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The glycosphingolipid MacCer promotes synaptic bouton formation in Drosophila by interacting with Wnt

Yan Huang1, Sheng Huang1,2†, Coralie Di Scala3, Qifu Wang1, Hans H Wandall4, Jacques Fantini5, Yong Q Zhang1*

1Key Laboratory for Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, CAS Center for Excellence in Brain Science and Intelligence Technology, Beijing, China; 2Sino-Danish College, Sino-Danish Center for Education and Research, Chinese Academy of Sciences, Beijing, China; 3INMED U1249, INSERM, Marseille, France; 4Copenhagen Center for Glycomics, Department of Cellular and Molecular Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark; 5UNIS UMR_S 1072, INSERM, Aix-Marseille Université, Marseille, France

Abstract Lipids are structural components of cellular membranes and signaling molecules that are widely involved in development and diseases, but the underlying molecular mechanisms are poorly understood, partly because of the vast variety of lipid species and complexity of synthetic and turnover pathways. From a genetic screen, we identify that mannosyl glucosylceramide (MacCer), a species of glycosphingolipid (GSL), promotes synaptic bouton formation at the Drosophila neuromuscular junction (NMJ). Pharmacological and genetic analysis shows that the NMJ growth-promoting effect of MacCer depends on normal lipid rafts, which are known to be composed of sphingolipids, sterols and select proteins. MacCer positively regulates the synaptic level of Wnt1/Wingless (Wg) and facilitates presynaptic Wg signaling, whose activity is raft-dependent. Furthermore, a functional GSL-binding motif in Wg exhibiting a high affinity for MacCer is required for normal NMJ growth. These findings reveal a novel mechanism whereby the GSL MacCer promotes synaptic bouton formation via Wg signaling.

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Introduction

The glycosphingolipids (GSLs) are particularly abundant in the nervous system and are essential for brain development (Fantini and Yahi, 2015; Yu et al., 2009). Specific deletion of GSLs in mouse brain leads to severe neural defects or lethality (Jennemann et al., 2005). Nevertheless, the molecular and cellular functions of GSLs in development are poorly understood, partially due to the complexity of GSL metabolism and the variety of GSL structures in vertebrates. Because of the comparatively simple metabolic pathways and the power of genetic studies in invertebrates (Bellen and Yamamoto, 2015; Zhu and Han, 2014), a mechanistic understanding of the role of GSLs in development and function of the nervous system is beginning to be established (Dahlgaard et al., 2012; Huang et al., 2016; Kniazeva et al., 2015; Yonamine et al., 2011). GSLs and sphingomyelins (SMs, another class of sphingolipids) assemble with sterol into detergent-resistant membrane microdomains known as lipid rafts, which are critical for signal transduction and membrane trafficking (Lingwood and Simons, 2010). In neural development, multiple processes such as neuronal proliferation, recognition, migration, and synapse formation are regulated by lipid rafts (Aureli et al., 2015). For instance, disruption of rafts by depletion of sphingolipid or sterol...
leads to enlargement and gradually loss of synapses in cultured hippocampal neurons (Hering et al., 2003). Many growth factors or their receptors are preferentially located within and functionally dependent on membrane rafts (Wang and Yu, 2013; Watanabe et al., 2009; Zhai et al., 2004). Specifically, GSLs interact with raft-associated signaling proteins, such as epidermal growth factor receptor (EGFR) and Notch, thereby facilitating signal transduction (Coskun et al., 2011; Hamel et al., 2010; Wang and Yu, 2013). Our previous study uncovered that the GSL mannosyl glucosylceramide (MacCer) promotes NMJ overgrowth (Huang et al., 2016). However, the mechanisms by which GSLs mediate in vivo neural development remain elusive.

Normal brain function depends on proper formation of synaptic connections. The Drosophila larval glutamatergic neuromuscular junction (NMJ) is an advantageous model for dissecting mechanisms underlying synaptic development (Bayat et al., 2011; Khuong et al., 2013; Khuong et al., 2010; Korkut and Budnik, 2009). To uncover potential functions of lipids at synapses, we used the Drosophila NMJ as a model synapse and performed a genetic screen targeting genes involved in lipid biosynthesis and turnover pathways. From this screen, we identified multiple genes involved in sphingolipid de novo synthesis affecting NMJ development. We further found that MacCer is both required and sufficient for promoting NMJ growth and bouton formation in presynaptic neurons. MacCer promotes NMJ growth in a raft-dependent manner. We revealed that MacCer positively regulates synaptic Wg level and the presynaptic activity of Wg signaling. Further multiple independent assays showed MacCer physically interacts with Wg via a previously unidentified GSL-binding motif in Wg. Mutations in this motif disrupt the MacCer-Wg binding and normal NMJ growth. These findings demonstrate that the GSL MacCer plays a crucial role in bouton formation and NMJ growth and uncover a novel regulatory mechanism of Wg signaling pathway by MacCer.

Results

Mutations in de novo sphingolipid synthetic enzymes affect NMJ growth

To gain novel insights into the role of lipids in regulating synaptic development, we carried out a genetic screen targeting genes involved in the biosynthesis and turnover of fatty acids, glycerophospholipids, and sphingolipids. We tested over 60 candidate genes by examining NMJ morphology (Supplementary file 1) and identified two enzymes, serine palmitoyltransferase 2 Lace and ceramide synthase Schlank, promoting NMJ bouton formation as mutations in either of the two proteins led to fewer and larger boutons (Figure 1B–F,H,I,K). Mutations in lace and schlank disrupt the de novo synthesis of ceramides, the central intermediate in sphingolipid synthesis/metabolism (Figure 1A; Adachi-Yamada et al., 1999; Bauer et al., 2009; Fyrst et al., 2004). These data indicate that depletion in de novo synthesis of ceramides inhibits bouton formation. In addition to the de novo ceramide synthesis, the ceramide precursor sphingosines can be phosphorylated by sphingosine kinase 2 (Sk2) to produce phosphorylated sphingosines (Figure 1A). In Sk2 mutants, the level of phosphorylated sphingosines is reduced, while sphingosines accumulate (Fyrst et al., 2004; Yonamine et al., 2011). We found that mutations in Sk2 resulted in more satellite boutons at NMJs (Figure 1G,J), in contrast to the fewer and larger bouton phenotype in lace and schlank mutants. These results indicate that the de novo synthesis of ceramides, their downstream derivatives, or both promote bouton formation and NMJ growth.

GSL and ceramide phosphoethanolamine (CerPE, the Drosophila analogue of SM) are major membrane sphingolipids modified from ceramides (Figure 1A). However, we did not study the effects of CerPE in NMJ growth because there are multiple genes encoding candidates of CerPE synthase in the Drosophila genome (Vacaru et al., 2013; Supplementary file 1).

