pathways, reduced glycogene genomic redundancy in this system, and host of techniques available at the well characterized glutamatergic neuromuscular synapse (Keshishian et al., 1996; Gagneux and

Varki, 1999). Using this model, we have shown recently that endogenous glycan-binding lectin [*mind the gap* (mtg); Rushton et al., 2009], heparan sulfate proteoglycan (HSPG) modifiers [heparan sulfate 6-O-endosulfatase (*sulf1*)/heparan sulfate 6-O-sulfotransferase (*hs6st*); Dani et al., 2012], and N-linked glycosylation [UDP-GlcNAc: α -3-D-mannoside- β 1,2-N-acetylglucosaminyltransferase I (*mgat1*); Parkinson et al., 2013] glycan mechanisms all act as potent regulators of *trans*-synaptic integrin, WNT (wingless-type MMTV integration site family), and bone morphogenetic protein (BMP) signaling.

To systematically pursue synaptic glycan mechanisms, we undertook a *Drosophila* whole-genome screen of glycogenes using RNAi-mediated knockdown of all N-/O-/glycosaminoglycan-linked enzymes, glycosyltransferases, and glycan-binding lectins, characterizing effects on neuromuscular junction (NMJ) structure and function using confocal microscopy and two-electrode voltage-clamp (TEVC) electrophysiology, respectively (Dani et al., 2012). This screen identified two α -N-acetylgalactosaminyltransferases, *pgant3* and *pgant35A*, that catalyze transfer of N-acetylgalactosamine (GalNAc) monosaccharides onto serine/threonine residues (GalNAc α 1-O-S/T) to form Tn antigens, as found within mucin-like O-linked glycans (Ten Hagen et al., 2003a). This most complexly regulated glycosylation is orchestrated by multiple GalNAc transferases (12 *pgants* in *Drosophila*) with distinct and overlapping peptide specificities (Yoshida et al., 2008; Tran and Ten Hagen, 2013). Pursuing our screen results with well characterized *pgant3* and *pgant35A* loss-of-function mutants (see Materials and Methods), we found elevated synaptic

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O-linked glycosylation, presynaptic/postsynaptic molecular assembly, presynaptic/postsynaptic ultrastructural elaborations, and neurotransmission strength, which are all corrected in double mutants that show none of these synaptic defects, identifying a novel suppressive genetic interaction.

In non-neuronal tissues, *Drosophila pgants* regulate integrin signaling and intercellular adhesion (Zhang and Ten Hagen, 2011). Importantly, we have shown that position-specific (PS) integrins, localized both presynaptically and postsynaptically, regulate NMJ morphogenesis (Beumer et al., 1999), synaptic scaffold/synaptomatrix adhesion molecules (Beumer et al., 2002), functional differentiation (Rohrbough et al., 2007), and activity-dependent plasticity (Rohrbough et al., 2000). Therefore, we hypothesized that *pgants* regulate integrin signaling at the synapse and consistently find suppressive downregulation of α PS2-containing integrin receptors (Beumer et al., 1999), RGD-containing tenascin (Ten-m) ligand (Mosca et al., 2012), and postsynaptic membrane adhesion defects in *pgant* mutants. Furthermore, we find integrin- and activity-dependent functional synaptic plasticity is suppressively regulated in *pgant* mutants. Importantly, we find that channelrhodopsin activity stimulation (Wang et al., 2011) disrupts downstream integrin association with Talin and pFAK signaling and elevates postsynaptic membrane adhesion defects. RGD peptide blockade of integrin function synergistically abolishes all activity-dependent synaptic plasticity in *pgant* mutants. These data show that two *pgants* suppressively regulate synaptic O-GalNAc glycosylation, synapse molecular assembly, neurotransmission strength, and activity-dependent plasticity via *trans*-synaptic integrin–tenascin signaling.

Materials and Methods

Drosophila genetics. All stocks were maintained at 25°C on standard food. Two independent mutant alleles isolated by ethyl methanesulfonate (EMS) mutagenesis were used for *pgant3*: (1) *pgant3^{mi}*, a C > T transition changing conserved arginine to cysteine at amino acid 130 resulting in failure to glycosylate substrates in enzymatic activity tests; and (2) *pgant3^{mi2}*, a G > A transition that creates a stop codon at amino acid 609, thereby deleting the C-terminal 59 aa and resulting in an unstable protein (Zhang et al., 2010). Similarly, the *pgant35A* mutations used included the following: (1) *pgant35A^{HG8}*, a C > T transition at nucleotide 265 resulting in a glutamine to stop codon change at amino acid 89; and (2) *pgant35A³⁷⁷⁵*, a T > A transversion at nucleotide 584 resulting in a premature stop codon at amino acid 195, both fully eliminating the catalytic domain (Ten Hagen and Tran, 2002). All mutants were placed in the *w¹¹¹⁸* genetic background, and *w¹¹¹⁸* was therefore used as the wild-type control. Rescue and overexpression experiments were performed with *UAS-pgant3* and *UAS-pgant35A* (Zhang et al., 2008) wild-type transgenes driven by neural (*elav-gal4*; Lin and Goodman, 1994), muscle (*24B-gal4*; Brand and Perrimon, 1993), and ubiquitous (*UHL-gal4*; Wodarz et al., 1995) drivers. Standard genetic techniques were used to generate recombinant and multiply mutant animals. Optogenetic studies were performed with the *UAS-Chief-tdTomato* channelrhodopsin (chimera with a crossover site at loop E-F and isoleucine 170 mutated to valine) transgene (Wang et al., 2011) driven by the neural-specific *elav-gal4* driver in animals raised on 0.25 mM all-trans retinal (Sigma) supplemented food. Animals used for experimentation were of either sex.

Immunocytochemistry. Wandering third instars were dissected in Ca^{2+} -free saline and then fixed in 4% paraformaldehyde for 10 min. Preparations were then washed in either permeabilizing PBST (PBS + 0.1% Triton X-100) or detergent-free PBS for extracellular labeling (Rushton et al., 2009). O-GalNAc glycans were visualized with TRITC-conjugated vicia villosa lectin (VVA; 1:250; EY Laboratories) and helix pomatia lectin (HPL; 1:250; Invitrogen) (Chia et al., 2014). Mouse antibodies obtained from the Developmental Studies Hybridoma Bank included anti- β PS (1:500), anti- α PS1 (1:200), anti- α PS2 (1:500), anti-*scab*

(1:200), anti-Talin (1:10), and anti-Disc large (DLG; 4F3; 1:250). Other sourced primary antibodies included the following: mouse anti-Ten-m (1:3000; Levine et al., 1994), mouse anti-Tiggrin (Tig; 1:200; Fogerty et al., 1994), guinea pig anti-LanA (1:200; Inoue and Hayashi, 2007), rat anti-Thrombospondin (Tsp; 1:200; Subramanian et al., 2007), rabbit anti-Wing-blister N-terminus (Wb-N; 1:500; Martin et al., 1999), rabbit anti- β v (1:300; Yee and Hynes, 1993), and rabbit anti-pFAK (pY397; 1:50; Invitrogen). All antibodies were incubated at 4°C overnight. Alexa Fluor-647-conjugated goat anti-HRP and secondary antibodies (Jackson ImmunoResearch) were incubated at 1:250 for 2 h at room temperature.

Image quantification. Control and mutant preparations for antibody and lectin studies were processed simultaneously for all intensity comparisons (Dani et al., 2012). To allow for direct comparisons of signal intensity levels, all genotypes were dissected, fixed, labeled, and imaged in parallel at the same time, with identical confocal settings and intensity measurements also made at the same time for all compared genotypes. Imaging was done on an upright Zeiss LSM 510 META laser-scanning confocal using a Plan Apo 63 $\times$ oil-immersion objective. NMJ structural quantification was done with anti-HRP imaging at muscle 6/7 in segment A3. All intensity analyses were done with NIH ImageJ software using the threshold function to outline Z-stack areas with the maximum projection function. All statistical comparisons were performed with one-way ANOVA analysis, followed by Dunnett's or Dunn's *post hoc* test for non-parametric data using Instat software (GraphPad Software). All data are presented as mean $\pm$ SEM. All images were projected in LSM Image Examiner (Zeiss) and exported to Adobe Photoshop.

Electrophysiology. TEVC records were made from NMJs of paired control and mutant wandering third instars as reported previously (Beumer et al., 1999). Briefly, recordings were performed in the following solution (in mM): 128 NaCl, 2 KCl, 4 MgCl₂, 1.0 CaCl₂, 70 sucrose, and 5 HEPES saline, pH 7.1. Recording electrodes (1 mm outer diameter capillaries; World Precision Instruments) filled with 3 M KCl had resistances of >15 M Ω . Evoked excitatory junction currents (EJCs) were recorded at 18°C using episodic recording from voltage-clamped (V_{hold} , -60 mV) muscle 6 in segment A3 with a TEVC amplifier (Axoclamp 2B; Molecular Devices). Excitatory junctional potentials (EJPs) were also recorded in parallel. Segmental nerves were stimulated with a glass suction electrode at a suprathreshold voltage (50% above threshold) for 0.5 ms duration at 0.5 Hz. For synaptic plasticity studies, the nerve was stimulated at 10 Hz for 60 s in 0.2 mM CaCl₂ saline (Rohrbough et al., 2000). EJCs were acquired via Clampex (Molecular Devices) and analyzed using Clampfit 9.0 by averaging 10 [during initial/posttetanic potentiation (PTP)] to 20 (during tetanus) consecutive responses. Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) integrin inhibition and Gly-Arg-Ala-Asp-Ser-Pro (GRADSP) control peptides (Sigma) were used at 0.2 mM, incubated for 1 h at 18°C. Statistical comparisons were done using one-way ANOVA analysis, followed by Dunnett's *post hoc* test with Instat software (GraphPad Software). Each $n = 1$ represents a recording from a different animal. All data are presented as mean $\pm$ SEM.

