Perlecan regulates bidirectional Wnt signaling at the Drosophila neuromuscular junction

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Abstract

Heparan sulfate proteoglycans (HSPGs) play pivotal roles in the regulation of Wnt signaling activity in several tissues. At the Drosophila melanogaster neuromuscular junction (NMJ), Wnt/Wingless (Wg) regulates the formation of both pre- and postsynaptic structures; however, the mechanism balancing such bidirectional signaling remains elusive. In this paper, we demonstrate that mutations in the gene of a secreted HSPG, perlecan/trol, resulted in diverse postsynaptic defects and overproduction of synaptic boutons at NMJ.

Introduction

Highly coordinated structuring of the pre- and postsynaptic apparatus is essential for synaptic development and plasticity. Trans-synaptic communications mediated by secreted and membrane-bound proteins, such as agrin, Wnt, and neuregulin, play pivotal roles in such coordination (Packard et al., 2003; Dean and Dresbach, 2006; Singhal and Martin, 2011). Among these, Wnt signalings have been implicated in the formation and plasticity of synaptic connections in the central nervous system (CNS) and neuromuscular junction (NMJ) of both vertebrates and invertebrates (Salinas and Zou, 2008; Budnik and Salinas, 2011). Recent genetic studies on Drosophila melanogaster began to elucidate the molecular mechanism of Wnt signaling in developing larval NMJs, a widely used model of glutamatergic synapse that shows many similarities to the excitatory synapses in mammalian CNS. At Drosophila NMJ, Wnt/Wingless (Wg) is secreted from the motor axon terminals and binds to the receptor Drosophila Frizzled-2 (DFz2), which is localized on both pre- and postsynaptic membranes (Packard et al., 2002). Wg presynaptically activates the local canonical pathway involving Dishevelled (Dsh) and Shaggy (Sgg), a Drosophila homologue of glycogen synthase kinase 3β, and promotes the proliferation of synaptic boutons (Miech et al., 2008). On the other hand, in the postsynaptic muscle fibers, Wg activates the Frizzled nuclear import (FNI) pathway, in which Wg induces the cleavage of the C terminus of DFz2 (DFz2-C). The DFz2-C is then transported into muscle nuclei and promotes postsynaptic differentiation (Mathew et al., 2005; Mosca and Schwarz, 2010). Thus, Wg signaling has bidirectional activity coordinating the development of pre- and postsynaptic apparatus during rapid growth of larval muscle fibers.

Complicated machinery is required to achieve such bidirectional signaling because Wg is highly hydrophobic owing to palmitoyl modifications. Recently, it was revealed that Wg is released from the presynaptic terminals by exosome-like vesicles containing the Wnt-binding protein Evenness interrupted (Evi; Korkut et al., 2009). Evi plays critical roles both in the presynaptic secretion of Wg via exosomes and in the postsynaptic trafficking of DFz2 to the muscle nuclei. However, little is known about the mechanism by which Wg proteins are properly

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Abbreviations used in this paper: BMP, bone morphogenetic protein; BRP, Bruchpilot; CNS, central nervous system; DFz2, Drosophila Frizzled-2; Dlg, Discs large; Dip, Dolly-like glypican; Dsh, Dishevelled; dsRNA, double-stranded hairpin RNA; eEPJ, evoked excitatory junction potential; Evi, Evenness interrupted; FNI, Frizzled nuclear import; Gbb, Glass bottom boat; HS, heparan sulfate; HSPG, HS proteoglycan; KO, knockout; mEPJ, miniature excitatory junction potential; NMJ, neuromuscular junction; sfl, sulfatelcss; Sgg, Shaggy; SSR, subsynaptic reticulum; Trol, terribly reduced optic lobes; UAS, upstream activation sequence; Wg, Wingless.

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In particular, cell surface HSPGs are required to form the stable long-range gradient of Wnt/Wg, which specifies distinct cell fates in a concentration-dependent manner during morphogenesis of various organs (Lin, 2004; Gallet et al., 2008; Kikuchi et al., 2011). Thus, we hypothesized that HSPGs transported across the synaptic clefts and allocated to both pre- and postsynaptic membranes at the NMJ.

Previous studies showed that Wnt/Wg proteins bind to heparan sulfate (HS) proteoglycans (HSPGs) via HS chains, the interactions of which play critical roles in Wnt/Wg signaling.
modulate Wnt/Wg signaling by regulating Wnt/Wg distribution in the synapses. To test this possibility, we examined the Drosophila NMJs of various mutants for HSPG-related genes and found that mutations in *terribly reduced optic lobes* (*trol*), encoding a secreted HSPG, perlecan, resulted in the characteristic abnormalities in the NMJ that were reminiscent of the phenotypes of Wg pathway mutants. Here, we show that, in the *trol* mutants, the presynaptic canonical Wg pathway was enhanced, whereas the postsynaptic FNI pathway was suppressed, presumably by the disrupted transport of Wg in the NMJ synapses. Thus, we suggest that Trol is required to distribute Wg proteins precisely in developing NMJ synapses, balancing the pre- and postsynaptic activities of Wg signaling. To our knowledge, this is a first study to demonstrate that HSPG contributes to the short-range bidirectional signal control of Wnt at synapses.

## Results

### Trol is required for functional NMJ formation

In vertebrates, two HSPGs, agrin and perlecan, play pivotal roles in NMJ formation (Singhal and Martin, 2011). Although no obvious orthologues of agrin have been identified in *Drosophila*, there is a single fly perlecan gene named *trol* (Voigt et al., 2002). To clarify the Trol function, null and hypomorphic alleles of *trol* mutants were used, *trol*<sup>null</sup> and *trol*<sup>7</sup>, which lack the entire coding region and the C terminus, respectively (Voigt et al., 2002; Park et al., 2003). We examined the morphology of larval NMJs located on muscle 6/7 by immunohistochemistry with antibodies against HRP (presynaptic marker) and Discs large (DLG), a *Drosophila* homologue of the mammalian PSD-95 (postsynaptic marker). In wild-type, synaptic boutons increased in number during larval development concomitant with the increase in muscle size (Fig. 1, A, E, and F; Schuster et al., 1996). We first noticed that muscle size was reduced in *trol*<sup>null</sup> mutants (Fig. 1 E).