GSL synthases Egh and Brn bi-directionally regulate NMJ growth presynaptically

In contrast to the complex CerPE synthesis, the first three steps of GSL synthetic pathway are catalyzed by single GSL synthases in Drosophila (Figure 2A; Chen et al., 2007; Schwientek et al., 2002; Wandall et al., 2003). Disruption of the first one glucosylceramide synthase (GlcT1) to block GSL biosynthesis by feeding larvae with a specific GlcT1 inhibitor D, L-threo-PDMP at 0.5 mg/ml (Delgado et al., 2006) resulted in fewer and larger boutons at NMJ synapses (Figure 2C,N), without
Figure 1. NMJ growth depends on de novo synthesis of ceramides. (A) Simplified de novo biosynthesis pathway of sphingolipid in Drosophila is shown. (B–G) Representative images of NMJ4 co-stained with anti-HRP (green) and anti-CSP (magenta) in wild type (B), lace<sup>ko5305</sup>/lace<sup>2</sup> (C), lace<sup>ko5305</sup>/Df (2L)Exel7063 (D), schlank<sup>GO489</sup>/Y (E), schlank<sup>GO061</sup>/Y (F) and Sk2<sup>2KG05894</sup>/Df(3L)BSC671 (G). Scale bar: 10 μm; Arrowheads indicate large boutons in different mutants. (H–J) Quantifications of bouton number (H), bouton size (I) and satellite bouton number (J) of NMJs in abdominal segments A3 or A4 of different genotypes. (K) Bouton number and size in schlank mutants were normalized to muscle surface area as schlank mutants showed decreased body size (Bauer et al., 2009). *p<0.05; **p<0.01, and ***p<0.001 by student’s t test between a test genotype and the wild-type control; n ≥ 10 larvae; error bars: s.e.m. Source data 1. Numerical data for the statistical graphs. The following figure supplement is available for Figure 1. DOI: https://doi.org/10.7554/eLife.38183.002
Figure 2. GSL synthases Egh and Brn bi-directionally regulates NMJ growth presynaptically. (A) GSL synthesis pathway in Drosophila. (B–M) Images of NMJ co-stained with anti-HRP (green) and anti-CSP (magenta) in wild type (B), larvae treated with 0.5 mg/ml D, L-threo-PDMP (C, egh62d18/Y (D), UAS-Egh/+; nSyb-Gal4/+ (E), egh62d18/Y; UAS-Egh/+; nSyb-Gal4/+ (F), UAS-Egh/OK6-Gal4 (G), egh62d18/Y; UAS-Egh/OK6-Gal4 (H) brn1.6P6 (I), UAS-brn-RNAi/+; nSyb-Gal4/UAS-brn (J), UAS-brn-RNAi/OK6-Gal4 (L), and OK6-Gal4/+; UAS-brn/+ (M). Scale bar: 10 µm; Arrowheads point

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at large boutons. (N–P) Quantifications of total bouton number, bouton size, and satellite bouton number of NMJs in different genotypes or treated with PDMP. “CT” denotes corresponding control in each multiple comparison. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey post hoc tests; n ≥ 12 larvae; error bars: s.e.m.

DOI: https://doi.org/10.7554/eLife.38183.005

The following source data and figure supplements are available for figure 2:

**Source data 1.** Numerical data for the statistical graphs.

DOI: https://doi.org/10.7554/eLife.38183.006

**Figure supplement 1.** Additional NMJ images and quantifications.

DOI: https://doi.org/10.7554/eLife.38183.004

**Figure supplement 2.** Alteration of egh and brn level in glia or muscle does not affect NMJ growth.

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affecting the developmental time and larval size (muscle 4 size was normal; Figure 2—figure supplement 1). GlcCer can be further converted into MacCer by Egghead (Egh) (Wandall et al., 2003). It has been previously shown that the enzymatic activity of Egh is reduced in egh<sup>62d18</sup> and egh<sup>7</sup> mutants (Wandall et al., 2005). Here we show that both homozygous egh<sup>62d18</sup> and hetero-allelic egh<sup>62d18</sup>/egh<sup>7</sup> mutants also displayed fewer and larger synaptic boutons (Figure 2,D,O and Figure 2—figure supplement 1; Huang et al., 2016). In contrast, neuronal (presynaptic) overexpression of Egh driven by the pan-neuronal nSyb-Gal4 or motor neuronal specific OK6-Gal4 led to synaptic overgrowth with more and smaller boutons (Figure 2, D–H and O and Figure 2—figure supplement 1), indicating that Egh promotes bouton formation. However, expression of egh in muscles (postsynaptic) driven by C57-Gal4 or in glia driven by Repo-Gal4 did not affect NMJ growth in both wild type and egh<sup>62d18</sup> mutant background (Figure 2—figure supplement 2). The cell-type specific manipulations of egh expression support that Egh promotes NMJ bouton formation in presynaptic motoneurons. Heterozygous mutation of egh significantly suppressed NMJ overgrowth in larvae neuronal overexpressing Egh, and homozygous mutation of egh in Egh-overexpressing background fully suppressed bouton number but not bouton size to the control level (Figure 2O), indicating that neuronal expression of egh promotes NMJ growth in a dose-dependent manner.

Brainiac (Brn) converts MacCer to GlcNAc-MacCer (Figure 2A; Schwientek et al., 2002). Hypomorphic brn<sup>6.107</sup> and brn<sup>1.6P6</sup> mutations display MacCer accumulation in egg chambers (Pizette et al., 2009; Wandall et al., 2005). We observed more boutons and satellite boutons in homozygous brn<sup>6.107</sup> and trans-allelic brn<sup>1.6P6</sup>/brn<sup>6.107</sup> mutants; neuronal knockdown of brn by an RNAi under the control of nSyb-Gal4 or OK6-Gal4 also resulted in NMJ overgrowth. Conversely, neuronal overexpression of Brn driven by nSyb-Gal4 or OK6-Gal4 led to fewer and larger boutons (Figure 2, I–M and P; Huang et al., 2016), recapitulating the phenotype of egh mutants. Furthermore, overexpression and knockdown of brn in muscles by C57-Gal4 or in glia by Repo-Gal4 did not affect NMJ growth (Figure 2—figure supplement 2). The cell type specific rescue and RNAi knockdown results demonstrate that both Egh and Brn act presynaptically. The opposite effect of Egh and Brn in regulating bouton number suggests that the GSL MacCer promotes NMJ growth.

**GSL MacCer promotes NMJ growth**

To verify if MacCer indeed promotes NMJ growth, we performed immunostaining and found that endogenous MacCer, detected by a specific anti-MacCer antibody (Wandall et al., 2003), was enriched at presynaptic NMJ boutons in a punctate pattern (Figure 3A). Compared to wild type, MacCer intensity at NMJs was reduced in egh mutant and brn-overexpressing larvae; Conversely, MacCer intensity was increased at NMJs of brn mutant, brn RNAi knockdown, and egh-overexpressing larvae (Figure 3, B–I), demonstrating the specificity of the anti-MacCer. These findings are consistent with previous reports showing that MacCer is decreased in egh mutants but increased in brn mutants by high performance thin layer chromatography (Hamel et al., 2010; Wandall et al., 2005), which we confirmed (Figure 8—figure supplement 1). Based on the fact that the presynaptic MacCer levels positively correlate with synaptic bouton number, we conclude that the GSL MacCer promotes NMJ growth presynaptically.

To characterize the synaptic structure in MacCer-deficient NMJs, we examined a few molecules that play important roles in synaptic growth and function, including the cell adhesion molecule...
Figure 3. MacCer staining intensity at NMJs is bi-directionally regulated by Egh and Brn. (A) Images of wild-type NMJ4 co-stained with anti-MacCer (green), anti-HRP (blue) and anti-DLG (red). MacCer puncta were apparent in presynaptic boutons. Scale bar: 10 µm. (B) Statistical results of normalized intensities of MacCer against anti-HRP staining within presynaptic boutons in different genotypes. **p<0.01 and ***p<0.001 by student’s t test between a test genotype and wild type; n ≥ 16 larvae, error bars: s.e.m. (C–H) Representative images of NMJ4 stained with anti-MacCer in wild type (C), egh62d18/Y (D), UAS-Egh/+; nSyb-Gal4/+ (E), brn1.6P6/brn939107 (F), UAS-bm-RNAi/+; nSyb-Gal4/+ (G) and nSyb-Gal4/UAS-Brn (H). Scale bar: 5 µm.