Electron microscopy. Ultrastructural analyses were performed as reported previously (Beumer et al., 1999). Briefly, staged third-instar preparations were fixed in 1.6% paraformaldehyde/2% glutaraldehyde (20 min), washed in 1 $\times$ PBS (10 min), and transferred to 2.5% glutaraldehyde in cacodylate buffer (12 h) with washes in the same buffer (30 min). Preparations were postfixed in 1% OsO₄ in cacodylate buffer (2 h) and then dehydrated in an ethanol series, followed by propylene oxide (30 min). Segment A3 muscle 6/7 was dissected free of the preparations and separately embedded in Araldite resin. Ultrathin (40 nm) sections were cut using a Leica ultracut UCT 54 ultramicrotome and then transferred to Formvar-coated slot grids. Sections were imaged using a Phillips CM10 transmission electron microscope at 80 kV, with images collected on a 4 megapixel CCD camera. Sample sizes are ≥ 10 independent NMJs, with the statistical analyses calculated using unpaired *t* tests. Images acquired from AMT Image Capture Software were exported to Adobe Photoshop. All data are presented as mean $\pm$ SEM.

Optogenetics. Wandering third instars were dissected in 0.2 mM Ca^{2+} saline on Sylgard-coated plates with the nervous system kept intact. An LEDD1B LED driver, M470L2 mounted LED at 470 nm affixed with an LA1951-A lens was used to stimulate channelrhodopsin activity (Gruntman and Turner, 2013). Preparations were subjected to a 60 s train of

light stimulation at 10 Hz, with a pulse duration of 60 ms, followed by immediate fixation and processing during continual stimulation, using the methods described above. At least eight independent NMJs were analyzed for each genotype and condition, with statistical tests for activity-dependent changes in fluorescence intensity and ultrastructure performed as described above in the immunocytochemistry and electron microscopy sections.

Results

pgants regulate synapse composition and transmission strength

An unbiased genetic screen of glycogenes identified synaptic function defects using inducible RNAi-mediated downregulation of two *pgants* (*pgant3* and *pgant35A*). This screen tested 130 glycan-related genes defined in eight function categories: N-glycan, O-glycan, and glycosaminoglycan biosynthesis; glycosyltransferases, glycan degrading/modifying enzymes; glycoprotein and proteoglycan core proteins; and sugar transporters and glycan-binding lectins. Using a combination of confocal microscopy and TEVC electrophysiology, NMJ morphology and functional transmission defects were tested in *Drosophila* wandering third-instar larvae following ubiquitous (*UH1-gal4*) RNAi knockdown. From this screen, 31 genes affected synapse structure (27 increased bouton number, two increased branching, and two increased NMJ area), and 13 affected synapse function (12 increased and one decreased). Only six gene knockdowns affected both structure and function. To investigate mucin-type O-linked glycosylation, nine available RNAi lines were used to test six *pgant* genes (*pgant2*, *pgant3*, *pgant4*, *pgant5*, *pgant6*, and *pgant35A*) and three additional GalNAc transferases (GalNAcT-1, GalNAcT-2, and C1GalTA). Of these, three *pgant* genes (*pgant3*, *pgant5*, and *pgant35A*) were identified to have increased neurotransmission strength during knockdown, and GalNAc-T2 showed increased NMJ area. The other five gene knockdowns caused no detectable NMJ phenotypes. Well characterized mutants are available for only *pgant3* and *pgant35A* (see Materials and Methods), which have been studied extensively in heteroallelic null combinations (Ten Hagen and Tran, 2002; Zhang et al., 2010). In this study, we pursued a full characterization of these two *pgant* genes using the same conditions.

To characterize synaptic mechanisms in *pgant3* and *pgant35A* null single and double mutant larvae, we performed nerve-evoked EJC recordings in the TEVC paradigm (Dani et al., 2012). Sample traces of 10 consecutive, superimposed responses are shown for four genotypes: the genetic background control (*w¹¹¹⁸*), *pgant3* (*pgant3^{m1}/pgant3^{m2}*) and *pgant35A* (*pgant35A^{HG8}/pgant35A³⁷⁷⁵*) single mutants in *w¹¹¹⁸* background, and the double null mutant (*pgant3^{m1},pgant35A^{HG8}/pgant3^{m2},pgant35A³⁷⁷⁵*). Neurotransmission is clearly and consistently elevated in both *pgant* mutants, increased 25–40% compared with controls (Fig. 1A). Quantification of mean EJC amplitudes shows that synapse strength is very significantly elevated in both *pgant3* (255.46 ± 8.12 nA, $n = 26$, $p < 0.001$) and *pgant35A* (277.62 ± 11.88 nA, $n = 22$, $p < 0.001$) single mutants compared with control (198.73 ± 7.77 nA, $n = 17$; Fig. 1A, right). Surprisingly, however, neurotransmission in the recombinant double null mutant is not significantly elevated compared with control (231.64 ± 7.24 nA, $n = 21$, $p > 0.05$; Fig. 1A, right), which behaves like the control. Thus, a similar phenotype occurs in the two *pgant* single mutants, which is absent in the double mutant. We use the term “suppression” throughout this study, as the simplest genetic term describing the observed interaction. Importantly, there is a synaptic function defect only, with no differences in NMJ morphology in

either of the *pgant* mutants. In quantifying synaptic branching, neither *pgant3* (5.00 ± 0.28 decrease, $n = 28$) nor *pgant35A* (5.79 ± 0.26 , $n = 24$) single mutants or the double mutants (6.26 ± 0.41 , $n = 23$) showed any significant difference from controls (5.58 ± 0.22 , $n = 26$). Likewise, NMJ bouton number is also not significantly affected in *pgant3* (90.89 ± 4.25 , $n = 28$) or *pgant35A* (92.29 ± 5.78 , $n = 24$) single mutants or double mutants (77.46 ± 4.18 , $n = 24$) compared with controls (85.96 ± 4.18 , $n = 28$). This finding was the first discovery of the suppressive action of *pgant* genes on synapse function.

To begin to determine how *pgant* corepressive regulation arises at the synapse, we labeled NMJs for presynaptic active zones with Bruchpilot (Brp) and postsynaptic glutamate receptors (GluRIID), marking the two sides of each individual synapse (Fig. 1B). There is a clear and consistent increase in Brp/GluRIID punctae in both *pgant3* and *pgant35A* single mutants, indicating a cooperative change on both presynaptic and postsynaptic sides of the synapse. Importantly, however, the double null mutant does not show any detectable increase in either synaptic marker (Fig. 1B). Quantification reveals significantly increased glutamate receptor field area and punctae number in *pgant3* [$82.59 \pm 6.77 \mu\text{m}^2$ ($p < 0.05$) and $358.0 \pm 16.20 \mu\text{m}^2$ ($p < 0.01$); $n = 14$] and *pgant35A* (81.02 ± 6.95 and $302.73 \pm 15.61 \mu\text{m}^2$; $n = 15$, $p < 0.05$) single mutants but with no differences in the double mutants in either parameter compared with controls (61.03 ± 3.99 and $233.25 \pm 12.33 \mu\text{m}^2$; $n = 16$; Fig. 1C). Likewise, presynaptic Brp active zone area and punctae number are increased at *pgant3* (54.80 ± 4.80 and $357.46 \pm 18.89 \mu\text{m}^2$; $n = 13$, $p < 0.05$) and *pgant35A* (52.96 ± 4.30 and $305.4 \pm 14.86 \mu\text{m}^2$; $n = 15$, $p < 0.05$) single mutants, with no differences in *pgant3,pgant35A* double mutants compared with controls (38.56 ± 3.03 and $242 \pm 31.22 \mu\text{m}^2$; $n = 16$; Fig. 1C). Both *pgant* mutants show no significant change in spontaneous miniature EJC (mEJC) frequencies (*pgant3*, 2.29 ± 0.17 Hz, $n = 10$; *pgant35A*, 2.06 ± 0.11 Hz, $n = 9$) compared with control (1.93 ± 0.13 Hz, $n = 10$) but do show small, significant decreases in mEJC amplitudes (*pgant3*, 0.68 ± 0.03 nA, $n = 10$, $p < 0.05$; *pgant35A*, 0.69 ± 0.03 nA, $n = 9$, $p < 0.05$) compared with control (0.80 ± 0.02 nA, $n = 10$). These results show that *pgant3* and *pgant35A* both upregulate neurotransmission strength through elevated presynaptic and postsynaptic assembly via a mutually suppressive mechanism that predominantly affects evoked function.

pgants regulate presynaptic vesicles and postsynaptic pocket size

The synaptic ultrastructure of the *Drosophila* NMJ has been well characterized by transmission electron microscopy (TEM), categorizing multiple synaptic vesicle (SV) pools in the presynaptic bouton and the complex architecture of the expansive subsynaptic reticulum (SSR) of the postsynaptic membrane (Rohrbough et al., 2007). Because no gross morphology differences were associated with observed neurotransmission elevations in *pgant* mutants, we next investigated synapse ultrastructure. On the presynaptic side, we measured bouton area, active zone architecture, overall SV density, and SV distribution in concentric rings (e.g., 250 nm, 500 nm) extending from each active zone (Fig. 2A,B; white arrows). On the postsynaptic side, we assayed SSR area, thickness on major and minor axes, density (folds/unit length), and postsynaptic pocket (PSP) size by measuring the distance from the presynaptic active zone to the first SSR membrane layer (Fig. 2A,B; dotted white lines). Expansion of the PSP is a hallmark of mutants defective in synaptomatrix resident, *trans-*