Growth defect of *trol* muscles was first detected at the second-instar larval stage and became pronounced at the third-instar stage, indicating that Trol is required for muscle growth. Furthermore, we observed that the subsets of synaptic boutons in *trol* mutants lacked postsynaptic DLG (Fig. 1, B, C, and G). These boutons are called “ghost boutons,” which have been identified in several Wg signaling mutants (Packard et al., 2002; Ataman et al., 2006; Mosca and Schwarz, 2010). The ghost boutons in *trol* mutants appeared as early as the first-instar larval stage. In addition to the postsynaptic defects, *trol* mutants showed overproduction of small synaptic boutons formed on the short branches that project from the main shaft of the NMJ (satellite boutons; Fig. 1, D and H). Although the muscles in *trol* mutants were remarkably small compared with those in wild-type animals, the total number of synaptic boutons in these mutants was comparable to that in the wild type owing to the presence of satellite boutons (Fig. 1 F).

Consistent with these morphological defects of the NMJ, the *trol*<sup>null</sup> mutant larvae showed abnormality in crawling behavior. We counted the number of peristaltic contractions...
Trol is localized at postsynaptic subsynaptic reticulum (SSR)

To examine where Trol functions at the larval NMJ, we used transgenic RNAi, a conditional knockdown system in *Drosophila* (Dietzl et al., 2007). We expressed double-stranded hairpin RNA (dsRNA) of *trol* specifically in muscle, neuron, and glia, respectively, using the upstream activation sequence (UAS)/Gal4 system. As shown in Fig. 3, muscle-specific expression of *trol* dsRNA (*24B>trol dsRNA*) reduced the muscle size and increased the numbers of satellite and ghost boutons, which were markedly similar to the *trol* mutant phenotype. On the other hand, neither neuronal (*elav>trol dsRNA*)- nor glial (*repo>trol dsRNA*)-specific *trol* RNAi affected the formation of both muscles and synaptic boutons (Fig. 3, A–C). Thus, it was suggested that muscle-derived Trol contributes to muscle growth and NMJ formation.

Next, to determine the subcellular localization of Trol at NMJ, we performed immunoelectron microscopic analyses (Fig. 3, D and E) using the FlyTrap transgenic fly line, ZCL1700, which expresses the Trol-GFP fusion protein under an endogenous promoter (Guha et al., 2009). We stained the *trol* FlyTrap line with the anti-GFP antibody using the preembedding nanogold silver intensification technique. Consistent with a previous study, immunogold signals were found to be localized at the basement membrane of various tissues, including muscle cells (Fig. 3 D, arrowheads; Friedrich et al., 2000). Notably, although the first-instar larvae of *trol* null mutants have normally sized muscles, these animals showed a significantly reduced number of contractions per minute. The locomotion defects of *trol* null animals were more obvious at the third-instar larval stage, at which point they stayed at the same position during our assay period without muscle contraction.

We next analyzed the electrophysiological properties of muscle 6 of *trol* null mutant larvae (Fig. 2, B–E). In the *trol* null mutants, the frequency of spontaneous miniature excitatory junction potentials (mEJPs) was remarkably decreased (Fig. 2, B and C), suggesting reduction of the probability of spontaneous vesicle release in the motor neuron axon terminals and/or the dysfunction of postsynaptic glutamate receptors. In addition, amplitudes of the mEJP and the evoked excitatory junction potential (eEJP) induced by nerve stimulation were also decreased (Fig. 2, B, D, and E). The quantal contents, the numbers of synaptic vesicles released with each stimulus, were not significantly different between wild-type and *trol* null animals (28.2 ± 6.4 compared with 37.2 ± 3.1 for wild type; P = 0.16), suggesting that the presynaptic neurotransmitter release machinery was not severely disrupted. These results suggested that postsynaptic responses were decreased in the NMJ of *trol* null mutant larvae, leading to their locomotion defects. However, we cannot exclude the possibility that the locomotion defects were also caused by abnormalities in the CNS of the *trol* null mutant.

![Figure 3. Trol is localized at postsynaptic SSR and functions postsynaptically.](image-url)

(A–C) Area of muscle 6/7 (A) and the numbers of ghost (B) and satellite boutons (C) in the NMJs of *24B>trol dsRNA, elav>trol dsRNA*, and *repo>trol dsRNA* animals, in which Trol was knocked down specifically in muscle, neuron, and glia, respectively, were quantified. Muscle-specific *trol* knockdown caused reduction of muscle area and increase of ghost and satellite boutons. Error bars represent SEM (*n* = 20; *, *P* < 0.01 and **, *P* < 0.001 relative to wild type). (D) and (E) Immunoelectron microscopy was performed for the synaptic bouton regions of the *trol* FlyTrap line. Localization of Trol was analyzed by immunostaining with the anti-GFP antibody. Arrows indicate Trol signals in the SSR. (E) White arrowheads indicate the localization of Trol signals at the perisynaptic region between pre- and postsynaptic membranes. (D) Trol signals were also detected on basement membranes (black arrowheads). Bars: (D) 1 µm; (E) 300 nm. B, synaptic bouton.

while larvae crawled on an agar plate (Fig. 2 A). Notably, although the first-instar larvae of *trol* null mutants have normalized muscles, these animals showed a significantly reduced number of contractions per minute. The locomotion defects of *trol* null animals were more obvious at the third-instar larval stage, at which point they stayed at the same position during our assay period without muscle contraction.
with the SSR, highly convoluted folds of the postsynaptic muscle plasma membranes (Fig. 3, D and E, arrows). The positive signals were also observed in the extracellular space between pre- and postsynaptic membranes (Fig. 3 E, arrowheads). Thus, it was suggested that Trol is secreted by the muscle cells and is accumulated mainly in the SSR membrane at the NMJ.