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The following source data and figure supplement are available for figure 3:

Source data 1. Numerical data for the statistical graphs.
DOI: https://doi.org/10.7554/eLife.38183.009

Figure supplement 1. Images and quantifications of Fas II and Syx1A staining at NMJs of different genotypes.
DOI: https://doi.org/10.7554/eLife.38183.008
MacCer promotes NMJ bouton formation in a lipid raft-dependent manner

GSLs are constituents of lipid rafts that are detergent-resistant membrane microdomains composed of sphingolipids, sterols and proteins. Like other GSLs, Drosophila MacCer is enriched in detergent-insoluble membrane microdomains (Rietveld et al., 1999). Consistently, MacCer substantially overlaps with Syx1A, a lipid raft-localized protein in human and Drosophila cells (Chamberlain et al., 2001; Zhai et al., 2004), at NMJs (Figure 4A). Our results also show that mutations in lace and schlank result in fewer boutons at NMJs (Figure 1), probably due to a disruption of lipid rafts by depleting total sphingolipids. Thus, we asked if synaptic development requires the formation of lipid rafts. The assembly of lipid rafts is sterol-dependent (Lingwood and Simons, 2010); sterol depletion by methyl-β-cycloexdextrin (MβCD), a cyclic oligosaccharide that absorbs sterols from the membrane, efficiently disrupts raft assembly in mammalian cells and Drosophila cells (Sharma et al., 2004; van Zanten et al., 2009; Zhai et al., 2004). Drosophila does not synthesize but takes sterols from food (Carvalho et al., 2010). Thus we used drug treatment rather than genetic means to deplete sterols. Feeding wild-type larvae with MβCD at concentrations higher than 25 mM led to early larval lethality, while MβCD treatment at 20 mM led to a mild developmental delay but normal larval and muscle size (Figure 4—figure supplement 1). MβCD treatment significantly reduced the co-localization of MacCer and Syx1A (Figure 4, B and C). We also treated larvae with another sterol binding drug filipin at 50 μg/ml, which did not affect larval developmental time but led to a mild but significant decrease in muscle size (Figure 4—figure supplement 1). Treatments with both drugs resulted in obviously fewer and larger boutons at NMJs, recapitulating the NMJ phenotype of sphingolipid-deficient and MacCer-deficient larvae (Figure 4, D–G and Figure 4—figure supplement 1). These results suggest that a proper level of sterols is required for bouton formation.

Depleting sterol with MβCD in egh-overexpressing background fully restrict the NMJ overgrowth to the level of the wild-type larvae treated with MβCD. Moreover, MβCD treatments in sphingolipid-deficient lace6035S/DF mutants and MacCer-deficient larvae (egh62d18 and nSyb-Gal4/UAS-brn) did not exacerbate the NMJ phenotype (Figure 4G, and Figure 4—figure supplement 1), suggesting that sterol may modulate NMJ growth in a common genetic pathway with sphingolipids and MacCer. We then determined if MβCD treatment suppresses NMJ growth via directly reducing the MacCer level. We found that MβCD completely suppressed the NMJ overgrowth without detectably reducing the synaptic MacCer level in wild-type and Egh-overexpressing larvae; similarly, Filipin treatment did not affect the synaptic level of MacCer (Figure 4, H–N). These data suggest that restriction of synaptic growth by MβCD may be due to defects of MacCer function by disrupting raft assembly rather than a reduction in the synaptic level of MacCer per se. This finding is consistent with a previous report that disrupting the formation of membrane rafts may compromise the function of raft-associated factors but not necessarily affecting their levels; more specifically, the localization of a raft protein LFA-1 was unaltered examined by confocal microscopy at subcellular level but altered by single-molecule near-field optical microscopy at molecular level upon raft disruption (van Zanten et al., 2009). Together, these data suggest that the NMJ growth-promoting effect of MacCer depends on normal lipid rafts.

MacCer promotes NMJ bouton formation via Wg signaling

NMJ development is controlled in part by growth factors and pathways (Bayat et al., 2011; Harris and Littleton, 2015; Korkut and Budnik, 2009). Bone morphogenetic protein (BMP) is an important growth promoting signaling in NMJs (Bayat et al., 2011). However, we found that the NMJ growth-promoting effect of MacCer is not mediated through the BMP signaling pathway (Huang et al., 2016). Wnt1/Wingless (Wg) activates another signaling pathway promoting NMJ growth (Korkut and Budnik, 2009). Because Wg is a lipid raft-associated protein (Zhai et al., 2004), we further investigated if Wg signaling play roles in MacCer-mediated NMJ growth. Previous reports showed that loss-of-function mutations of Wg signaling components, including the ligand Wg, the receptor dFrizzled2 (Fz2), the co-receptor Arrow (Arr), and the Wg-binding protein Evenness.
Interrupted/Wntless (Evi/Wls), decrease synaptic bouton number, while overexpression of Wg in motor neurons results in synaptic overgrowth (Korkut et al., 2009; Miech et al., 2008; Packard et al., 2002). In addition, the proper secretion, binding with the receptor Frizzled, and activation of Wg requires lipidation at the conserved serine at amino acid 239 (Janda et al., 2012; Takada et al., 2006). Specifically, the synaptic level of Wg and the downstream signaling are
positively regulated by the acyltransferase Porcupine (Packard et al., 2002), which activates the lipidation and raft-association of Wg (Zhai et al., 2004). We hypothesized that the NMJ growth-promoting effect of Wg depends on its association with membrane rafts. Indeed, raft disruption by MJICD treatment which depletes sterol significantly reduced Wg staining intensity at NMJs and constrained NMJ overgrowth in Wg-overexpressing larvae (Figure 5—figure supplement 1), suggesting that normal Wg signaling at NMJs requires intact lipid rafts.

Next, we determined whether Wg signaling cooperates with MacCer in promoting NMJ growth. Genetic analysis showed that loss of one copy of egfiny had no effect on NMJ morphology but fully suppressed the NMJ overgrowth phenotype of Wg-overexpressing larvae. Homozygous mutation of egf or overexpression of brn on a Wg-overexpressing background led to a decrease in bouton number, recapitulating the phenotype of egf mutants and brn-overexpressing larvae (Figure 5, A–E, K and Figure 5—figure supplement 1). These results together indicate that the NMJ growth-promoting effect of Wg signaling depends on the proper level of MacCer. Conversely, mutation in wg/2/wgC14 restricted NMJ overgrowth in Egh-overexpressing larvae to the wg mutant level (Figure 5, G–I and K). In addition, although bouton number was normal in wg+ and egfiny single heterozygotes (wg+/+ and egfiny/++), there were fewer and larger boutons in egfiny/++; wg+/+ transheterozygotes. Similarly reduced bouton numbers were observed in egfiny/++; arri–/+] and egfiny/++; ev2+/+ transheterozygotes (Figure 5, J, K and Figure 5—figure supplement 1), suggesting that Egh and Wg signaling act in the same genetic pathway. Together, these results demonstrate that MacCer works synergistically with Wg signaling in promoting NMJ growth and bouton formation.