Figure 1. Null *pgant* mutants suppressively elevate neurotransmission strength. **A**, Representative evoked EJC records from genetic control (w^{1118}), *pgant3* ($pgant3^{m1}/pgant3^{m2}$) and *pgant35A* ($pgant35A^{H68}/pgant35A^{3775}$) single mutants, and double mutants ($pgant3^{m1}, pgant35A^{H68}/pgant3^{m2}, pgant35A^{3775}$). Ten consecutive EJCs traces shown at 0.2 Hz stimulation from the muscle 6 NMJ in segment A3. Calibration: 10 ms, 50 nA. Right, Histogram of mean EJC amplitudes, with sample sizes ≥ 17 for each genotype. **B**, Representative NMJ boutons for the above four genotypes labeled for postsynaptic glutamate receptors (GluRIID, green) and presynaptic active zone Brp (red), with split channels shown for clarity. White arrows indicate GluRIID/Brp paired punctae in single mutants. Scale bar, 2 μm . **C**, Quantification of GluRIID/Brp areas and punctae number per NMJ terminal. Statistical differences are calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., No significance. Error bars indicate SEM.

synaptic signaling ligands (Packard et al., 2002) and HSPG extracellular regulators of *trans*-synaptic signaling (Kamimura et al., 2013).

Presynaptic bouton appearance (Fig. 2A) and area in both *pgant* mutants (*pgant3*, $7.53 \pm 0.85 \mu\text{m}^2$, $n = 10$; *pgant35A*,

$7.76 \pm 0.80 \mu\text{m}^2$, $n = 12$) are not significantly different from the genetic control (w^{1118} , $9.51 \pm 1.78 \mu\text{m}^2$, $n = 7$), although there is a trend toward smaller boutons. Likewise, active zone architecture and t-bar dimensions are not measurably affected by loss of

Figure 2. Null *pgant* mutants suppressively alter presynaptic/postsynaptic ultrastructure. **A**, Representative TEM images of synaptic boutons from muscle 6 NMJ in segment A3 of genetic control (w^{1118} ; top), *pgant3* ($pgant3^{m1}/pgant3^{m2}$) and *pgant35A* ($pgant35A^{H68}/pgant35A^{3775}$) single mutants (middle), and double mutants ($pgant3^{m1},pgant35A^{H68/3775}/pgant3^{m2},pgant35A^{3775}$; bottom). Labels indicate SV pools in presynaptic bouton, SSR of muscle membrane, and presynaptic active zones (arrowheads). Scale bar, 500 nm. **B**, High-magnification images of single active zone synapses in all four genotypes. Labels indicate presynaptic t-bars (arrowheads) and PSPs (white dotted lines). Scale bar, 100 nm. **C**, Quantification of SV pools (<250 nm (top), <500 nm (middle) from active zone t-bar) and PSP depth (bottom). Sample size is ≥ 10 boutons for each of the four genotypes. Statistical differences calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$. N.S., Not significance. Error bars indicate SEM.

pgant activity (Fig. 2B). In contrast, the density and distribution of SV pools is clearly aberrant in both *pgant* single mutants, although the double null mutant is not detectably different from the control (Fig. 2A,B). Immediately adjacent to active zone

t-bars (Fig. 2B, arrows), SV clustering is increased in both *pgant* single mutants but not in the double mutant combination. Quantifying SV number within 250 nm of the t-bar shows a consistent density in controls (10.5 ± 0.91), which is significantly increased

in both *pgant3* (15.22 ± 0.99 , $p < 0.01$) and *pgant35A* (15.53 ± 0.78 , $p < 0.01$) single mutants but back at the control level in double null mutants (9.4 ± 0.67 , $n = 15$; Fig. 2C, top). Likewise, at a distance of 500 nm from the active zone t-bar, SV number increases in *pgant3* (36.3 ± 1.66 , $p < 0.01$) and *pgant35A* (43.31 ± 1.21 , $p < 0.01$) single mutants but not in double mutants (26.06 ± 1.44 , $n = 15$) compared with controls (26 ± 2.18 ; Fig. 1C, middle). Thus, presynaptic vesicle pool distribution is suppressively regulated by *pgant3* and *pgant35A*, in line with changes in synaptic function.

Postsynaptic SSR appearance, area, thickness, and density are normal in all *pgant* mutants (Fig. 2A). Quantification of normalized SSR area (*pgant3*, 1.26 ± 0.16 ; *pgant35A*, 1.13 ± 0.17 ; *pgant3,pgant35A*, 1.09 ± 0.17), thickness (*pgant3*, 1.12 ± 0.10 ; *pgant35A*, 0.84 ± 0.08 ; *pgant3,pgant35A*, 0.80 ± 0.07), and normalized density (*pgant3*, 0.83 ± 0.05 ; *pgant35A*, 1.10 ± 0.08 ; *pgant3,pgant35A*, 1.11 ± 0.08) all show no significant changes normalized to controls (Fig. 2A). In contrast, however, there is a striking expansion in both *pgant* mutants of the PSP (Packard et al., 2002; Kamimura et al., 2013). This compartment has been defined as “a postsynaptic area immediately apposed to an active zone containing amorphous material” (Packard et al., 2002), which is spatially localized between the postsynaptic membrane and SSR (Ren et al., 2009). The PSP compartment has been shown to be expanded in *trans*-synaptic signaling disrupted mutants, including WNT wingless, BMP glass bottom boat, HSPG perlecan, and HSPG sulfateless mutants (Packard et al., 2002; Tian and Ten Hagen, 2007; Ren et al., 2009; Nahm et al., 2010; Kamimura et al., 2013). Both *pgant3* and *pgant35A* single mutants similarly display an enlarged PSP compartment, although the double null mutant is not detectably different from the control (Fig. 2B, dotted white lines). As a quantifiable PSP parameter, pocket depth from the presynaptic active zone to the next adjacent postsynaptic SSR membrane was measured in all four genotypes. Compared with controls (mean PSP depth, 121.17 ± 4.95 nm, $n = 10$), both *pgant* single mutants display a more than twofold expanded PSP (*pgant3*, 254.7 ± 35.25 nm, $p < 0.01$; *pgant35A*, 233.96 ± 41.83 nm, $p < 0.05$; Fig. 2B,C, bottom). In sharp contrast, the double null mutants show no significant increase in PSP depth compared with controls (169.09 ± 15.46 nm, $n = 12$; Fig. 2C, bottom). Thus, we observe suppressive regulation by *pgant* genes of SV pools in the presynaptic compartment as well as postsynaptic compartment expansion, paralleling the changes in neurotransmission strength.

Neuronal and muscle *pgant3* and *pgant35A* modulate neurotransmission

To determine cell-specific requirements of *pgant3* and *pgant35A*, we used the inducible Gal4–UAS binary system (Brand and Perrimon, 1993) to express UAS–*pgant3* or UAS–*pgant35A* wild-type transgenes in neurons (*elav–gal4*) or muscles (*24B–gal4*) in respective single mutant backgrounds and assayed for phenotype rescue (Fig. 3). Sample EJC traces show an average of 10 consecutive nerve-evoked responses for both rescue conditions in both *pgant* nulls (Fig. 3A). We find that functional neurotransmission strength is restored to control levels when *pgant3* is expressed in either neurons (*pgant3^{ml}/pgant3^{ml};UAS–pgant3/elav*, 181.61 ± 11.85 nA, $n = 14$) or muscles (*pgant3^{ml}/pgant3^{ml};UAS–pgant3/24B*, 187.47 ± 12.72 nA, $n = 11$) in the otherwise *pgant3* null background compared with controls (*w¹¹¹⁸*, 193.34 ± 8.69 nA, $n = 14$; Fig. 3C, top). Similarly, *pgant35A* expression in neurons (*pgant35A^{HG8}/pgant35A³⁷⁷⁵;UAS–pgant35A/elav*, 197.50 ± 14.26 nA, $n = 9$) or muscles (*pgant35A^{HG8}/pgant35A³⁷⁷⁵;UAS–*

pgant35A/24B, 211.42 ± 17.06 nA, $n = 10$) in the *pgant35A* mutant background likewise rescues neurotransmission strength to control levels (Fig. 3C, top).

To test whether this functional rescue correlates with corrected synaptic molecular assembly, NMJs were labeled for presynaptic Brp and postsynaptic GluRIID (Fig. 3B). Brp punctae number in *pgant3* neuronal (392.56 ± 22.86 , $n = 9$) or muscle (321.13 ± 17.84 , $n = 15$) rescue conditions and *pgant35A* neuronal (340 ± 25.63 , $n = 8$) or muscle (409.58 ± 27.71 , $n = 12$) rescue conditions does not differ significantly from control (358.84 ± 18 , $n = 19$; Fig. 3B,C). Similarly, GluRIID punctae number is also restored to control levels (360.73 ± 19.16 , $n = 19$), with *pgant3* neuronal (395.89 ± 23.95 , $n = 9$) or muscle (327.6 ± 18.22 , $n = 15$) rescue and *pgant35A* neuronal (336 ± 24.71 , $n = 8$) or muscle (414.17 ± 26.98 , $n = 12$) rescue. The same result is reflected in Brp area measurements, in which *pgant3* neuronal (85.67 ± 6.78 , $n = 10$) or muscle (75.66 ± 7.99 , $n = 15$) rescue and *pgant35A* neuronal ($79.12 \pm 8.23 \mu\text{m}^2$, $n = 10$) or muscle ($88.80 \pm 8.29 \mu\text{m}^2$, $n = 12$) rescue is similar to control values ($74.35 \pm 4.69 \mu\text{m}^2$, $n = 18$; Fig. 3B,C). Similarly, postsynaptic GluRIID area measurements in *pgant3* neuronal ($173.75 \pm 10.38 \mu\text{m}^2$, $n = 10$) or muscle ($142.47 \pm 10.80 \mu\text{m}^2$, $n = 15$) rescue and *pgant35A* neuronal ($155.61 \pm 9.97 \mu\text{m}^2$, $n = 10$) or muscle ($156.99 \pm 11.89 \mu\text{m}^2$, $n = 12$) rescue are not significantly different from control ($148.20 \pm 7.01 \mu\text{m}^2$, $n = 18$; Fig. 3C). These results show that both *pgant3* and *pgant35A* can function either presynaptic or postsynaptically to regulate synaptic assembly and neurotransmission strength.