Both the core protein and HS moieties of Trol contribute to NMJ development

Perlecan regulates diverse signaling pathways by binding to many proteins through both the core protein and HS chains (Whitelock et al., 2008). So, we next tried to get information regarding the parts of Trol that contribute to NMJ formation. During biosynthesis of HS, the enzyme encoded by sulfateless (sfl), a homologue of mammalian glucosaminyl N-deacetylase-N-sulfotransferase, initiates the polysaccharide modifications by generating N-sulfate groups (Maeda et al., 2011). Because N-sulfation is a prerequisite for the subsequent HS modifications, defects in sfl lead to generation of undersulfated polysaccharides, which would show reduced affinity for most HS-binding proteins (Toyoda et al., 2000).

Thus, we knocked down sfl specifically in muscles and examined its effects on trol mutations. Muscle-specific expression of sfl dsRNA (24B>sfl dsRNA) in wild-type animals significantly reduced the muscle size, suggesting that HS plays important roles in muscle growth (Fig. 4 G). However, the same manipulation did not affect the muscle size of trol null mutants (trolnull; 24B>sfl dsRNA). This suggested that HS chains attached to the core proteins other than that of Trol play minor roles in muscle growth. Muscle-specific expression of the secreted form of Dally, a Drosophila glycosylphosphatidylinositol-anchored HSPG, glypican, did not rescue the muscle phenotype of trol null mutants (trolnull; 24B>sec-dally), supporting the importance of Trol core protein and its HS modifications (Fig. 4 G). The ghost bouton phenotype of trol null mutants was augmented by muscle-specific expression of sfl dsRNA, although the difference was not statistically significant (Fig. 4, E and H). This might be interpreted that HS chains on the core proteins other than that of Trol suppressed the ghost bouton phenotype of trol null mutants. We found consistently that muscle-specific expression of sec-dally rescued the ghost bouton phenotype of trol null mutants, suggesting that HS chains rather than specific core protein portions might be important for this phenotype (Fig. 4, F and H). On the other hand, neither sfl dsRNA nor sec-dally affected the satellite bouton phenotype of trol null mutants, suggesting that this phenotype is mainly dependent on the core protein portion of Trol (Fig. 4 I).

trol mutants show diverse postsynaptic defects

We analyzed the morphology of trol null NMJ using transmission electron microscopy (Fig. 5, A–I). As reported previously, the basement membrane of trol null mutant was closely apposed to the muscle cell surface compared with the wild type, but the overall structure of muscles appeared to be normal in this mutant (Fig. 5, A and B; Pastor-Pareja and Xu, 2011). However, mutations in trol caused severe structural defects in the NMJ. We found that the area of postsynaptic SSR was dramatically reduced in trol null mutants (Fig. 5, C, D, and G). The SSR of wild-type animals appeared as complex alternating layers of clear extracellular and opaque intracellular spaces (Fig. 5, C and E). In the trol null mutants, the SSR layers were densely packed, and the number of layers was also decreased, leading to the small SSR phenotype (Fig. 5, D and F). In contrast to the drastic changes in the postsynaptic structure, many parameters of presynaptic terminals, such as the density of synaptic vesicles (Fig. 5 I) and the number of mitochondria (0.12 ± 0.01/µm2 compared with 0.10 ± 0.02/µm2 for wild type; P = 0.46), were normal in the trol null mutants. However, the size of presynaptic boutons appeared to be slightly reduced because of the presence of small satellite boutons (2.4 ± 0.2 µm2 compared with 2.7 ± 0.1 µm2 for wild type; P = 0.21), although the difference was not statistically significant.

Next, we analyzed the active zones, the electron-dense thickening of presynaptic membranes, and the T bar, T-shaped electron-dense projections (Fig. 5, E and F, arrowheads). The number of active zones (0.09 ± 0.01/µm2 compared with 0.11 ± 0.02/µm2 for wild type; P = 0.08) and the morphology of T bar were normal in trol null mutants; however, the postsynaptic area immediately apposed to the active zones was remarkably altered. Although this postsynaptic area contained a shallow pocket of amorphous materials in the wild-type animals, this pocket was dramatically enlarged in the trol null mutants (Fig. 5, E, F, and H).

We next immunohistochemically stained the larval NMJ using antibodies against a subunit of glutamate receptor (GlutRIIA) and Bruchpilot (BRP), an active zone marker. In the wild type, GlutRIIA signals were localized at the postsynaptic sites apposed to the BRP signals (Fig. 5 J). In the trol null mutants, the intensity of GlutRIIA signals was dramatically reduced in the majority of boutons (Fig. 5, K and L). On the other hand, the number and intensity of BRP signals were not affected (Fig. 5 L), consistent with the aforementioned ultrastructural experiments, indicating that the number and morphology of active zones were normal in the trol null mutants. Thus, our analyses revealed the diverse postsynaptic defects in the NMJ of trol null mutants and suggested that the defects in the synaptic transmission were caused mainly by these postsynaptic dysfunctions.

Trol promotes the FNI pathway of Wg signaling during postsynaptic development

It has been revealed that wg loss-of-function mutations lead to diverse pre- and postsynaptic defects at larval NMJ, including decreases in the number of active zones and the SSR area, increases in the enlarged postsynaptic pockets and the ghost boutons, and altered active zone structure and GlutRIIA distribution (Packard et al., 2002). Because the defects in the postsynaptic structure of trol mutants were extremely similar to those of wg loss-of-function mutants, we examined whether Trol regulates the activity of Wg signaling during postsynaptic development (Fig. 6). Previous studies indicated that presynaptically released Wg activates the postsynaptic Dfz2, leading to the cleavage of the C terminus of the receptor (DFz2-C), which is translocated...
trol mutations affect the translocation of DFz2-C into muscle nuclei using the antibody against the DFz2-C. In wild type, DFz2-C signals showed a punctate pattern in the muscle nuclei (Fig. 6 A, arrowheads), in addition to robust signals at the...