MacCer facilitates local presynaptic Wg signaling at NMJs

To reveal the molecular mechanism by which MacCer promotes NMJ growth via Wg signaling, we first examined if there was colocalization between MacCer and Wg. Indeed, a substantial overlap between MacCer and Wg immunoreactivity was observed in NMJ boutons (Figure 6A and E). It is known that Wg secretion from presynaptic terminals requires the recycling endosomal small GTPase Rab11 (Koles et al., 2012; Korkut et al., 2009). It is also known that recycling endosomes are enriched with components of lipid rafts including Lactosylceramide (LacCer), the vertebrate analog of MacCer (Balasubramanian et al., 2007; Gagescu et al., 2000; Hortsch et al., 2010). Consistently, we found strong colocalization of MacCer with Rab11 at NMJ terminals (Figure 6, C and E). In contrast, MacCer puncta did not overlap with the early endosomal marker Rab5-YFP or the late endosome protein Spinster-GFP (Spin-GFP). Although GSLs are synthesized in the Golgi apparatus, there were few overlaps between MacCer and the Golgi marker Mannose II-GFP (Figure 5—figure supplement 1).

The specific colocalization of MacCer with Wg and Rab11 suggests that the trafficking or distribution of Wg at NMJ synapses may be altered upon MacCer reduction. Indeed, the staining intensity of endogenous Wg was significantly decreased in egfiny mutant boutons and brn-overexpressing NMJs; Conversely, Wg intensity was increased in egfiny-overexpressing and brn-RNAi knockdown boutons (Figure 6B, F–I and Figure 6—figure supplement 2). Thus, MacCer is both required and sufficient for Wg level at NMJ synapses. Though the total protein level of Wg in egf mutant brains appeared normal by Western assay, we found an increase of Wg in the central neuropil but not in peripheral axons within segmental nerves of egf mutants (Figure 6—figure supplement 3), suggesting a defect in axonal transport of Wg. In contrast to reduced synaptic Wg, the staining intensities of Rab11 and the Wg receptor Fz2 were largely normal in egf NMJs (Figure 6D and Figure 6—figure supplement 4). Conversely, MacCer staining was normal in wg or rab11 mutants (Figure 6—figure supplement 4). These results together show that MacCer positively regulates Wg level, but not vice versa, at NMJs.

Wg coordinates pre- and postsynaptic signaling cascades that modulate synapse development. The presynaptic Wg signaling pathway promotes formation of Futsch-associated microtubule loops without transcriptional regulation (Korkut and Budnik, 2009; Miech et al., 2008). Futsch is a microtubule-associated protein, and the formation of Futsch-positive microtubule loops is essential for synaptic growth (Roos et al., 2000). In wg and arr mutants with impaired Wg signaling, the number of Futsch loops is reduced while the percentage of boutons containing unbundled Futsch is increased (Miech et al., 2008; Packard et al., 2002). We observed similar Futsch staining phenotype in brn-overexpression larvae, egfiny mutants and egfiny/++; wg+/+ transheterozygotes, i.e., a
Figure 5. MacCer is required for the NMJ growth-promoting effect of Wg signaling. (A–J) Representative images of NMJ4 co-stained with anti-HRP (green) and anti-CSP (magenta) in egh62d18+/+ (control, (A), UAS-Wg-HA/+; nSyb-Gal4/+ (B), egh62d18+/+; UAS-Wg-HA/+; nSyb-Gal4/+ (C), egh62d18+/+; UAS-Wg-HA/+; nSyb-Gal4/+ (D), egh62d18+/+; UAS-Wg-HA/+; nSyb-Gal4/+ (E), wg1/+/ (F), wg1/cx4 (G), nSyb-Gal4/UAS-Egh (H), wg1/cx4, UAS-Egh/wg1/+/ (I), and egh62d18+/+; wg1/+/ (J). Scale bar: 10 μm. (K) Quantifications of bouton number and bouton size of NMJs in different genotypes. ‘CT’ denotes corresponding control.

Figure 5 continued on next page.
significant increase in the percentage of boutons containing unbundled and punctate Futsch signals, especially in large boutons (Figure 6, J–M and Figure 6—figure supplement 2), indicating that MacCer and Wg cooperate in controlling the formation of Futsch loops. Together, these results support that MacCer facilitates presynaptic Wg signaling during NMJ development.

MacCer is not required for postsynaptic differentiation

Distinct from the presynaptic Wg pathway, Wg regulates postsynaptic differentiation and bouton formation by activating the Fz2 nuclear import pathway in postsynaptic muscles (Ataman et al., 2006; Mathew et al., 2005; Mosca and Schwarz, 2010); defects in this pathway lead to a reduced number of boutons, altered distribution of the postsynaptic type A glutamate receptor (GluRIIA), a decrease of subsynaptic reticulum (SSR) with increase of ‘ghost bouton’ that lacks of Discs large (DLG) postsynaptic staining, and enlarged pockets in SSR juxtaposed to active zones (Mosca and Schwarz, 2010; Packard et al., 2002). However, we did not find these abnormalities in egh62d18 mutants except that the GluRIIA intensity was significantly but slightly increased (Figure 7, A, B and E); rather, the ghost bouton number, the GluRIIA cluster size, as well as the SSR ultrastructure were largely normal (Figure 7, C–I), suggesting that the postsynaptic Wg signaling remains unaffected upon MacCer reduction.

Wg contains a functional MacCer-binding domain

The results shown above suggest that MacCer may interact with Wg and thus modulate its signaling activity. Previous in vitro studies show that GSLs are able to specifically bind proteins containing a structurally conserved GSL-binding motif (GBM), which contains both basic (Lys, Arg) and turn-inducing (Gly, Ser) residues (Fantini and Yahi, 2015; Hamel et al., 2010). By in silico analysis, we identified a potential GBM of Wg between amino acid residues 233 and 247, therein Lys and Met are required for the interaction with MacCer (Figure 8A). Interestingly, this region contains a palmitoylation site at Ser-239 by a palmitoleate (C16:1; Figure 8A and C) (Herr and Basler, 2012; Kakugawa et al., 2015; Takada et al., 2006), which is known to affect the association of membrane proteins to lipid rafts (Resh, 2004). Structure homology modeling and a series of molecular dynamics simulations showed that this acylated GBM is fully exposed on the protein surface and perfectly fit with MacCer. A remarkable feature of the molecular complex is that the ceramide part of MacCer interacts with the acyl chain of the GBM, whereas the sugars of MacCer bind the peptidic part of the GBM (Figure 8B and C).