Presynaptic/postsynaptic balance of *pgant3* and *pgant35A* regulate neurotransmission

Given the *pgant* suppressive mechanism and coupled presynaptic/postsynaptic roles of *pgant3* and *pgant35A*, we next tested whether the balance of *pgant3* and *pgant35A* is required to properly regulate neurotransmission. We generated allelic combinations for UAS–*pgant3* wild-type transgene expression in neurons (*elav–gal4*), muscles (*24B–gal4*), or ubiquitously (*UH1–gal4*) in the *pgant3,pgant35A* double mutant background and tested effects on neurotransmission strength. Representative EJC traces for each genotype are shown in Figure 4A. Compared with the control mean EJC amplitude (*pgant3^{ml},pgant35A^{HG8}/pgant3^{ml},pgant35A³⁷⁷⁵;UAS–pgant3/+*, 211.42 ± 11.94 nA, $n = 8$), neuronal (*pgant3^{ml},pgant35A^{HG8}/pgant3^{ml},pgant35A³⁷⁷⁵; elav/UAS–pgant3*, 258.99 ± 9.59 nA, $n = 9$, $p < 0.05$), muscle (*pgant3^{ml},pgant35A^{HG8}/pgant3^{ml},pgant35A³⁷⁷⁵; 24B/UAS–pgant3*, 276.52 ± 11.19 nA, $n = 9$, $p < 0.05$), and ubiquitous (*pgant3^{ml},pgant35A^{HG8}/pgant3^{ml},pgant35A³⁷⁷⁵;UH1/UAS–pgant3*, 254.47 ± 13.59 nA, $n = 13$, $p < 0.05$) *pgant3* expression in the double mutant background all significantly elevated neurotransmission strength (Fig. 4B). Thus, restoring *pgant3* to either neuron or muscle effectively reveals the *pgant35A* single mutant phenotype (Fig. 1A).

In parallel, we overexpressed both *pgant* genes alone to test the effect on neurotransmission strength. Representative EJC traces for each genotype are shown in Figure 4C. Compared with control (*UAS–pgant3/+*, 205.55 ± 8.77 nA, $n = 22$), *pgant3* overexpression in neurons (*UAS–pgant3/elav*, 154.99 ± 11.99 nA, $n = 11$, $p < 0.01$), muscles (*UAS–pgant3/24B*, 165.62 ± 11.13 nA, $n = 10$, $p < 0.05$), or ubiquitous (*UAS–pgant3/UH1*, 164.80 ± 9.79 nA, $n = 10$, $p < 0.05$) all similarly decreased mean EJC amplitudes (Fig. 4D, left). Likewise, compared with control (*UAS–pgant35A/+*, 235.15 ± 10.77 nA, $n = 19$), *pgant35A* overexpression in neurons (*UAS–pgant35A/elav*, 193.50 ± 13.39 nA, $n = 18$, $p < 0.05$) and ubiquitous overexpression (*UAS–pgant35A/UH1*,

Figure 3. *pgants* function in neurons and muscle to regulate neurotransmission. **A**, Representative EJC records from genetic control (*w¹¹¹⁸*), *pgant3* mutant with neuronal (*pgant3^{m1}/pgant3^{m2}; elav/UAS-pgant3*) and muscle (*pgant3^{m1}/pgant3^{m2}; 24B/UAS-pgant3*) *pgant3* expression, and *pgant35A* mutant with neuronal (*pgant35A^{H68}/pgant35A³⁷⁷⁵; elav/UAS-pgant35A*) and muscle (*pgant35A^{H68}/pgant35A³⁷⁷⁵; 24B/UAS-pgant35A*) *pgant35A* expression. Ten consecutive EJC traces are shown at 0.2 Hz stimulation from the muscle 6 NMJ in segment A3. Calibration: 10 ms, 50 nA. Sample size is $n \geq 8$ animals for all conditions. **B**, Representative NMJ boutons for the above five genotypes colabeled for both postsynaptic glutamate receptors (GluRIID; green) and presynaptic active zone Brp (red), with split channels shown for clarity. Scale bar, 2 μ m. **C**, Histograms show normalized EJC amplitude, punctae number, and GluRIID/Brp area per NMJ terminal for the above five genotypes. Sample size is $n \geq 10$ animals for all conditions. Statistical differences are calculated using one-way ANOVA with Dunnett's *post hoc* test. N.S. indicates no significance. Error bars indicate SEM.

187.52 ± 9.43 nA, $n = 10$, $p < 0.05$) both decrease neurotransmission transmission, although muscle overexpression alone has no significant effect (*UAS-pgant35A/24B*, 224.99 ± 8.77 nA, $n = 20$; Fig. 4D, right). Overall, *pgant* overexpression has the opposite consequence of *pgant* loss of function (Fig. 1A). Thus, the proper balance of *pgant3/pgant35A* in neurons and muscle bidirectionally regulates the strength of synaptic transmission.

Activity-dependent synaptic plasticity is impaired in *pgant* mutants

In the non-neuronal context of the *Drosophila* wing disc, *pgant* mutants specifically impair integrin signaling to cause intercellular de-adhesion (Zhang et al., 2010). Similarly, the above synaptic ultrastructure defects in *pgant* mutants recalls synaptic integrin signaling, which we have shown is required for activity-dependent

Figure 4. Pre/postsynaptic *pgant3/35A* balance regulates neurotransmission. Representative EJC records (**A**) and mean amplitudes (**B**) for *pgant3* expression in the double mutant background. The four genotypes include genetic control (*pgant3*^{m1}, *pgant35A*^{HG8} / *pgant3*^{m2}, *pgant35A*³⁷⁷⁵; + / UAS-*pgant3*), neuronal (*pgant3*^{m1}, *pgant35A*^{HG8} / *pgant3*^{m2}, *pgant35A*³⁷⁷⁵; elav / UAS-*pgant3*), muscle (*pgant3*^{m1}, *pgant35A*^{HG8} / *pgant3*^{m2}, *pgant35A*³⁷⁷⁵; 24B / UAS-*pgant3*), and ubiquitous (*pgant3*^{m1}, *pgant35A*^{HG8} / *pgant3*^{m2}, *pgant35A*³⁷⁷⁵; UH1 / UAS-*pgant3*) *pgant3* expression in the double mutant. Sample size is $n \geq 10$ animals for all conditions. Representative EJC records (**C**) and mean amplitudes (**D**) for *pgant3* and *pgant35A* overexpression conditions. The eight genotypes include *pgant3* genetic control (UAS-*pgant3* / +), neuronal (UAS-*pgant3* / elav), muscle (UAS-*pgant3* / 24B), and ubiquitous (UAS-*pgant3* / UH1) *pgant3* overexpression, and *pgant35A* genetic control (UAS-*pgant35A* / +), neuronal (UAS-*pgant35A* / elav), muscle (UAS-*pgant35A* / 24B), and ubiquitous (UAS-*pgant35A* / UH1) *pgant35A* overexpression. Sample size is $n \geq 10$ animals for each genotype. Statistical differences are calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$. N.S., No significance. Error bars indicate SEM.

synaptic plasticity (Rohrbough et al., 2000). Therefore, we next investigated the multiple phases of activity-dependent plasticity in *pgant* mutants, including immediate facilitation and maintained augmentation during a tetanic stimulus train (10 Hz, 60 s), and initiation and maintenance of PTP after return to basal stimulation. In this paradigm, EJCs are recorded initially at 0.5 Hz for 30 s, followed by the tetanic train, and then returned to basal 0.5 Hz for a total of 5 min recording (Rohrbough et al., 2000). Figure 5A shows representative traces for control (w^{1118}), single mutants (*pgant3*^{m1}/*pgant3*^{m2} and *pgant35A*^{HG8}/*pgant35A*³⁷⁷⁵), and double mutants (*pgant3*^{m1}, *pgant35A*^{HG8}/*pgant3*^{m2}, *pgant35A*³⁷⁷⁵).