Figure 4. Both the core protein and HS moieties of Trol are required for NMJ formation. (A–F) Larval NMJs of wild type (A), trolnull (B), 24B>sfl dsRNA (C), 24B>sec-dally (D), trolnull; 24B>sfl dsRNA (E), and trolnull; 24B>sec-dally (F) were stained with anti-HRP (magenta) and anti-DLG (green) antibodies. Arrowheads in B and E indicate ghost boutons. Bars, 50 µm. (G–I) Area of muscle 6/7 (G) and the numbers of ghost (H) and satellite (I) boutons in animals of indicated genotypes were measured. Muscle-specific expression of sfl dsRNA reduced muscle size (24B>sfl dsRNA). The ghost bouton phenotype of the trolnull mutant was rescued by muscle-specific expression of sec-dally (trolnull; 24B>sec-dally) but not by sfl dsRNA (trolnull; 24B>sfl dsRNA). Expression of neither sfl dsRNA nor sec-dally affected the satellite bouton phenotype of the trolnull mutant. Error bars represent SEM (n = 18; *, P < 0.01).
synaptic boutons (Fig. 6 A, arrow). We found that trolnull mutations dramatically reduced the DFz2-C signals in the muscle nuclei without affecting their localization at the synaptic boutons (Fig. 6, B and C), suggesting that Trol is required for the activation of Wg signaling in the postsynaptic cells.

We next investigated whether the postsynaptic defects of trol mutants arise from the reduced activities of Wg signaling. We restored the FNI pathway of Wg signaling in the trolnull muscles by expressing the C terminus of DFz2 fused to an NLS (DFz2-C–NLS; Mosca and Schwartz, 2010). We investigated four parameters related to NMJ development: number of ghost boutons, SSR structures, number of enlarged postsynaptic pockets, and localization of GluRIIA (Fig. 6, D–J). As shown in Fig. 6 G, the expression of DFz2-C–NLS transgene in trolnull muscles (trolnull; 24B>DFz2-C–NLS) reduced the number of ghost boutons to the wild-type level. Furthermore, DFz2–C–NLS expression significantly rescued the phenotypes of small SSR and the postsynaptic enlarged pockets around active zones in trolnull mutants (Fig. 6, F, H, and I). These results provide strong evidence that the defects of postsynaptic structure in trol mutants arose from the reduced activity of Wg signaling in the muscles.

Although the small SSR area of trol mutants was significantly restored by the DFz2–C–NLS expression, the thickness of each SSR layer remained thin in these transgenic animals (Fig. 6 F). In particular, it is noteworthy that the amounts of extracellular space around folds remained small in these animals. Furthermore, the expression of DFz2–C–NLS did not rescue the defects of GluRIIA localization and electrophysiological properties of trolnull NMJ (Fig. 6 J and Fig. S1). Mosca and Schwartz (2010) have shown that importin-B11/a2 is required for the translocation of DFz2 to the muscle nuclei, but its mutation did not affect the localization of GluRIIA at NMJ, suggesting that Wg regulates the GluRIIA localization by an FNI-independent pathway. It is thus considered that Trol controls the GluRIIA localization and so controls synaptic transmission by the Wg signaling that is independent of the FNI pathway or by another signaling pathway unrelated to Wg. Finally, the GluRIIA localization does not seem to be critically dependent on the sulfation of HS because the NMJs of sffnull mutants showed normal levels of GluRIIA expression (Fig. S2).

Satellite bouton phenotype of trol mutants is suppressed by reduced Wg signaling

The ultrastructural organization of presynaptic terminals, such as the number and morphology of active zones, was apparently normal in trol mutants, which was a marked contrast to the wg loss-of-function mutants showing various presynaptic defects in addition to the postsynaptic ones (Packard et al., 2002). Instead, trol mutants showed the overproduction of type I synaptic boutons (Fig. 7 D). It is noteworthy that this increase of satellite boutons in trol mutants was not caused by the reduced FNI signaling in the postsynaptic cells because expression of DFz2–C–NLS in trolnull muscles did not rescue their satellite bouton phenotype (Fig. 7 A). Recent studies have shown that the satellite boutons are induced by the excess signaling activities of Wg and/or Glass bottom boat (Gbb), a Drosophila homologue of bone morphogenetic protein (BMP; Packard et al., 2002; Miech et al., 2008; O’Connor-Giles et al., 2008). Thus, to examine the possibility that trol mutations up-regulate the presynaptic activities of Wg and/or Gbb, we investigated whether mutations in these signaling pathways affect the satellite bouton phenotype of trol mutants (Fig. 7, B–F). The heterozygous mutations of wg significantly suppressed the satellite bouton phenotype of trolnull animals (trolnull; wgCX4/+; Fig. 7, B and E). This effect was specific to the wg mutations because the heterozygous mutation of gbb did not show similar effects (trolnull; gbb+/+; Fig. 7, B and F). We additionally studied the contribution of FGF receptor Heartless, which is essential for muscle development (Shishido et al., 1997). As shown in Fig. S3, the heterozygous mutations of heartless (htr14/+) did not affect the ghost and satellite bouton phenotypes of trol mutants.

To examine whether presynaptic canonical Wg signaling was increased in the trol mutants, we next tried to reduce its activity specifically in the motor neurons. sgg and dsh are negative and positive regulators of the canonical Wg signaling, respectively (Axelrod et al., 1998; Bourouis, 2002; Miech et al., 2008), and thus, we expressed the active form of sgg and the dominant-negative form of dsh in trolnull motor neurons using OK6-Gal4 driver (trolnull; OK6>sggact and trolnull; OK6>dshDIX, respectively). These manipulations completely suppressed the satellite bouton phenotype of trolnull mutants (Fig. 7, B, G, and H), although the ghost bouton phenotype was not rescued (Fig. 7, G and H, arrowheads). These results suggested that the canonical Wg signaling was elevated in the presynaptic motor neurons of trol mutants.

Previous studies have shown that Wg signaling regulates the cytoskeletal organization in presynaptic terminals (Packard et al., 2002; Miech et al., 2008). During NMJ development, Futsch, a microtubule-associated protein, associates with loops of bundled microtubules within subsets of boutons. This Futsch loop is essential for synaptic growth, and its formation is promoted by activation of Wg signaling (Miech et al., 2008). As expected, we found that the number of Futsch loops was increased in the NMJ of trolnull mutants (Fig. 7, I–K), suggesting that Trol suppresses the presynaptic activity of Wg signaling during NMJ formation.