We then analyzed the interaction of a synthetic peptide derived from the predicted GBM of Wg with monolayers of purified GSLs by Langmuir microtensiometer experiments (Di Scala et al., 2014). The amino acid sequence of the peptide contains four Cys residues with several possibilities of forming two disulfide bridges may not fully stable under our experimental conditions. Thus we designed a synthetic peptide in which the Cys residues were replaced by isosteric Ser residues (referred to as Wg233-247). This Cys/Ser substitution respects the electrostatic distribution of partial charges at the surface of the peptide. The Wg233-247 peptide showed high affinity with LacCer (Figure 8—figure supplement 1).
Figure 6. MacCer is required for the local presynaptic Wg signaling at NMJs. (A–D) Confocal images of single slice of NMJ4 boutons triple-labeled with anti-MacCer (red), anti-HRP (blue) and anti-Wg (green; A–B) or anti-Rab11 (green; C–D) in wild type and egh^{62d18} mutants. MacCer puncta showed substantial colocalization (arrowheads) with Wg and Rab11 in boutons. Arrows indicate Rab11-positive puncta without MacCer staining in the egh^{62d18} mutants. Images were processed by deconvolution. Scale bar: 2 μm. (E) Pearson’s coefficients of colocalization between MacCer and indicated proteins. n = 51 and 44 boutons (from ten wild-type larvae each) for colocalization of MacCer with Wg and Rab11, respectively. (F–H) Representative images of NMJ4 co-labeled with anti-Wg (green) and anti-HRP (magenta) in wild type (F), egh^{62d18} mutants (G) and UAS-Egh/+; nSyb-Gal4/+ (H). Scale bar: 5 μm. (I) Quantification of intensities of endogenous Wg normalized to HRP intensities in different genotypes. n ≥ 15 larvae; ns, no significance; **p<0.01 and ***p<0.001 by student’s t test; error bars: s.e.m. (J–L) Representative images of NMJ6/7 labeled with anti-Futsch (green) and anti-HRP (magenta). Scale bar: 10 μm.
We further analyzed the interaction of Wg\textsuperscript{233-247} with GSLs extracted from Drosophila larvae of different genotypes. In agreement with previous findings (Hamel et al., 2010; Wandall et al., 2005), wild-type larvae expressed a broad range of GSLs including tetra- and penta-hexosylceramides, while egh\textsuperscript{62d18} mutants lacked MacCer and brn\textsuperscript{1.6P6} mutants contained almost exclusively MacCer (Figure 8—figure supplement 1). As expected, Wg\textsuperscript{233-247} showed strong interaction with MacCer-enriched GSLs from brn mutants but no interaction with MacCer-deficient GSLs from egh mutants (Figure 8D). In contrast, a GBM mutant containing mutations at key Lys and Met (K234E and M238R; Figure 8A) showed no interaction to GSLs from brn mutants (Figure 8—figure supplement 1). Moreover, we examined whether full-length Wg binds to MacCer-enriched GSLs. Myc-tagged full-length Wg was produced by an in vitro translation kit containing rabbit reticulocyte lysates and purified by anti-Myc immunoprecipitation. Wild-typed Wg showed high affinity to GSLs from brn but not egh mutants; in contrast, Wg with GBM deleted or mutated showed no affinity to GSLs from brn mutants (Figure 8E and F).

We further validated the GSL binding ability of Wg by pull-down assay. Wild-type Myc-Wg but not Wg with mutated GBM was substantially pulled down by LacCer-coated beads (Figure 8G). Taken together, these data support that Wg has an intrinsic affinity for MacCer/LacCer via GBM.

**Wg GBM is critical for its colocalization with MacCer and normal NMJ growth**

To further test the physiological role of the Wg GBM, we generated double site-directed point mutations of K234E and M238R in Wg (Figure 8A) by CRISPR/Cas9-mediated mutagenesis. This double point-mutation wg allele was named \( \text{wg}_{\text{GBM}} \). Homozygous \( \text{wg}_{\text{GBM}} \) or in combination with a null allele \( \text{wg}_{\text{CX4}} \) (\( \text{wg}_{\text{GBM}}/\text{wg}_{\text{CX4}} \)) led to larval lethality before 3rd instar stage. However, \( \text{wg}_{\text{GBM}} \) in combination with a hypomorphic allele \( \text{wg}^\prime \) (\( \text{wg}^\prime/\text{wg}_{\text{GBM}} \)) resulted in escapers survived to adults. The Wg protein level in \( \text{wg}^\prime/\text{wg}_{\text{GBM}} \) mutants was largely normal in whole larvae by western analysis and at NMJs by immunostaining compared with \( \text{wg}^\prime \) heterozygotes (\( \text{wg}^\prime/\text{wg}^\prime \)) (Figure 9, A–D). MacCer level was not altered in \( \text{wg}^\prime/\text{wg}_{\text{GBM}} \) mutants compared with that of \( \text{wg}^\prime/\text{wg}^\prime \) heterozygous control (Figure 9, A–C). However, the colocalization between Wg and MacCer was significantly decreased in \( \text{wg}^\prime/\text{wg}_{\text{GBM}} \) mutants compared with that of \( \text{wg}^\prime/\text{wg}^\prime \) heterozygous and wild-type control (Figure 9, E–H), suggesting that \( \text{wg}_{\text{GBM}} \) mutation, presumably a null allele, affect proper localization of Wg in synaptic boutons.

To determine if Wg GBM interacts with MacCer in regulating Wg signaling and bouton formation, we examined the genetic interaction between \( \text{wg}_{\text{GBM}} \) and egh mutation. We found that \( \text{wg}_{\text{GBM}}/\text{wg}^\prime \) mutants and egh\textsuperscript{62d18}/+; \( \text{wg}_{\text{GBM}}/+ \) transheterozygotes showed fewer and larger boutons, and the
Figure 7. Postsynaptic differentiation is normal in egh mutants. (A and B) Representative images of NMJ4 from wild type and egh\(^{62d18}\) mutants co-stained with anti-HRP (magenta) and anti-GluRIIA (green). Scale bar: 2 μm. (C and D) Images of NMJ6/7 from wild type and egh\(^{62d18}\) mutants co-stained with anti-HRP (magenta) and anti-DLG (green). An arrowhead points at a ghost bouton. Scale bar: 20 μm. (E and F) Quantification of normalized intensities and area of GluRIIA against anti-HRP staining of NMJ4 (E) and ghost bouton number of NMJ6/7 (F) in wild type and egh\(^{62d18}\) mutants. ns, no significance, *p<0.05 by student’s t test; n ≥ 12 larvae; error bars: s.e.m. (G and H) Ultrastructure images of NMJ6/7 boutons from wild type and egh\(^{62d18}\) mutants. The subsynaptic reticulum (SSR; green) is a folded membrane network that surrounds the presynaptic bouton (b). The SSR region juxtaposed to the active zone (AZ) is indicated by asterisks in red. Scale bar: 1 μm. (I) Quantification of various bouton parameters of NMJ 6/7 synapses Figure 7 continued on next page

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phenotype in \( w^{\text{GBM}}/w^{1} \) mutants was rescued by neuronal expression of Wg but not by expression of Egh (Figure 9I–N). The NMJ phenotype of \( w^{\text{GBM}}/w^{1} \) was insensitive to egh overexpression (Figure 9L and N), supporting that wg is epistatic to egh. We also observed significantly fewer boutons with Futsch loops and more boutons with unbundled Futsch staining in both \( w^{\text{GBM}}/w^{1} \) mutants and \( \text{egh}^{62a18}/+; w^{\text{GBM}}/+ \) transheterozygotes (Figure 9O-R). Together, these findings suggest that the intact Wg GBM is critical for proper Wg localization, Wg signaling and thus bouton formation.

**Discussion**
In the present study, based on genetic analysis of cell-type specific manipulations of Egh, Brn and Wg expression, together with immunochemical, genetic interaction and lipid-protein interaction analysis, we demonstrate that MacCer promotes NMJ bouton formation by interacting with Wg, thereby facilitating presynaptic Wg signaling. This is the first report defining a critical role of a specific class of sphingolipids in synapse development.