For quantification, consecutive EJCs are averaged throughout the stimulation phases to display mean amplitudes normalized to the starting level (Fig. 5B). Controls show immediate, rapid facilitation leading to a sixfold augmentation during tetanic stimulation, followed by twofold initial PTP phase, later maintained as an ~50% elevation for the duration of the recording (Fig. 5A, B). In contrast, both *pgant* single mutants show very significantly impaired initial facilitation and blunted fourfold augmentation during tetanic stimulation (Fig. 5B, solid bar labeled C). For example, at 20 s into the tetanic train, EJC amplitudes show augmentation decreases of ≤65% in *pgant3* ($p < 0.01$, $n = 10$) and ≤55% in *pgant35A* ($p < 0.01$, $n = 11$) mutants. In contrast, the double null mutant is clearly less impaired than either single *pgant* mutant (Fig. 5A, B). At 20 s into the tetanic train, the double mutants exhibit a reduced impairment of ≤40% compared with controls ($p < 0.05$, $n = 11$; Fig. 5C). After this initial facilitation phase, double mutants reach control levels of augmentation, whereas the single *pgant* mutants remain impaired (Fig. 5A, B), showing a suppressive interaction. After the tetanic train, potentiation in double mutants is indistinguishable from controls, whereas both single mutants (*pgant3* and *pgant35A*) show strong loss of PTP initiation (Fig. 5B, solid bar labeled D). Quantification shows ≥50% decrease in *pgant3* ($p < 0.001$, $n = 10$) and ≥35% decrease in *pgant35A* ($p < 0.05$, $n = 11$) single mutants compared with controls but no detectable decrease in the double null mutants (Fig. 5D). Thus, *pgant* mechanisms regulate activity-dependent facilitation, augmentation, and potentiation.

pgants suppressively regulate integrin signaling

Synapses sandwich heavily glycosylated transmembrane and extracellular proteins that regulate synaptic function and plasticity (Dani and Broadie, 2012). For example, we have shown previously that O-linked heparan sulfate glycosaminoglycans bidirectionally regulate WNT and BMP trans-synaptic signaling to modulate neurotransmission strength (Dani et al., 2012). To directly visualize changes in synaptic O-GalNAc glycosylation in *pgant* mutants, we used fluorescently conjugated VVA–TRITC and HPL–Alexa Fluor-488 to label NMJ terminals (Fig. 6). Non-detergent conditions were used throughout to examine only the glycosylation state of the extracellular synaptomatrix (Dani et al., 2012). Representative images show the halo-like VVA (Fig. 6A, top left) and HPL (Fig. 6A, middle left) labeling surrounding the anti-HRP marked synaptic boutons. Compared with controls, O-linked glycan expression is very significantly increased in both *pgant3* ($31.65 \pm 5.61\%$, $n = 9$, $p < 0.01$) and *pgant35A* ($58.50 \pm 4.39\%$, $n = 14$, $p < 0.01$) single mutants, but there is no significant change in the double mutants ($13.54 \pm 5.04\%$, $n = 6$, $p > 0.05$) (Fig. 6B). Similarly, quantified HPL labeling is very significantly elevated in both *pgant3* ($33.13 \pm 6.39\%$, $n = 21$, $p < 0.01$) and *pgant35A* ($41.06 \pm 7.83\%$, $n = 18$, $p < 0.01$) single mutants, but no significant difference occurs in the double null mutants

($7.49 \pm 6.85\%$, $n = 18$, $p > 0.05$) compared with controls (Fig. 6B). Thus, two independent approaches highlight the suppressive regulation of synaptic O-GalNAc modification by these two *pgant* genes.

Studies in non-neuronal tissues have shown that *pgant* mutants misregulate integrin signaling (Zhang et al., 2010). Consistently, we have identified previously presynaptically/postsynaptically localized PS integrin receptors at the *Drosophila* NMJ, containing multiple different α and β subunits (Beumer et al., 1999, 2002; Rohrbough et al., 2000, 2007; Rushton et al., 2009). Therefore, we tested the multiple integrin receptor subunits, including α PS1 (*mew*), α PS2 (*if*), α PS3 (*scab/volado*), β PS (*mys*; Brower et al., 1984), and β_v (Yee and Hynes, 1993). The two β subunits show an interesting *pgant*-specific change, with β PS increased in *pgant35A* (1.39 ± 0.08 , $n = 14$, $p < 0.01$) and β_v increased in *pgant3* (1.21 ± 0.03 , $n = 18$, $p < 0.05$) single mutants but no significant change of either β subunit in the double mutant (β PS, 1.17 ± 0.64 , $n = 10$, $p > 0.05$; β_v , 0.91 ± 0.06 , $n = 10$, $p > 0.05$) normalized to control. Most of the α receptor subunits show no consistent changes in the *pgant* mutants, including α PS1 (*pgant3*, 1.27 ± 0.07 , $n = 18$; *pgant35A*, 1.10 ± 0.05 , $n = 18$) and α PS3 (*pgant3*, 1.05 ± 0.02 , $n = 8$; *pgant35A*, 1.15 ± 0.06 , $n = 12$; all $p > 0.05$ with respect to control). The sole exception is α PS2, which sharply decreases in both *pgant3* ($39.19 \pm 6.75\%$, $n = 10$, $p < 0.01$) and *pgant35A* ($34.37 \pm 5.69\%$, $n = 11$, $p < 0.01$) single mutants but does not change in double mutants compared with controls (Fig. 6B, right).

We next examined a host of characterized integrin ligands for changes in *pgant* single and double mutants (Zhang and Ten Hagen, 2011), including Tig (Fogerty et al., 1994), laminin α subunits LanA (Inoue and Hayashi, 2007), and Wb (Martin et al., 1999), Tsp (Subramanian et al., 2007), and Ten-m (Levine et al., 1994). Most of these ligands show no consistent changes in *pgant* single and double mutants compared with control: Tig (*pgant3*, 1.08 ± 0.05 , $n = 11$; *pgant35A*, 1.03 ± 0.05 , $n = 14$; *pgant3*, *pgant35A*, 1.07 ± 0.03 , $n = 8$, compared with control, $p > 0.05$), LanA (*pgant3*, 1.25 ± 0.07 , $n = 11$, $p < 0.05$; *pgant35A*, 1.16 ± 0.07 , $n = 14$, $p > 0.05$; double mutant, 1.00 ± 0.05 , $n = 8$, $p > 0.05$), Wb-N (*pgant3*, 1.00 ± 0.05 , $n = 10$; *pgant35A*, 1.12 ± 0.03 , $n = 11$; double mutant, 1.11 ± 0.04 , $n = 7$; all $p > 0.05$), and Tsp (*pgant3*, 1.02 ± 0.07 , $n = 9$; *pgant35A*, 1.12 ± 0.06 , $n = 10$; double mutant, 1.13 ± 0.05 , $n = 8$; $p > 0.05$), all normalized to control. The sole exception was the RGD domain-containing, transmembrane Ten-m (Levine et al., 1994). Ten-m localizes in a halo-like ring around HRP-labeled synaptic boutons in controls but is consistently reduced in both *pgant* single mutants (Fig. 6A, middle). Compared with controls, Ten-m levels are very significantly decreased in *pgant3* ($21.88 \pm 3.47\%$, $n = 15$, $p < 0.01$) and *pgant35A* ($20.84 \pm 3.91\%$, $n = 16$, $p < 0.01$) single mutants but show no change in double null mutants (Fig. 6A, right). Thus, the two *pgant* genes suppressively downregulate α PS2 integrin/Ten-m ligand at the synapse.

Neuronal and muscle *pgants* regulate O-glycosylation and integrin signaling

To determine whether changes in synaptic O-linked glycosylation and trans-synaptic Ten-m/ α PS2 integrin signaling are directed by presynaptic or postsynaptic *pgant* function, we next tested both *pgant3* and *pgant35A* rescue in neurons and muscle in their respective null mutant backgrounds (Fig. 7). Representative NMJs showing VVA lectin, α PS2 integrin, and Ten-m ligand labeling are shown for all genotype conditions in Figure 7A. Both *pgant3* neuronal (1.02 ± 0.05 , $n = 15$) and muscle (1.05 ± 0.05 , $n = 15$) and *pgant35A* neuronal (1.13 ± 0.03 , $n = 13$) and muscle

Figure 5. Impaired activity-dependent synaptic plasticity in *pgant* mutants. **A**, Representative TEVC records from genetic control (w^{1118} , top), *pgant3* ($pgant3^{m1}/pgant3^{m2}$) and *pgant35A* ($pgant35A^{H68}/pgant35A^{3775}$) single mutants (middle two traces), and double mutants ($pgant3^{m1},pgant35A^{H68}/pgant3^{m2},pgant35A^{3775}$; bottom). The stimulation paradigm is 0.5 Hz for 30 s (solid bar), 10 Hz for 60 s (hatched bar), and then a return to 0.5 Hz (solid bar) for 5 min total recording time. Calibration: 10 s, 20 nA. **B**, Mean EJC amplitudes over time normalized to initial mean EJC amplitude for control (filled circle), *pgant3* (open square) and *pgant35A* (open triangle) single mutants, and double mutant (open circle). Bars labeled C and D are expanded below, showing normalized EJC amplitudes during tetanic stimulation (**C**) and early posttetanic stimulation periods (**D**). Sample sizes are $n \geq 10$ independent NMJs for each of the four genotypes. Statistical differences are calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., No significance. Error bars indicate SEM.