Trol regulates the extracellular distribution of Wg

We next expressed the Wg-GFP fusion protein in the motor neurons of trolnull mutants and investigated the extracellular levels of Wg-GFP at the NMJ using an extracellular staining protocol (Strigini and Cohen, 2000). In wild type, the extracellular Wg was diffusely distributed at the surroundings of presynaptic terminals (Fig. 8, A, D, and F). At the trolnull boutons, the extracellular levels of Wg were significantly reduced without affecting the total Wg-GFP level, indicating that Trol regulates the extracellular localization of Wg at NMJ (Fig. 8, B, C, E, and F). Detailed analyses of Wg localization at the single bouton level showed that the extracellular Wg did not spread and was deposited closely around the presynaptic membranes in trolnull mutants (Fig. 8, D–F). This finding suggested that Trol regulates...
Figure 5. Trol is required for formation of postsynaptic structures and glutamate receptor localization at postsynaptic sites. (A–F) Electron micrographs of wild-type (A, C, and E) and trolnull mutant (B, D, and F) tissues are presented. In trolnull mutants, the cell surface of muscle is located closer to the basement membrane (arrowheads in B) than in the wild type (A). Mutations in trol caused small SSRs (D) and abnormally enlarged pockets in the postsynaptic region apposed to active zones (asterisks in D and F). The structure of T bar appeared normal in the trolnull mutant (arrowheads in E and F). Arrowheads in A indicate the cell surface of the muscle. An asterisk in E indicates the normal postsynaptic area apposed to the active zone. (G–I) Morphometric analyses of type Ib boutons showed that the SSR area was decreased (G), and the number of enlarged pockets increased (H) in trolnull mutants. On the other hand, the number of synaptic vesicles was not altered in trolnull mutants (I). To count the number of synaptic vesicles, we selected those within 250 nm of the active
The trafficking of Wg from presynaptic terminals to the postsynaptic membrane.

A previous study has indicated that HSPGs bind to Wnt via HS chains (Reichsman et al., 1996). To directly examine whether HS regulates the localization of Wg at the NMJ, we degraded HS with heparitinase and evaluated its effects on the extracellular Wg distribution at NMJs (Fig. 8, G–I). About 60% of HS was degraded after heparitinase treatment as judged by the staining intensity of 10E4 anti-HS monoclonal antibody (Fig. S4). Under this condition, the extracellular Wg levels on

zones. Error bars represent SEM (n = 15; *, P < 0.0001). (J–L) Synaptic regions of wild-type (J) and tro null mutants (K) were stained with the antibodies against active zone marker BRP and GluRIA. (J) BRP and GluRIA clusters were located on apposed membranes at the synaptic boutons in wild-type larvae. (K) In tro null mutants, the levels of GluRIA, but not BRP, were dramatically decreased compared with those of wild-type animals. (L) Bar graphs show the mean staining intensity for GluRIA and BRP at the NMJ. Error bars represent SEM (n = 20; ***, P < 0.001, and ****, P < 0.0001). Bars: (A) 15 µm; (J and K) 15 µm. AZ, active zone.

Figure 6. Trol regulates Wg-mediated postsynaptic development. (A–C) Muscle 6 in wild-type (A) and tro null (B) animals was stained with antibodies to the C terminus of Dfz2 (green) and α-Tubulin (magenta). Immunoreactive puncta in the nuclei (arrowheads in dashed circles) were markedly reduced in tro null mutants. Dfz2-C localization in the synaptic boutons was not affected by mutations in trol (arrows). (C) The numbers of Dfz2-C nuclear puncta in third-instar larval muscles of wild-type and tro null animals were counted. (D–F) Electron micrographs of synaptic type I boutons of wild-type (D), tro null (E), and tro null; 24B>DFz2-C–NLS (F) animals are presented. The SSR region is colored brown. (G–J) Expression of DFz2-C–NLS in tro null muscles rescued their ghost bouton (G), small SSR (H), and enlarged postsynaptic pocket (I) phenotypes. However, DFz2-C–NLS expression did not rescue the abnormal localization of GluRIA at tro null synaptic boutons (J). Error bars represent SEM (n = 20; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001). Bars: (A) 15 µm; (D) 1 µm. AZ, active zone.
Figure 7. Reduction of presynaptic Wg signaling suppresses satellite bouton phenotype of tro1 mutants. (A) Activation of FNI pathway in the muscles (tro1<sup>null</sup>; 24B>DF2-C–NLS) did not rescue the satellite bouton phenotype of tro1<sup>null</sup> mutants. (B–H) The numbers of satellite boutons in muscle 6/7 of the indicated genotypes were counted (B). Larval NMJs of wild type (C), tro1<sup>null</sup> (D), tro1<sup>null</sup>; wg<sup>CX4/+</sup> (E), tro1<sup>null</sup>; gbb<sup>1/+</sup> (F), tro1<sup>null</sup>; OK6>sgg<sup>act</sup> (G), and tro1<sup>null</sup>; OK6>dsh<sup>DIX</sup> (H) were stained with anti-HRP (magenta) and anti-DLG (green) antibodies. Heterozygous mutation of wg (E), but not that of gbb (F), suppressed the satellite bouton phenotype of tro1<sup>null</sup> animals (B). Presynaptic suppression of canonical Wg signaling (G and H) rescued the satellite bouton phenotype (B), but not the ghost bouton phenotype, of tro1<sup>null</sup> animals. Arrows and arrowheads indicate the satellite and ghost boutons, respectively. (I–K) The NMJs of wild-type (I)
analyses showed that, in the NMJ of trol mutants, canonical Wg signaling was increased in the presynaptic motor neurons, producing satellite boutons. In contrast, the activity of the FNI Wg pathway was decreased in the postsynaptic muscle cells, which led to the abnormal postsynaptic structures. These results indicated that the balance of Wg signaling between pre- and postsynaptic compartments was disturbed in the trol NMJ.

We observed that Wg was localized around the presynaptic membranes and did not diffuse into the SSR regions in the synaptic boutons were decreased by ~20%, although total Wg levels were not altered, suggesting that HS plays an important role in Wg localization at NMJ.