**A crucial role for sphingolipids in NMJ synapse development**
Sphingolipids are essential components of lipid rafts which regulate multiple signaling pathways and neural functions. The present study provides compelling evidence implicating sphingolipids in regulating NMJ growth (Figure 1 and Figure 2; Supplementary file 1). Previous studies revealed that various mutations in either egh or brn result in similar ovarian defects and neuronal hypertrophy during embryogenesis (Goode et al., 1996). The similarity of egh and brn mutant phenotypes suggests that both are caused by a lack of complex GSLs downstream of brn. A more recent study showed that egh mutations result in overgrowth of subperineurial glia through elevated phosphatidylinositol-3 kinase (PI3K) signaling; the glial phenotype is due to the accumulation of GlcCer, the substrate of Egh (Dahlgaard et al., 2012). At NMJs, however, our data demonstrate that the NMJ defects in egh mutants are caused by lack of MacCer.

Do other classes of sphingolipids besides MacCer also regulate synapse development? Many sphingolipids, such as phosphorylated sphingosines and CerPE, the most abundant sphingolipid in lipid rafts, play a diverse array of functions in the nervous system (Fantini and Yahi, 2015; Yonamine et al., 2011). It will be intriguing to define the molecular mechanisms by which these structural and signaling sphingolipids regulate synapse development.

**MacCer promotes synaptic growth via presynaptic Wg signaling**
*Drosophila* NMJ development is mediated by multiple signaling cascades. How does MacCer function in synaptic growth? Consistent with previous findings that GSL/lipid rafts are enriched in recycling endosomes (Balasubramanian et al., 2007; Gagescu et al., 2000; Hortsch et al., 2010), we observed a substantial colocalization between MacCer and recycling endosomal marker Rab11 at NMJ synapses (Figure 6). We note, however, the impact of MacCer and Rab11 on NMJ growth is opposite. The NMJ bouton formation is positively regulated by MacCer but negatively regulated by Rab11 (Huang et al., 2016; Khodosh et al., 2006; Liu et al., 2014; this study). The opposite effect on NMJ growth of the two might be caused by the fact that MacCer and Rab11 regulate different signaling pathways. For example, Rab11 inhibits BMP signaling (Huang et al., 2016; Liu et al., 2014) while MacCer facilitates Wg signaling (this study) at NMJ terminals.

Our results showed that MacCer facilitates the presynaptic Wg signaling in promoting NMJ growth (Figure 5). In contrast to the abnormal microtubule cytoskeleton (Figure 6), egh mutants...
Figure 8. Wg has a functional MacCer-binding domain (A) Schematic representation of Wg protein and amino acid sequence of a putative GSL-binding motif (GBM) and mutated GBM with asterisks denoting the mutated amino acids. (B) A 3D model of Wg residues 85–277 with the location of the GBM, which exposes on the protein surface. (C) Energy-minimized model of the acylated (C16:1ω7) GBM in Wg binding with MacCer. (D and E) Surface pressure change (ΔT) by the addition of Wg233-247 peptide (GBM peptide; D) and full-length Wg produced by in vitro translation system (E) on a Figure 8 continued on next page
monolayer of GSLs purified from wild type, egh^{241Ab} and brn^{16Pr} larvae. GBM peptide and full-length Wg showed specific high affinity to MacCer enriched GSLs. (F) Surface pressure change (dσ) by the addition of Myc-tagged wild-type Wg (Wg^{WT}) and Wg with GBM deleted (Wg^{GBM deleted}) or mutated (Wg^{GBM mutated}) on a monolayer of GSLs purified from brn^{16Pr} larvae. All surface pressure measurements were performed in triplicate and a representative curve is shown. (G) Wild-type Wg (Wg^{WT}) but not Wg with mutated GBM (Wg^{GBM mutated}) was pulled-down by LacCer-beads. Five biological repeats were carried out for the pull-down assay and a representative blot is shown. 

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The following figure supplement is available for figure 8:

Figure supplement 1. Additional monolayer data and GSL profiles of egh and brn mutants.
DOI: https://doi.org/10.7554/eLife.38183.025

Figure 8 continued

How does MacCer affect Wg signaling at the molecular and cellular levels? Wg associates with lipid rafts via its acylation (Zhai et al., 2004), enabling its access to the raft-component MacCer. Upon lipid raft disruption by sterol depletion, Wg de-localizes from the lipid rafts (detergent-resistant, cholesterol-rich membrane microdomains) revealed by biochemical fractionation (Zhai et al., 2004). We envisage that delocalization of Wg does not allow its binding to MacCer, compromises its transport to the NMJ terminals (Figure 5—figure supplement 1), and in turn restricts NMJ development (Figure 2—figure supplement 2).

The association of Wg with membrane rafts requires the acylation at Ser-239 mediated by Porcupine, a process conserved from Drosophila to vertebrates (Galli et al., 2007; Herr and Basler, 2012; Zhai et al., 2004). Importantly, the acylation by Porcupine is essential for the recognition of Wg by Evi thereby affecting the transport and secretion of Wg through recycling endosomes and exosomes (Herr and Basler, 2012; Koles et al., 2012; Korkut et al., 2009). Furthermore, both Porcupine and Evi are known to regulate the synaptic level of Wg and the signaling activity (Korkut et al., 2009; Packard et al., 2002). In the present study, we showed a reduction in Wg level at NMJ terminals but an increase in the neuropil of the ventral nerve cord of egh mutants (Figure 6 and Figure 6—figure supplement 2), similar to that in evi mutants with impaired Wg transport and secretion (Korkut et al., 2009). These findings suggest that the transport and trafficking of Wg via endosomes may be compromised when MacCer is deficient. The detailed mechanism by which MacCer regulates Wg transport and trafficking remains to be further investigated. As Wnt signaling, which is widely involved in various developmental processes and diseases including neurological and psychological diseases, is evolutionally conserved (Herr et al., 2012; Korkut and Budnik, 2009; Salinas, 2012), our finding that MacCer facilitates Wg signaling transduction suggests a new target for intervening Wnt signaling associated pathogenesis.
Figure 9. Wg GBM is required for Wg-MacCer colocalization and NMJ growth. (A and B) Representative images of NMJ4 co-labeled with anti-Wg (green), anti-MacCer (red) and anti-HRP (blue) in wg\(^{1/+}\) (A) and wg\(^{GBM/wg^{1}}\) mutants (B). (C) Quantification of Wg and MacCer intensities normalized to HRP intensities in different genotypes. \(n > 15\) larvae; ns, no significance by one-way ANOVA with Tukey post hoc tests; error bars: s.e.m. (D) Western results of Wg and Actin from 3rd instar larvae of wg\(^{1/+}\) and wg\(^{GBM/wg^{1}}\) mutants. Actin was used as a loading control. Western blots were performed in triplicate and a representative image is shown. (E-G) Representative images of NMJ4 boutons triple-labeled with anti-Wg (green), anti-MacCer (red) and anti-HRP (blue) in wild type (E), wg\(^{1/+}\) (F) and wg\(^{GBM/wg^{1}}\) mutants (G). Arrowheads indicate puncta positive for both MacCer and Wg signals; arrows indicate puncta without obvious overlap of MacCer and Wg. Images were processed by deconvolution. Scale bar: 2 μm. Plot profiles of relative intensity of MacCer and Wg along the dashed lines were shown. (H) Pearson’s coefficients of colocalization between MacCer and Wg. \(n = 58/10\), 57/8, 57/8, 57/8. Figure 9 continued on next page.
and 46/8 (from left to right, boutons/animals). ns, no significance, **p<0.001 by one-way ANOVA with Tukey post hoc tests; error bars: s.e.m. (l–M) Representative images of NMJ co-stained with anti-HRP (green) and anti-CSP (magenta) in wz<sub>GBM</sub>+ (I), wz<sub>GBM</sub>/wz<sub>1</sub> (J), UAS-Wg-HA, wz<sub>GBM</sub>/wz<sub>1</sub>; nSyb-Gal4/+ (K), UAS-Egh, wz<sub>GBM</sub>/wz<sub>1</sub>; nSyb-Gal4/+ (L), and egh<sub>62d18</sub>/+; wz<sub>GBM</sub>/+ (M). Scale bar: 10 μm. (N) Quantifications of bouton number and bouton size of NMJs in different genotypes. n ≥ 12 larvae; ns, no significance, **p<0.01; ***p<0.001 by one-way ANOVA with Tukey post hoc tests, error bars: s.e.m. (O–Q) Representative images of NMJ6/7 labeled with anti-Futsch (green) and anti-HRP (magenta) in wild type (O), wz<sub>GBM</sub>/wz<sub>1</sub> (P), and egh<sub>62d18</sub>/+; wz<sub>GBM</sub>/+ transheterozygotes (Q). Insets show higher magnification images of Futsch staining of a single bouton (asterisk). Arrows indicate boutons displaying unbundled Futsch; arrowheads denote boutons with Futsch loops. Scale bar: 10 μm. (R) Quantifications of percentage of Futsch loops and unbundled Futsch staining in different genotypes. n ≥ 12 larvae; ns, no significance, **p<0.01 by one-way ANOVA with Tukey post hoc tests, error bars: s.e.m. (S) A schematic presentation of the role for MacCer in promoting synapse growth via interacting with Wg in lipid rafts. The presynaptic Wg signaling is denoted by a curved arrow.