(1.01 ± 0.03 , $n = 22$) expression restored VVA lectin labeling to control levels (1.00 ± 0.03 , $n = 18$; Fig. 7B). Similarly, α PS2 integrin abundance is also rescued with *pgant3* neuronal (0.94 ± 0.05 , $n = 19$) or muscle (0.99 ± 0.06 , $n = 20$) expression and

pgant35A neuronal (0.90 ± 0.04 , $n = 21$) or muscle (0.86 ± 0.04 , $n = 25$) expression compared with control (1.00 ± 0.03 , $n = 20$; Fig. 7B). Interestingly, only neuronal *pgant3* (0.99 ± 0.04 , $n = 10$) and *pgant35A* (0.97 ± 0.04 , $n = 8$) expression could restore

Figure 6. Synaptomatrix O-glycan and integrin signaling defects in *pgant* mutants. **A**, NMJ synaptic boutons from control (w^{1118} ; left column), *pgant3* ($pgant3^{m1}/pgant3^{m2}$) and *pgant35A* ($pgant35A^{H68}/pgant35A^{3775}$) single mutants (middle 2 columns), and the double mutant ($pgant3^{m1},pgant35A^{H68}/pgant3^{m2},pgant35A^{3775}$; right column) show colabeling for synaptic O-linked glycosylation markers (VVA and HPL), integrin receptor (α PS2), and transmembrane integrin ligand (Ten-m) relative to the presynaptic marker anti-HRP. Scale bar, 2 μ m. **B**, Histograms show fluorescence intensities for all four labels (VVA, HPL, α PS2, and Ten-m) normalized to each genetic control (dotted red line). Sample size is $n \geq 10$ independent NMJs for each label for all four genotypes. Statistical differences calculated using one-way ANOVA with Dunnett's *post hoc* test: ** $p < 0.01$. Error bars indicate SEM.

synaptic Ten-m levels to control levels (1.00 ± 0.03 , $n = 21$), whereas muscle *pgant3* (0.88 ± 0.04 , $n = 17$, $p < 0.05$) and *pgant35A* (0.82 ± 0.03 , $n = 14$, $p < 0.01$) remained significantly decreased normalized to control (Fig. 7B, right).

Thus, both presynaptic and postsynaptic *pgant3* and *pgant35A* are sufficient to properly regulate synaptic O-linked glycosylation and integrin levels, but regulation of the Ten-m ligand requires *pgant* function in the presynaptic neuron.

Figure 7. Presynaptic/postsynaptic *pgant3/35A* regulate O-GalNAc and integrin signaling. **A**, NMJ synaptic boutons colabeled for synaptic O-linked glycosylation marker (VVA), integrin receptor (α PS2; top 3 panels), and Ten-m relative to the presynaptic marker anti-HRP (bottom 2 panels), with split channels shown for clarity. The five genotypes shown include genetic control (w^{1118}), neuronal (*pgant3^{m1}/pgant3^{m2};elav/UAS-pgant3*) and muscle (*pgant3^{m1}/pgant3^{m2};24B/UAS-pgant3*) *pgant3* expression, and neuronal (*pgant35A^{H68}/pgant35A³⁷⁷⁵;elav/UAS-pgant35A*) and muscle (*pgant35A^{H68}/pgant35A³⁷⁷⁵;24B/UAS-pgant35A*) *pgant35A* expression conditions. Scale bar, 2 μ m. **B**, Histograms showing the relative fluorescence intensities for all three labels (VVA, α PS2, and Ten-m) normalized to each genetic control (dotted red line). Sample size is $n \geq 8$ independent NMJs for each label for all five genotypes. Statistical differences calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$. Error bars indicate SEM.

pgants regulate activity-dependent integrin signaling at the synapse

With striking activity-dependent effects on synaptic plasticity in *pgant* mutants (Fig. 5), we next queried activity-dependent changes in integrin signaling (Fig. 8). Channelrhodopsin-mediated optogenetic stimulation was used to drive presynaptic activity, followed by confocal microscopy examination for molecular changes at the NMJ synapse. The neuronal driver (*elav-gal4*; Lin and Goodman, 1994) was used to target UAS-ChIEF-tdTomato (Wang et al., 2011) in genetic control, single mutants, and double mutants. Channelrhodopsin targeting was confirmed by visualizing tdTomato expression (Fig. 8A) and eliciting EJPs with 5 Hz blue light ($\lambda = 460$ nm, 60 ms duration)

stimulation (Fig. 8B). Guided by the plasticity stimulation paradigm (Fig. 5), preparations were illuminated with 60 ms light pulses at 10 Hz for 60 s and then immediately fixed for imaging (Fig. 8C). VVA-TRITC O-GalNAc labeling did not detectably change in unstimulated controls compared with optogenetically stimulated preparations (*pgant3*, 1.09 ± 0.04 ; *pgant35A*, 1.09 ± 0.03 ; double mutant, 0.96 ± 0.04 as normalized to controls; $n \geq 13$; all $p > 0.05$). Similarly, we observe no change in levels of integrin ligand Ten-m (*pgant3*, 1.00 ± 0.04 ; *pgant35A*, 1.02 ± 0.04 ; double mutant, 1.14 ± 0.05 ; $n \geq 17$; all $p > 0.05$) or integrin receptor α PS2 levels (*pgant3*, 0.94 ± 0.04 ; *pgant35A*, 0.88 ± 0.04 ; double mutant, 0.84 ± 0.07 as normalized to controls; $n \geq 8$; all $p > 0.05$). Therefore, we investigated integrin downstream sig-

Figure 8. Activity-dependent integrin signaling changes in *pgant* mutants. **A**, Representative image of neurally targeted (*elav-gal4*) channelrhodopsin expression (ChIEF-tdTomato) in third-instar ventral nerve cord. The native fluorescence of the tagged light-activated channel (red) is shown without amplification. Scale bar, 50 μ m. **B**, Representative EJP trace from the muscle 6 NMJ induced by blue light (470 nm) stimulation of ChIEF-expressing neurons. **C**, Schematic of the optogenetic stimulation paradigm used to assay changes in integrin signaling. Basal stimulation at 0.5 Hz for 30 s to establish baseline, followed by 10 Hz blue light pulses for 60 s to induce activity-dependent changes. Samples were then immediately fixed and labeled for analyses. **D**, Representative images of pFAK (top) and Talin (bottom) labeling, with HRP colabeling, showing split channels to compare unstimulated control (left) and light-stimulated (10 Hz, 60 s) synaptic boutons. The genotypes are ChIEF-expressing control (*w¹¹¹⁸;UAS-ChIEF/elav-gal4*; top), *pgant3* (*pgant3^{m1}/pgant3^{m2};UAS-ChIEF/elav*) and *pgant35A* (*pgant35A^{H68}/pgant35A³⁷⁷⁵;UAS-ChIEF/elav-gal4*) single mutants (middle 2 rows), and the double mutant (*pgant3^{m1},pgant35A^{H68}/pgant3^{m2},pgant35A³⁷⁷⁵;UAS-ChIEF/elav-gal4*) (far right). **E**, Histograms show relative fluorescence intensities of pFAK (left) and Talin (right) normalized to genetic control for all four genotypes expressing ChIEF. Sample size is $n \geq 8$ independent NMJs for each label and condition for all genotypes. Statistics calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$. Error bars indicate SEM.

naling by assaying Talin and pFAK abundance (Devenport et al., 2007; Tsai et al., 2012a).

To determine whether activity-dependent integrin signal transduction is affected, we investigated channelrhodopsin-dependent changes in Talin recruitment and downstream pFAK production (Fig. 8D). Interestingly, when compared with respective unstimulated genotype controls, optogenetic stimulation drives a striking increase in Talin levels in both control ($49.41 \pm 18.96\%$, $n = 8$, $p < 0.05$) and *pgant* double mutants ($80.1 \pm 7.93\%$, $n = 13$, $p < 0.05$) compared with unstimulated conditions, whereas neither *pgant3* nor *pgant35A* single mutants showed any significant activity-dependent change in Talin recruitment to the synapse (Fig. 8D, bottom row, E, right). Moreover, we find an activity-dependent decrease pFAK levels in stimulated controls (*UAS-ChIEF/elav*, $17.85 \pm 4.23\%$, $n = 49$, $p < 0.05$), with no change in stimulated *pgant3* (*pgant3^{m1}/pgant3^{m2};UAS-ChIEF/elav*, $8.15 \pm 6.28\%$, $n = 43$, $p > 0.05$), *pgant35A* (*pgant35A^{HG8}/pgant35A³⁷⁷⁵;UAS-ChIEF/elav*, $7.76 \pm 3.94\%$, $n = 61$, $p > 0.05$), and double mutant *pgant3,pgant35A* (*pgant3^{m1},pgant35A^{HG8}/pgant3^{m2},pgant35A³⁷⁷⁵;UAS-ChIEF/elav*, $3.61 \pm 2.61\%$, $n = 27$, $p > 0.05$) conditions (Fig. 8D, top row, E, left). We conclude that both integrin recruitment of Talin and downstream production of pFAK is activity dependent and under *pgant* dependent suppressive regulation.

pgants regulate activity-dependent PSP size

Misregulated integrin signaling leads to intercellular de-adhesion and subsequent wing blistering in *pgant* mutant wing discs (Zhang et al., 2010). Moreover, mutants in *trans*-synaptic WNT/BMP and HSPG extracellular pathways manifest enlarged PSPs at the NMJ (Packard et al., 2002; Kamimura et al., 2013). Because we have shown that *pgant* mutants suppressively regulate basal and activity-dependent integrin signaling and PSP expansion, we next examined optogenetic activity-dependent synaptic ultrastructural changes, with a particular focus on the PSP. In the above channelrhodopsin-expressing mutants and controls, we adopted the same light stimulation paradigm, followed by fixation and transmission electron microscope (TEM) examination of synapse ultrastructure (Fig. 9).