**Discussion**

During larval development of *Drosophila*, the muscle fibers grow rapidly; therefore, coordinated synapse formation is required to achieve proper levels of muscle depolarization. Our analyses showed that, in the NMJ of trol mutants, canonical Wg signaling was increased in the presynaptic motor neurons, producing satellite boutons. In contrast, the activity of the FNI Wg pathway was decreased in the postsynaptic muscle cells, which led to the abnormal postsynaptic structures. These results indicated that the balance of Wg signaling between pre- and postsynaptic compartments was disturbed in the trol NMJ.

We observed that Wg was localized around the presynaptic membranes and did not diffuse into the SSR regions in...
the NMJ of trol mutants, leading to a model in which Trol regulates the bidirectional activity of Wg by distributing the appropriate levels of Wg on both pre- and postsynaptic membranes (Fig. 9). Because Trol is highly expressed in the SSR, it is likely that Trol promotes accumulation of Wg in SSRs, which efficiently activates postsynaptic DFz2 (Fig. 9, left). In the absence of Trol, it is considered that Wg is not efficiently transported into SSRs and may be localized excessively around presynaptic boutons, leading to satellite bouton phenotype (Fig. 9, right). Our results suggested that this phenotype mainly depends on the core protein portion of Trol. In addition to the abnormal localization of Wg, the extracellular level of this protein was also significantly decreased in the NMJ of trol mutants, which may lead to ghost bouton phenotype. Because it is well known that HS protects many heparin-binding proteins from proteolytic degradation in the ECM (Saksela et al., 1988), this may be caused by the promoted proteolysis of Wg in the absence of Trol. In fact, we observed that at least a part of Wg bound to HS in the NMJ. These protective effects may not depend on the Trol core protein because ghost bouton phenotype of trol mutants was rescued by the secreted form of Dally.

Because Wg is a highly hydrophobic protein caused by acylation with palmitic acid, the diffusion and/or transport of this protein is not a simple process. Recently, it was proposed that Wg is transported from presynaptic membrane to SSR by exosome-like vesicles containing the Wnt-binding transmembrane protein Evi (Korkut et al., 2009). In this model, the exosomes carrying Wg are released from the presynaptic terminals into the synaptic clefts and are then transported to the SSR, where Wg proteins on the exosomes bind to DFz2. Importantly, Evi-containing exosomes were observed in the cisternae of SSRs (Koles et al., 2012), where immunohistochemical Trol signals were also intensely detected, suggesting the possibility that Trol supports the transport of Wg-bound exosomes in SSRs. In addition, several studies revealed that HSPGs, including perlecan, are essential for the internalization of diverse ligand molecules, such as growth factors and lipoproteins (Fuki et al., 2000; Deguchi et al., 2002). Therefore, there is a possibility that Trol directly participates in the internalization of Wg/DFz2 by muscle fibers. Further study is necessary to examine whether Trol regulates transport of exosomes as well as internalization of Wg/DFz2 in the SSR.

Besides Wg, perlecan also binds to ECM proteins, such as laminin and dystroglycan (Whitelock et al., 2008). We found that the thickness of the extracellular space of SSR layers remained thin even after Wg activation in the muscle cells of trol mutants, although most of the postsynaptic phenotype was rescued. This suggests that Trol is structurally required to construct ECM in the SSR, probably through interaction with these ECM components. Furthermore, Rohrbough et al. (2007) showed that mutations in mind-the-gap led to various abnormalities in glutamate receptor localization and postsynaptic development, which were similar to the phenotypes of trol mutants. Mind-the-gap is a secreted putative glycosaminoglycan-binding protein, which regulates the ECM–integrin interface at NMJ. Therefore, there is a possibility that Trol contributes to the ECM formation in coordination with Mind-the-gap, and such a precisely constructed ECM may be necessary to transport and localize Wg in the NMJ correctly. In this context, it is noteworthy that the satellite bouton phenotype of trol mutants mainly depends on the core protein portion of Trol. The core protein, rather than HS, may be essential for ECM construction and transport of Wg in the SSR.

Some studies on vertebrates and Drosophila showed that perlecan/Trol binds to various secreted molecules, such as BMP, FGF, and Hedgehog (Park et al., 2003; Lindner et al., 2007). Among these, Drosophila BMP orthologue Gbb expressed by muscle cells retrogradely regulates synapse growth at the NMJ (McCabe et al., 2003). gbb loss-of-function mutations caused reduced NMJ size, decreased neurotransmitter release, and abnormal presynaptic structure, which is a marked contrast to the phenotypes of trol mutants showing overgrowth of NMJ and relatively normal presynaptic structure. Because it has been revealed that HSPGs negatively regulate BMP signaling in some circumstances (Gumienny et al., 2007), presynaptic Gbb signaling might be activated without Trol. Alternatively, presynaptically expressed HSPGs may contribute to the Gbb signaling at NMJ, and postsynaptically expressed Trol may not play major roles in this signaling pathway. Our experiments indicated
that down-regulation of Gbb signaling in the trol mutants did not rescue the satellite bouton phenotype, supporting the latter possibility. In the *Drosophila* NMJ, syndecan and Dally-like glypican (Dlp) are expressed by pre- and postsynaptic cells, respectively (Johnson et al., 2006). It has been revealed that these HSPGs associate with DLAR, a presynaptically expressed receptor-type protein tyrosine phosphatase, and regulate synapse growth and active zone stabilization (Johnson et al., 2006). Accordingly, syndecan in the presynaptic membrane may also contribute to the Gbb signaling at NMJ. Furthermore, postsynaptically expressed Dlp may support residual Wg signaling in the trol mutants because it is known that this HSPG plays critical roles in the trafficking and gradient formation of Wg in *Drosophila* larval wing disc (Kirkpatrick et al., 2004; Kreuger et al., 2004).