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The following source data is available for figure 9:

**Source data 1.** Numerical data for the statistical graphs.

DOI: https://doi.org/10.7554/eLife.38183.028

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### Materials and methods

#### Key resources table

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**Drosophila strains and genetics**

Flies were cultured on standard cornmeal media at 25°C. w^{1118} was used as the wild type control, unless otherwise indicated. egli^{626A18}, brn^{1.6P6}, UAS-Egh-Myc (Wandall et al., 2005) and UAS-Brn (Chen et al., 2007) were provided by S. M. Cohen and S. Pizette. evi^{2} was from V. Budnik (Korkut et al., 2009). UAS-ManII-GFP was from Y. N. Jan (Ye et al., 2007). UAS-Spin-GFP was from G. Davis (Sweeney and Davis, 2002). The following fly lines were obtained from the Bloomington Stock Center: Df(2L)Exel7063, Df(3L)BSC671, Sk^{2KGOS894}, lace^{2}, lace^{65305}, schlank^{G0489}, schlank^{G0611}, egh^{7}, brn^{107}, arr^{2}, arr^{408131}, wg^{CX4 (wg^{l177}), wg^{1}, rab11^{93B6}, nSyb-Gal4, OK6-Gal4, CS7-Gal4, Repo-Gal4, UAS-Wg-HA, UAS-brn-RNAi, UAS-YFP-Rab5 and act-Cas9. For fly strains used in the genetic screen, UAS-GalNAc-TA and UAS-GalNAc-TB were provided by S. M. Cohen (Chen et al., 2007). desat1^{1GA}, desat1^{114A}, desat1^{1114A} and desat1^{1197679} were provided by E. Hafen (Köhler et al., 2009). Other fly lines were obtained from the Bloomington Stock Center, Kyoto Stock Center and the Tsinghua Drosophila RNAi Center.

**Site-directed mutation in wg**

We generated double site-directed point mutations at K234E and M238R in Wg to test the function of GBM. The CRISPR/Cas9-mediated targeted mutagenesis of wg^{1118} was performed largely according to previously published homology-directed repair procedures (Gratz et al., 2014; Port et al., 2014) at Fungene Biotech (http://www.fgbiotech.com). sgRNA (synthetic guide RNA) targets were designed with CRISPR Optimal Target Finder (Gratz et al., 2014). Two sgRNAs recognized the region containing the mutations (gcgacaggagtgcaaatgccaa, the last three bases ngg denotes proto-spacer adjacent motif) were cloned into U6b-sgRNA vector (Ren et al., 2013). For donor vector construction, a 1.8 kb wg gene region containing the site-directed mutation was cloned into pBluescript SK (-) vector. The donor vector with the site-directed mutation and the sgRNA vector were injected into act-Cas9 transgenic embryos. PCR sequencing was performed to identify if the offspring flies carried the designed mutation.

**Immunohistochemical analysis**

Immunochemistry of larval preparations was performed as previously described (Liu et al., 2014). For most antibody staining, specimens were dissected in Ca^{2+}-free HL3 saline, fixed in 4% paraformaldehyde for 30 min, and washed in 0.2% Triton X-100 in PBS. For MacCer staining, specimens were fixed in 4% paraformaldehyde at 4°C for 60 min and permeabilized with 0.1% Triton X-100 in cold PBS, incubated with undiluted anti-MacCer hybridoma supernatants for 18 hr at 4°C and detected with Fluor 568 conjugated goat anti-mouse IgM (1:1000; Invitrogen). For GluRIIA staining, specimens were fixed in cold methanol for 5 min. We used the following antibodies: mouse anti-cysteine string protein (CSP) (1:300; 6D6 from DSHB), mouse anti-Wg (1:10; 4D4 from DSHB), mouse anti-Futsch (1:50; 22C10 from DSHB), mouse anti-Rab11 (1:50; BD Biosciences), mouse anti-DLG (1:500; 4F3 from DSHB), mouse anti-GluRIIA (1:200; 8B4D2 from DSHB), mouse anti-Fas II (1:50; 1D4 from DSHB), mouse anti-Syx1A (1:20; 8C3 from DSHB), mouse anti-dFz2 (1:5; 12A7 from DSHB), mouse anti-HA (1:1000; MBL International), mouse anti-Myc (1:300; CWBIO, MBL International), rabbit anti-GFP (1:200; MBL International), and fluorescence-conjugated anti-horseradish peroxidase (HRP) (1:200; Jackson ImmunoResearch). Primary antibodies were visualized using corresponding secondary antibodies conjugated to Fluor 488, Cy3 (both at 1:1000; Invitrogen) or DyLight 649 (1:500; Jackson ImmunoResearch).
Western analysis was performed as previously described (Liu et al., 2014). Briefly, 3rd instar larval brains were dissected in cold PBS and homogenized in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40) with protease inhibitors Set I (Calbiochemistry) on ice. Samples were then subjected to SDS-PAGE and immunoblotting according to standard procedures. The following antibodies were used: mouse anti-Wg (1:50; 4D4 from DSHB), mouse anti-Syx1A (1:1000; 8C3 from DSHB), and mouse anti-actin (1:50000; Millipore Bioscience Research Reagents). All Western blotting were performed at least for three independent biological replicates.