In optogenetically stimulated synaptic terminals, there is an obvious decrease in SV density in all four genotypes compared with unstimulated controls (Fig. 9A). At <250 nm away from the active zone, quantification of SV number shows an $\sim 30\%$ decrease in controls and a similar $\sim 30\%$ decrease in stimulated *pgant3* mutants ($n = 13$, $p < 0.001$ compared with unstimulated condition; Fig. 9B). Both the *pgant35A* single mutant ($n = 19$) and the double null mutant ($n = 16$) behave similarly. Furthermore, there are no significant differences in suppressive regulation under basal conditions (Fig. 2) or unstimulated *UAS-ChIEF* carrying lines (Fig. 9) i.e., single mutants (*pgant3^{m1}/pgant3^{m2}*, 15.22 ± 0.99 vesicles; *pgant35A^{HG8}/pgant35A³⁷⁷⁵*, 15.54 ± 0.78 ; $p < 0.05$) are elevated compared with the control (*w¹¹¹⁸*, 10.5 ± 0.91) and the double mutant (*pgant3^{m1},pgant35A^{HG8}/pgant3^{m2},pgant35A³⁷⁷⁵*, 9.4 ± 0.67). Similarly, in unstimulated single mutants carrying the channelrhodopsin transgene, SVs are elevated (*pgant3^{m1}/pgant3^{m2};UAS-ChIEF/elav*, 9.53 ± 0.63 , $p < 0.01$; *pgant35A^{HG8}/pgant35A³⁷⁷⁵;UAS-ChIEF/elav*, 8.95 ± 0.49 , $p < 0.05$) with respect to control (*UAS-ChIEF/elav*, 7.13 ± 0.57) and double mutant (*pgant3^{m1},pgant35A^{HG8}/pgant3^{m2},pgant35A³⁷⁷⁵;UAS-ChIEF/elav*, 7.68 ± 0.39). Thus, activity drives SV cycling in all four genotypes comparably (Fig. 9B).

In contrast, optogenetically stimulated control NMJ synapses show an activity-dependent increase in PSP depth that does not

occur in either *pgant3* and *pgant35A* single mutant, although the double mutant is indistinguishable from control (Fig. 9A, dotted lines). Quantification of these differences reveal an activity-dependent PSP depth increase of $>50\%$ in control ($p < 0.05$ compared with unstimulated condition) and $\sim 35\%$ increase in the double null mutants ($n = 14$, $p < 0.05$) but no significant change in either single *pgant* mutant (Fig. 9C). Furthermore, comparing basal genotypes with unstimulated ChIEF carrying controls and mutants show no significant difference in PSP depth, for controls (*w¹¹¹⁸*, 120.47 ± 7.46 nm, $n = 12$ vs unstimulated control, 147.41 ± 15.69 nm, $n = 14$, $p > 0.05$), single mutants (*pgant3*, 272.36 ± 45.83 nm, $n = 13$ vs unstimulated *pgant3*, 253.26 ± 37.70 nm, $n = 14$, $p > 0.05$; *pgant35A*, 246.84 ± 46.63 nm, $n = 12$ vs unstimulated *pgant35A*, 232.97 ± 24.88 nm, $n = 15$, $p > 0.05$) and the double mutant (*pgant3,pgant35A*, 182.13 ± 23.76 nm, $n = 14$ vs. unstimulated *pgant3,pgant35A*, 227.31 ± 18.56 nm, $n = 25$, $p > 0.05$). Thus, presynaptic vesicle number decreases in all genotypes with acute optogenetic stimulation, but *pgants* suppressively regulate activity-dependent PSP expansion, consistent with the dysregulated integrin-mediated signaling.

Integrin inhibition blocks activity-dependent synaptic plasticity in *pgant* mutants

We have shown previously that blocking integrin signaling with RGD peptides interferes with synaptic plasticity at the *Drosophila* NMJ, comparably with integrin mutations (Bahr et al., 1997; Rohrbough et al., 2000). Furthermore, the Ten-m integrin ligand that is found to be suppressively regulated by *pgants* contains an RGD sequence. Hence, as a direct test of integrin signaling requirements in *pgant*-dependent facilitation, augmentation, and potentiation phases of tetanic stimulus train-induced synaptic plasticity, we used RGD integrin inhibitory peptides and scrambled RAD controls in the genetic background control, *pgant* single mutants, and the double mutant (Rohrbough et al., 2000). Using our established protocols for peptide incubation (Rohrbough et al., 2000), we recorded EJCs using the same stimulation paradigm used above (Fig. 5). Recordings were normalized to the mean basal EJC amplitude in sham/RGD/RAD-treated controls (Fig. 10A), *pgant3* (Fig. 10B) and *pgant35A* (Fig. 10C) single mutants, and *pgant3,pgant35A* (Fig. 10D) double mutants. Consecutive EJCs were averaged during the 0.5 and 10 Hz stimulation phases, respectively, for data presentation and quantification.

In RGD-treated compared with RAD-treated control (*w¹¹¹⁸*) synapses, a $>50\%$ elevation occurs in synaptic augmentation during the tetanic stimulus train, and $>30\%$ increase occurs in PTP after stimulation (Fig. 10A). In striking contrast, *pgant* single and double mutants show a synergistic interaction with integrin blockade to exhibit a loss of both phases of activity-dependent plasticity (Fig. 10B–D, left). Quantification of EJC amplitudes during the tetanic phase shows a significant increase in RGD-treated compared with RAD-treated control synapses ($p < 0.05$, $n \geq 9$; Fig. 10A, right). However, EJC amplitudes actually decrease $\sim 60\%$ in *pgant3* ($p < 0.05$, $n \geq 9$; Fig. 10B, right), *pgant35A* ($p < 0.05$, $n \geq 6$; Fig. 10C, right), and *pgant3,pgant35A* ($p < 0.05$, $n \geq 4$; Fig. 10D) after RGD treatment. During PTP phases, RGD treatment again causes a highly significant EJC amplitude increase compared with RAD-treated controls ($p < 0.05$, $n \geq 9$; Fig. 10A, right). Remarkably, RGD treatment instead causes $>50\%$ decreases in *pgant3* ($p < 0.05$, $n \geq 9$; Fig. 10B), *pgant35A* ($p < 0.05$, $n \geq 6$; Fig. 10C), and *pgant3,pgant35A* mutants ($p < 0.05$, $n \geq 4$; Fig. 10D) compared with RAD-treated synapses. Importantly, there are no significant differences be-

Figure 9. Activity-dependent changes in synapse ultrastructure in *pgant* mutants. **A**, Representative active zone synapses of control ($w^{1118};UAS-ChIEF/elav-gal4$; top), *pgant3* ($pgant3^{m1/m2};UAS-ChIEF/elav$), and *pgant35A* ($pgant35A^{H68/3775};UAS-ChIEF/elav-gal4$) single mutants (middle two rows), and double mutant ($pgant3^{m1};pgant35A^{H68/3775};UAS-ChIEF/elav-gal4$, bottom) when comparing the unstimulated (left column) and light-stimulated (10 Hz, 60 s; right column) conditions. Dotted white line indicates the PSP depth for each condition and genotype. Scale bar, 100 nm. **B**, **C**, Histograms showing quantification of activity-dependent changes for normalized SV number (**B**) and PSP depth (**C**) for all four genotypes under unstimulated (black bars) and stimulated (gray bars) conditions. Sample size is $n \geq 15$ independent boutons for each genotype and condition. Statistical differences are calculated using one-way ANOVA with Dunnett's *post hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., No significance. Error bars indicate SEM.

tween RAD-treated synapses and sham controls (Fig. 10A–D). We conclude that integrin signaling blockade coupled to the loss of *pgant* function causes a complete loss of activity-dependent facilitation, augmentation, and potentiation, consistent with a requirement of *pgant* activity in integrin-mediated functional synaptic plasticity.

Discussion

Across species, glycans are increasingly being recognized as key regulators of synaptic function and plasticity (Dani and Broadie, 2012; Scott and Panin, 2014). Classically, Gal(β1,4)GlcNAc, Gal(β1,3)GalNAc, CT carbohydrate antigen, heparin, heparan

sulfate, and sialic acid are all known to modulate the *trans*-synaptic agrin signal mediating postsynaptic acetylcholine receptor stabilization at mammalian NMJs (Wallace, 1990; Parkhomovskiy et al., 2000). Similarly, the *Drosophila* Mtg glycan-binding lectin regulates the stabilization/organization of postsynaptic glutamate receptors and establishes the extracellular matrix–integrin interface at the NMJ (Rohrbough et al., 2007; Rushton et al., 2009). Other *Drosophila* glycan regulating genes, including sialyltransferase, sialic acid transporter Fuseless, and Mgat1, also modulate ion channels, presynaptic/postsynaptic organization, and neurotransmission strength at the NMJ (Long et al., 2008; Repnikova

Figure 10. Integrin inhibition blocks all synaptic plasticity in *pgant* mutants. TEVC recordings from sham-treated, RAD control, and RGD integrin-blocking peptide applications in genetic control (*w¹¹¹⁸*; **A**), *pgant3* (*pgant3^{m1}/pgant3^{m2}*; **B**) and *pgant35A* (*pgant35A^{H68}/pgant35A³⁷⁷⁵*; **C**) single mutants, and the double mutant (*pgant3^{m1},pgant35A^{H68}/pgant3^{m2},pgant35A³⁷⁷⁵*; **D**). Left column, The stimulation paradigm is indicated on the x-axis with EJC amplitudes normalized to the basal EJC amplitude in each condition for sham control (filled circle), RAD control (open square), and integrin-blocking RGD (open circle) peptide applications. Right column, Histograms show normalized EJC amplitudes for sham/RAD/RGD peptide treatments for the indicated time periods. Samples size is $n \geq 5$ independent NMJs for each genotype and treatment condition (>60 recordings total). Statistical differences calculated using one-way ANOVA with Dunnett's *post hoc* test: $*p < 0.05$. Error bars indicate SEM.

et al., 2010). Our RNAi glycogene screen recently identified a pair of genes (*hs6st* and *sulf1*) that regulate HSPG sulfation state to modulate the bidirectional *trans*-synaptic WNT/BMP signaling driving presynaptic/postsynaptic assembly and synapse function (Dani et al., 2012; Parkinson et al., 2013). Another gene pair,

pgant3 and *pgant35A*, catalyzing early steps of mucin O-glycan (GalNAc α 1-O-S/T) posttranslational modification as *N*-acetyl-galactosaminyl transferases (Schwientek et al., 2002; Ten Hagen et al., 2003b), was identified to have neurotransmission effects in the same screen.