The frequency of mEJP and the amplitude of eEJP were significantly reduced in trol mutants. Mutations in sft and tout-velu, which encodes a *Drosophila* homologue of HS polymerase, EXT1, also reduced mEJP frequency (Ren et al., 2009). However, in contrast to the trol mutants, these HS biosynthetic mutants showed an increase of eEJP amplitude. Furthermore, the NMJ of these mutants displayed far broader abnormalities than that of trol mutants, such as disruptions of mitochondrial localization in the muscles, and an increase of stimulus-dependent endocytosis in the motoneurons. HS chains of all HSPGs, including syndecan, Dlp, and Trol, were affected in the NMJ of these mutants, which may explain such broader defects. In this study, our data suggested that both the Trol core protein and HS chains contribute to the muscle growth and NMJ development. Thus, the interplay between the core proteins and their attached HS chains might determine the specificity of HSPG activities. Furthermore, HS chains are structurally diverse polysaccharides that display different affinities for various morphogens and growth factors (Maeda et al., 2011). Each synaptic HSPG may be decorated with HS chains with specific structures and thus regulates a distinct set of signaling molecules.

Irie et al. (2012) produced Ext1 conditional knockout (KO) mice, in which HS biosynthesis was disrupted selectively in the glutamatergic neurons in the forebrain. In the pyramidal neurons of these KO mice, the evoked excitatory postsynaptic currents were remarkably depressed, and the frequency and amplitude of the miniature excitatory postsynaptic current were also reduced. Furthermore, the level of postsynaptically localized AMPA-type glutamate receptors was significantly reduced in this KO mouse. Such postsynaptic defects are similar to those of trol mutants observed in this study. The postsynaptic HSPGs expressed by the pyramidal neurons may regulate postsynaptic glutamate sensitivity by modulating Wnt signaling and/or ECM structures.

In the vertebrate NMJ, many Wnt family members are expressed by both motor neurons and muscle fibers, which play important roles in the motor growth cone guidance and acetylcholine receptor clustering at the NMJ (Strohlic et al., 2012; Zhang et al., 2012). However, perlecan-null mice showed only limited defects at the NMJ. Although acetylcholinesterase was absent at the NMJ of these KO mice, the early innervation pattern and acetylcholine receptor clustering were not significantly affected (Arikawa-Hirasawa et al., 2002). Because two HSPGs, agrin and perlecan, are attached to the postsynaptic membrane through dystroglycan (Singhal and Martin, 2011), agrin may provide compensatory support to Wnt signaling at the NMJ in perlecan-null mice. In fact, Henriquez et al. (2008) demonstrated that Wnt3 induces acetylcholine receptor aggregation at vertebrate neuromuscular synapses in collaboration with agrin. Recently, it has been proposed that Wnt and BMP/TGF-β signalings are involved in various neurodegenerative diseases, such as amyotrophic lateral sclerosis and Huntington’s disease (Inestrosa and Arenas, 2010; Bayat et al., 2011). HSPGs, including perlecan, might be involved in the pathological mechanism of these diseases by modulating BMP/TGF-β and Wnt signalings. In this context, trol mutants may be useful as a model system to elucidate the involvement of HSPGs in the pathogenesis of neurodegenerative diseases.

In conclusion, our analyses demonstrate that Trol spatially regulates Wg activity in the NMJ synapses. There is growing evidence that neuronal activity induces the release of Wnt proteins at synapses in both vertebrate hippocampal neurons and *Drosophila* NMJs (Atamna et al., 2008; Gogolla et al., 2009). It is attractive to speculate that Trol/perlecan controls neuronal activity-dependent signaling of Wg/Wnt. Additional studies will be required to elucidate the roles of Trol in Wg-mediated synapse plasticity.

**Materials and methods**

**Fly stocks**

Flies were raised on standard media at 25°C. wgRNAi, htlRNAi, elav-Gal4, OK6-Gal4, 24B-Gal4, repo-Gal4, and UAS-sggRNAi (Bourouis, 2002) flies were obtained from the Bloomington *Drosophila* Stock Center. trol conditional knockout (KO6-Gal4/UAS-GFP-Wg) flies were obtained from the Drosophila Genetic Resource Center (Ghata et al., 2009), UAS-trol dsRNA and UAS-sfl dsRNA were purchased from the Vienna *Drosophila* RNAi center, UAS-GFP-wg flies were obtained from S. Cohen (Institute of Molecular and Cell Biology, Singapore), trol flies were obtained from H. Jäckle [Max Planck Institute for Biophysical Chemistry, Göttingen, Germany; Voigt et al., 2002], trol flies were provided by S. Datta (Texas A&M University, College Station, TX; Park et al., 2003), UAS-sec-daily flies were obtained from H. Nakato (University of Minnesota, Minneapolis, MN; Takeo et al., 2005), gbb flies were obtained from K.A. Wharton [Brown University, Providence, RI], UAS-dshRNA was obtained from J. Axelrod (Stanford University School of Medicine, Stanford, CA; Axelrod et al., 1998), and UAS-myc-NLS-DFz2-C flies were provided by V. Budnik (University of Massachusetts Medical School, Worcester, MA; Mathew et al., 2005). All experiments were conducted in accordance with the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute of Medical Science, 2011).

**Immunocytochemistry**

Wandering third-instar larvae were used unless otherwise specified. Larval body wall muscles were dissected in Ca^2+^-free HL3 saline (70 mM NaCl, 5 mM KCl, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, and 5 mM Hepes, pH 7.2) and fixed in either Bouin’s fixative or 4% paraformaldehyde/PBS for 20 min. Larvae were incubated overnight at 4°C in primary antibodies and then for 2 h at room temperature in secondary antibodies. All incubation and washing steps were performed in PBT (PBS with 0.3% Triton X-100). Extracellular labeling of Wg protein was performed according to Strigini and Cohen (2000). In brief, dissected body wall muscles of OK6-Gal4/UAS-GFP-wg were incubated for 2 h at 4°C with anti-GFP antibody (1:10 dilution; MBL International) in HL3 saline. Body wall muscles were rinsed three times with ice-cold HL3 and fixed for 20 min in ice-cold PBS containing 4% paraformaldehyde. Subsequent processing was the same as in the conventional protocol except...
Behavioral assay and electrophysiology
First- and third-instar larvae of wild type and tro^null were placed on an agar plate. The number of body wall contractions during 1 min was counted. Electrophysiology was performed on late third-instar larvae of wild-type and tro^null mutants. Filleted larvae were washed in HL3 saline containing 1 × 10^(-4) M Na$_2$CO$_3$ before recording. Recording electrodes were heat-pulled glass capillaries with resistances between 10 and 40 MΩ, filled with 3 M KCl. mEJPs and eEJPs were recorded in the bridge mode using an amplifier (AxoClamp-2B; Axon Instruments). The low pass filter was set at 1 kHz on the amplifier. Recordings were performed on muscle 6 in abdominal segments 2 and 3. Only those muscles with a resting membrane potential less than ~60 mV throughout the recording were considered. Spontaneous events were recorded in the presence of 10 μM tetrodotoxin for 3 min, and these mEJP traces were analyzed by hand using the program of pClamp 9.2 software (Axon Instruments). Quantal content was estimated by dividing the mean eEJP by the mean mEJP.