**Image collecting and statistical analysis**

Image collecting was carried out as previously described (Liu et al., 2014). NMJ images were collected with an Olympus Fluoview FV1000 confocal microscope using a 40x/1.42 NA or 60 x/1.42 NA oil objective and FV10-ASW software, or with a Leica confocal microscope using a 40x/1.25 NA oil objective and LAS AF software. All images of muscle 4 or muscle 6/7 glutamatergic type Ib NMJ of abdominal segments A2 to A4 for a specific experiment were captured using identical settings for statistical comparison among different genotypes. High resolution confocal images were processed with the deconvolution software AutoQuant X2 (Media Cybernetics). Brightness, contrast, and color were adjusted using Photoshop CS5 (Adobe). NMJ morphological features were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

For quantification analysis of NMJ growth, individual boutons were defined according to the discrete staining signal of anti-CSP. Satellite boutons were defined as extensions of five or fewer small boutons emanating from the main branch of the NMJ terminals. For quantification of bouton sizes, synaptic areas (μm²) were measured by assessing HRP-positive boutons, and normalized to bouton numbers. Ghost boutons were defined as boutons positive for HRP staining but negative for DLG staining. NMJ bouton number and size of schlank mutants were normalized to muscle surface area. For quantification of protein levels at NMJ, staining intensities of each protein were measured within HRP-positive NMJs using ImageJ. For colocalization analysis, single slices were analyzed using WCIF-ImageJ ‘Colocalization Analysis’ within HRP-labeled synaptic boutons. Statistical comparisons were performed using GraphPad Prism 6. Data of NMJ features are expressed as mean ± standard error of the mean (s.e.m.). Statistical significance between each genotype and the controls was determined by two tailed Student’s t test, whereas multiple comparisons between genotypes were determined by one-way ANOVA with a Tukey post hoc test. Asterisks above a column indicate comparisons of a specific genotype to wild type (denoted WT) or genetic control (denoted CT), whereas asterisks above a bracket denote comparisons between two specific genotypes. ns denotes p>0.05; * indicates p<0.05; ** denotes p<0.01; *** indicates p<0.001.

**Drug treatment of larvae**

Larvae of different genotypes were raised on vehicle- or drug-containing media from egg hatching. D,L-threo-PDMP (Matreya) and filipin III (Cayman) were dissolved in DMSO and then individually added to standard media at specific concentrations. All the treatments used DMSO vehicle at a concentration of 0.5%. MβCD (Sigma-Aldrich) was resolved in standard media at a final concentration of 20 mM.

**Molecular simulation**

Docking of Wg onto MacCer was performed with the Hyperchem 8.0 program as described previously (Fantini and Yahi, 2011). Homology modeling of Wg fragment of 85 – 277 amino acids was based on the published crystal structure of XWnt8 (Janda et al., 2012).

**Lipid–protein interaction assay**

Synthetic peptides of GBM (95%) were purchased from Sangon Biotech. Myc-tagged full-length Wg were produced by an in vitro translation kit containing rabbit reticulocyte lysates (Promega). pCDNA-Myc-Wg was expressed in the translation system at 30°C for 90 min. Myc-Wg was immunoprecipitated with anti-Myc affinity resin (Millipore), eluted with a Myc-peptide solution (Sigma), and concentrated using a 30kD filter column (Amicon). GSL–protein interactions were determined with the Langmuir-film balance technique as previously described (Di Scala et al., 2014). It was monitored by surface pressure with a fully automated...
microtensiometer (μTrough SX; Kibron Inc.). All experiments were performed at 20 ± 1°C. Monomolecular films of GSLs were spread on pure water subphases (800 μl) from chloroform/methanol (1:1 vol/vol). The initial surface pressure of the monolayers was 12–15 mN/m. The accuracy of our experimental conditions was ±0.25 mN/m. Real-time measurements kinetically followed the increase in the surface pressure after injecting the peptide or protein (final concentration of 10 μM) into the aqueous phase underneath the GSL monolayer until equilibrium was reached. The data were analyzed with the FilmWareX program (version 3.57; Kibron Inc.).

Lipid–protein pull-down assay was modified following a previous protocol (Kunisaki et al., 2006). Wild-type and mutated Myc-Wg were produced by an in vitro translation kit containing rabbit reticulocyte lysates (Promega). The reticulocyte lysates were suspended in 400 μL lipid-binding/washing buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.35% NP-40) and incubated with 20 μL slurry of control or LacCer-coated beads (Echelon) at 4°C for 3 hr under rotary agitation. After extensive washing with lipid-binding/washing buffer, the bound proteins were eluted from beads with 2× Laemmli sample buffer and subjected to Western analysis with mouse anti-Myc (1:1000; CWBIO).

GSL extraction and analysis
GSL extraction was performed as described (Hamel et al., 2010; Wandall et al., 2005). 3rd instar larvae were homogenized in 1.5 ml solvent A (2-isopropanol/hexane/water; 55:25:20 vol/vol/vol). The homogenate was centrifuged at 2,000 rpm and the supernatant was collected. This step was repeated with 1.5 ml solvent B (chloroform/methanol; 1:1 vol/vol), 1.5 ml solvent A, and finally 1.5 ml solvent B. The four solvent extracts were combined, evaporated under a nitrogen flux, and re-suspended in chloroform/methanol (2:1 vol/vol) at a lipid concentration of 1 mg/ml. The extracts were again evaporated, then re-suspended in 5 ml methanol containing 0.1 M NaOH, and incubated for 1 hr at 37°C under agitation to remove most glycerolipids. The samples were evaporated and re-extracted in chloroform/methanol (2:1 vol/vol). Neutral GSLs were finally purified on a column (DEAE-Sephadex A-25; Sigma-Aldrich) and eluted with chloroform/methanol/water (30:60:8 vol/vol/vol). GSL extracts were analyzed by high performance thin layer chromatography (HPTLC) using silica gel 60 HPTLC plates (Merck) in chloroform/methanol/water (60:35:8 vol/vol/vol). The HPTLC plates were sprayed with orcinol and heated at 110°C for GSL detection. GSLs bands were identified by their mobility relative to GSL standards (Matreya).

Electron microscopy
Electron microscopy (EM) was performed as described (Liu et al., 2014). Briefly, dissected 3rd instar larvae were fixed overnight at 4°C in 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), and post-fixed in 1% OsO4 in cacodylate buffer for 90 min at room temperature. Samples were stained in saturated aqueous uranyl acetate for 1 hr, dehydrated in a graded acetone series and embedded in Spurr resin (Electron Microscopy Sciences). Type 1b boutons from NMJ 6/7 in abdominal segments A2 to A4 were serially sectioned with a Leica UC6 ultramicrotome, stained with uranyl acetate and Sato’s lead, and observed using a JEOL 1400 electron microscope. The number of synaptic vesicles within a 200 nm radius of the active zones (AZs) in egh mutants and wild type was quantified. Other features were quantified as previously described (Packard et al., 2002).

Acknowledgements
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Additional information

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Author contributions

Yan Huang, Conceptualization, Funding acquisition, Investigation, Writing—original draft, Project administration, Writing—review and editing; Sheng Huang, Coralie Di Scala, Qifu Wang, Investigation; Hans H Wandall, Resources; Jacques Fantini, Supervision, Investigation, Writing—original draft; Yong Q Zhang, Conceptualization, Supervision, Funding acquisition, Validation, Writing—review and editing

Author ORCIDs

Yan Huang [http://orcid.org/0000-0003-3833-9248](http://orcid.org/0000-0003-3833-9248)
Coralie Di Scala [http://orcid.org/0000-0003-0655-7056](http://orcid.org/0000-0003-0655-7056)
Hans H Wandall [http://orcid.org/0000-0003-0240-9232](http://orcid.org/0000-0003-0240-9232)
Jacques Fantini [http://orcid.org/0000-0001-8653-5521](http://orcid.org/0000-0001-8653-5521)
Yong Q Zhang [https://orcid.org/0000-0003-0581-4882](https://orcid.org/0000-0003-0581-4882)

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Additional files

Supplementary files
- Supplementary File 1. Genetic screen for NMJ morphology by manipulating selected genes involved in synthesis and turnover of membrane lipids.
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All data generated or analysed during this study are included in the manuscript and supporting files.

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