In *Drosophila*, *pgant3* is characterized to regulate integrin–ligand secretion and intercellular adhesion and *pgant35A* for appropriate intercellular septate junction formation (Tian and Ten Hagen, 2007; Zhang et al., 2008). Microarray analyses have identified *pgant3* and *pgant35A* transcripts in the developing nervous system and musculature (Tian and Ten Hagen, 2006; Chintapalli et al., 2007), and our lectin analyses show NMJ O-GalNAc modifications dependent on both *pgant3* and *pgant35A*. Null mutants display increased presynaptic active zone *brp* (ELKS/CAST) and postsynaptic glutamate receptor bad reception (GluRIID) assembly (Featherstone et al., 2005; Wagh et al., 2006) and elevated evoked neurotransmission strength, and genetic rescue experiments show *pgant3* and *pgant35A* function in both neurons and muscle. All synaptic defects occurring in single *pgant* nulls are absent in double mutants, which are essentially indistinguishable from controls. Similar observations have been described as “reciprocal suppression” in the context of physically interacting proteins, respectively (Honts et al., 1994). However, because the basis of the *pgant3/pgant35A* interaction is as yet unknown, we have opted here for the conservative suppression genetic interaction definition. This suppressive regulation presumably arises from balanced *pgant3/pgant35A* function. Consistently, when a single wild-type transgene (*UAS-pgant3*) is expressed (presynaptic or postsynaptically) in the double mutant (*pgant3,pgant35A*), the other mutant phenotype (*pgant35A*) reemerges. Moreover, overexpression of either *pgant3* or *pgant35A* individually in neuron or muscle decreases neurotransmission strength, which is the opposite consequence of single loss of function. These results reveal a *pgant3/pgant35A* suppressive mechanism dependent on the balance between these two genes on both sides of the synapse.

The *pgant* suppressive mechanism regulates synaptic ultrastructural organization, including presynaptic vesicle pools and PSP size. Like other synaptic phenotypes, PSP size is elevated in single *pgant3/pgant35A* mutants but normal in double mutants. Importantly, PSP compartments apposed to presynaptic active zones are expanded in *trans*-synaptic WNT/BMP signaling ligand mutants (Packard et al., 2002; Tian and Ten Hagen, 2007; Ren et al., 2009; Nahm et al., 2010; Kamimura et al., 2013), as well as mutants affecting extracellular HSPG regulators of *trans*-synaptic signaling (Packard et al., 2002; Tian and Ten Hagen, 2007; Ren et al., 2009; Nahm et al., 2010; Kamimura et al., 2013). Consistently, we identified the *trans*-synaptic Ten-m/ α PS2 integrin signaling pair (Mosca et al., 2012) to be suppressively regulated by the *pgant3/pgant35A* mechanism. Ten-m/ α PS2 integrin interactions are known to drive intercellular adhesion (Graner et al., 1998), and *pgant3* is known to regulate integrin–ligand secretion and promote adhesion in the developing *Drosophila* wing (Zhang et al., 2010). At the *Drosophila* NMJ, both Ten-m ligand and α PS2 integrin are localized presynaptically and postsynaptically (Mosca et al., 2012). Based on these extensive established interactions, we interpret the enlarged PSP in *pgant3* and *pgant35A* single mutants to a consequence of impaired Ten-m/integrin signaling. Because the spacing between presynaptic and postsynaptic membranes is not affected and normally apposed presynaptic/postsynaptic membranes occur with enlarged PSPs, we consider this to be a postsynaptic defect. This is not surprising because α PS2/Ten-m are both transmembrane proteins, and integrin signaling is well known to bridge to the cytoskeleton (Delon and Brown, 2007). Thus, an enlarged PSP can manifest on the inside of the postsynaptic membrane as a result of impaired integrin signaling. The levels of Ten-m and α PS2, as well as PSP size, are all suppressively regulated by the *pgant3/pgant35A* mechanism.

Synaptic O-GalNAc abundance is likewise suppressively regulated by *pgant3* and *pgant35A*, with levels elevated in single mutants and normal in double mutants. Like mammalian *pgants* (GalNAc-Ts or ppGalNAcTs), *Drosophila* *pgants* (12 total) are thought to function hierarchically, competing for naked or glycosylated substrates to regulate final O-GalNAc density (Ten Hagen et al., 2003a). The observed suppressive mechanism suggests that *pgant3* and *pgant35A* may function at the same tier of glycosylation. Alternatively, with the imbalance induced by *pgant* mutations, other *pgant* family members may be dysregulated, leading to increased O-GalNAc synaptic glycosylation. Normally, Golgi-resident *pgants* relocated to the ER are known to increase O-GalNAc glycosylation (Gill et al., 2010), dependent on Src activation downstream of integrin signaling (Mitra and Schlaepfer, 2006), which is misregulated in *pgant* mutants. In addition to well described α/β integrins functions at the mammalian NMJ, $\alpha 3$ integrin affects hippocampal dendrite stability and function (Kerrisk et al., 2013), whereas $\beta 3$ integrin associates with GluA2 AMPA receptors (Pozo et al., 2012). In *Drosophila*, we have shown that α PS1– α PS13 and β PS regulate synapse assembly and neurotransmission strength (Beumer et al., 1999; Rohrbough et al., 2000), agreeing with *pgant* roles shown here in the presynaptic vesicle pool and postsynaptic glutamate receptor regulation. In synaptic plasticity, $\alpha 3/5/8$ and $\beta 1$ integrin knockdown all impair hippocampal long-term potentiation (Chan et al., 2003, 2006). Similarly, *Drosophila* α PS3 (Volado) and β PS mutants show impaired augmentation and PTP (Rohrbough et al., 2000), agreeing with *pgant* roles shown here in maintaining both plasticity phases. In addition to the joint Ten-m/ α PS2 downregulation in *pgant3* and *pgant35A*, each mutant also displays distinct misregulation of integrin signaling components (β_v and β PS, respectively), with roles in neurotransmission and synaptic plasticity (Rohrbough et al., 2000; Tsai et al., 2012a; Tran and Ten Hagen, 2013).

All phases of synaptic plasticity (facilitation, augmentation, and potentiation) are suppressively regulated by *pgant3* and *pgant35A*. To investigate mechanisms of these activity-dependent changes, we used optogenetic stimulation to test acute subcellular ultrastructure and integrin signaling effects (Fenno et al., 2011). Classical studies coupling traditional electrical nerve stimulation to ultrastructural analysis at frog NMJ revealed dynamic vesicle fusion after single stimuli (Heuser and Reese, 1981) and vesicle depletion after a prolonged train of 10 Hz stimulation (Ceccarelli et al., 1972). Recent studies using channelrhodopsin (ChIEF) optogenetic stimulation identified an ultrafast endocytic mechanism at the *Caenorhabditis elegans* NMJ (Watanabe et al., 2013a), which was subsequently validated in hippocampal synapses (Watanabe et al., 2013b), but did not assay effects on vesicle pools. Using the same ChIEF optogenetic tool in *Drosophila*, we find that a brief, high-frequency light train (10 Hz, 60 ms pulses for 60 s) drives a depression of vesicles in distinct pools around presynaptic active zones. We also find activity-dependent expansion of PSPs in controls, which fails in both *pgant* single mutants but is restored in double mutants, again showing a suppressive mechanism. Consistently, we identify suppressive activity-dependent elevation of integrin downstream Talin signaling in only control and double mutant conditions, supported by known roles of Talin-mediated α PS2 integrin signaling (Devenport et al., 2007). Moreover, we find a lack of activity-dependent pFAK regulation, supported by previous studies showing activity-dependent decreases in pFAK signaling at the *Drosophila* NMJ (Tsai et al., 2012a). Importantly, RGD treatment perturbing integrin signaling and synaptic plasticity also alters synaptic pFAK

levels (Stäubli et al., 1998; Rohrbough et al., 2000; Russo et al., 2013). Consistent with this mechanism, RGD treatment acts synergistically with *pgant* mutations to prevent the manifestation of a synaptic plasticity.

In summary, this is the first investigation of synaptic *pgant* roles, which combines molecular, electrophysiological, electron microscopy, and optogenetic approaches. We identify here a novel suppressive mechanism between two *pgant* family members (*pgant3* and *pgant35A*) regulating synaptomatrix O-GalNAc glycosylation state, coupled presynaptic active zone and postsynaptic glutamate receptor assembly, transmission strength, integrin signaling and postsynaptic adhesion, and the appearance of activity-dependent plasticity. Future studies will seek to determine whether Ca^{2+} and/or CaMKII signaling mechanisms (Tsai et al., 2012a) are misregulated during *pgant* synaptic dysfunction, as the leading causal link between activity and observed synaptic changes. Based on recent reports that show that O-GalNAc levels regulate proteolytic cleavage and ligand secretion (Zhang et al., 2014), we will test whether the *pgant* suppressive mechanism may reflect interactions between additional *pgants* or within other enzymatic classes. A final priority will be investigation of *pgant*-mediated regulation of disease-related synaptic proteins, including Dystroglycan (Henry et al., 2001) and Neurofascin (Tsai et al., 2012b), to test the hypothesis that heritable neurological and neuromuscular disorders are causally related to the *pgant* synaptic glycan mechanism.

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