Electro microscopy and immunoelectron microscopy
Body wall muscles of third-instar larvae of wild type and tro^null were prepared for transmission electron microscopy as described previously (Jia et al., 1993; Packard et al., 2002). In brief, body wall muscles were fixed in 1% glutaraldehyde, 4% paraformaldehyde, and 0.1 M sodium phosphate buffer, pH 7.4, for 40 min at room temperature and then 4°C overnight. After washing in 0.1 M sodium phosphate buffer, pH 7.4, samples were postfixed for 1 h in 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in Quetol 812 (Nissin EM). Ultrathin sections (60–80 nm thickness) were cut from the embedded samples with an ultramicrotome (EM UC7; Leica), collected on Formvar-coated copper grids, and contrasted with lead citrate. Synaptic boutons between muscles 6 and 7 were examined using a transmission electron microscope (JEM-1400, JEOL) with an accelerating voltage of 80 kV and imaged with an ultramicrotage-coupled device camera at 100,000x magnification. The size of the bouton and SSR was measured using ImageJ software. Immunoelectron microscopy of tro^null third-instar larval larvae was performed using a preembedding technique. In brief, dissected larvae were fixed in 4% paraformaldehyde/0.1% glutaraldehyde/ PBS and incubated with the anti-GFP antibody (1:300) followed by anti–rabbit IgG (1:50; Nanoprobes). After intensification using HQ silver reagents (Nanoprobes), the samples were processed for electron microscopy as described in this paragraph.

Statistical analysis
Statistical analyses, we used Prism 4 (GraphPad Software). Numerical data are presented as means ± SEM. The nonparametric Mann–Whitney test was used to determine the differences between two groups, and Kruskal–Wallis nonparametric analysis of variance and Dunn’s post hoc test were used for multiple group comparisons.

Online supplemental material
Fig. S1 shows that the expression of Dfz2-C–NLS did not rescue the defects of larval locomotion of tro^null and electrophysiological properties of their NMJs. Fig. S2 shows that NMJs of sfl^null mutants showed normal levels of GluRIIA expression. Fig. S3 shows that reduction of FGFR signaling did not affect the tro^null mutant phenotype. Fig. S4 shows that ~60% of HS was degraded after hepatirinase treatment of the NMJ. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201207036/DC1.

We thank Drs. J. Axelrod, V. Budnik, S. Cohen, S. Datta, H. Jackle, H. Nakato, and K.A. Wharton and the Developmental Studies Hybridoma Bank, Bloomington Drosophila Stock Center, Drosophila Genetic Resource Center, and Vienna Drosophila RNAi Center for fly stocks and reagents. We are also grateful to the Center for Basic Technology Research at the Tokyo Metropolitan Institute of Medical Science for assistance with electron microscopy.

This work was partly supported by the Takeda Science Foundation, the Naito Foundation, and the Ministry of Education, Culture, Sports, Science and Technology of Japan/the Japan Society for the Promotion of Science KAKENHI grants 24110521 and 22570149.

Submitted: 5 July 2012
Accepted: 14 December 2012

References

Downloaded from on June 26, 2017
Supplemental material

Kamimura et al., http://www.jcb.org/cgi/content/full/jcb.201207036/DC1

Figure S1. Activation of postsynaptic Wg signaling did not rescue the locomotion and electrophysiological defect of trol mutant larvae. (A) Locomotion of wild-type, trolnull, and trolnull; 24B>DFz2-C-NLS third-instar larvae. (B) Representative traces of mEJP (left) and eEJP (right) of wild-type, trolnull, and trolnull; 24B>DFz2-C-NLS animals. (C–E) Bar graphs show the mEJP frequency (C) and amplitude (D) and eEJP amplitude (E) of the indicated genotypes. Expression of DFz2-C-NLS in muscle cells did not affect these physiological defects of trolnull larvae. Error bars represent SEM (n = 10).
Figure S2. sfl mutations did not affect the glutamate receptor localization at postsynaptic sites. (A and B) Synaptic regions of wild-type and sfl9B4/sfl03844 animals were stained with the antibodies against HRP (magenta) and GlurIIA (green). Bar, 10 µm. (C) Bar graph shows the GlurIIA intensity at boutons of wild-type and sfl9B4/sfl03844 animals. Loss of sulfate groups of HS did not affect the GlurIIA levels at postsynaptic sites. Error bars represent SEM (n = 20).

Figure S3. Reduction of FGF signaling did not affect the NMJ morphologies of trol mutants. (A–D) Larval NMJs of wild type (A), htlAB42/+ heterozygotes carrying the null allele of heartless (B), trolnull (C), and trolnull; htlAB42/+ (D) were stained with the antibodies against HRP (magenta) and DLG (green). Bar, 50 µm. (E–G) Bar graphs show the muscle size and the numbers of ghost and satellite boutons in larval NMJ of the indicated genotypes. The heterozygous mutations of htl did not affect the trol NMJ phenotypes. Error bars represent SEM (n = 10).
Figure S4. **Heparitinase treatment reduced the extracellular levels of HS at NMJ.** (A and B) Live body wall tissues of control and heparitinase-treated larvae were incubated with anti-HRP (magenta) and anti-HS (10E4; green) antibodies. Bar, 20 µm. (C) Bar graph shows the 10E4 signal intensity at boutons of control and heparitinase-treated larvae. Treatment of larval NMJ with heparitinase remarkably decreased the level of extracellular HS. Error bars represent SEM (n = 10; *, P < 0.0001). HSase, heparitinase.