Retrograde neurotrophin signaling through Tollo regulates synaptic growth in Drosophila

Shannon L. Ballard, Daniel L. Miller, and Barry Ganetzky

Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53705

Toll-like receptors (TLRs) are best characterized for their roles in mediating dorsoventral patterning and the innate immune response. However, recent studies indicate that TLRs are also involved in regulating neuronal growth and development. Here, we demonstrate that the TLR Tollo positively regulates growth of the Drosophila melanogaster larval neuromuscular junction (NMJ). Tollo mutants exhibited NMJ undergrowth, whereas increased expression of Tollo led to NMJ overgrowth. Tollo expression in the motoneuron was both necessary and sufficient for regulating NMJ growth.

Introduction

Accurate processing of information in neural circuits requires proper development of synaptic connections between neighboring cells. Even after their establishment, synapses must maintain the ability to undergo morphological and functional modifications in response to altered activity, experience, or environment throughout an organism's life (Budnik et al., 1990; Prakash et al., 1999; Featherstone and Broadie, 2000; Packard et al., 2003; Sigrist et al., 2003; Collins and DiAntonio, 2007). Although many neurological disorders including autism, epilepsy, and neurodegeneration are associated with synaptic defects (Prakash et al., 1999; Torroja et al., 1999; Zhang et al., 2001; Johnston, 2004; Chang et al., 2008; Jeibmann and Paulus, 2009), the genetic and molecular mechanisms governing synaptic growth and plasticity remain to be fully elucidated.

The Drosophila melanogaster larval neuromuscular junction (NMJ) is a powerful system for dissecting mechanisms regulating synaptic growth and plasticity, and many of the known regulators are conserved with vertebrates (Keshishian et al., 1996; Featherstone and Broadie, 2000; Packard et al., 2003; Ruiz-Cañada and Budnik, 2006; Collins and DiAntonio, 2007). In both vertebrates and invertebrates, secreted growth factors and their downstream components have been implicated in regulating processes such as patterning of the nervous system, neuronal proliferation, axon guidance, neurite outgrowth, and synapse formation (Charron and Tessier-Lavigne, 2007; Mizutani and Bier, 2008; Guillemot and Zimmer, 2011; Park and Poo, 2013; Rosso and Inestrosa, 2013). Development and growth of the larval NMJ is mediated in part by growth factors such as TGF-β–bone morphogenic protein (BMP) and Wnt/Wg. The TGF-β–BMP pathway positively regulates NMJ growth in a retrograde manner, wherein the ligand (Glass bottom boat) is secreted from muscle and signals through receptors at the presynaptic terminal (McCabe et al., 2003; Marqués, 2005). The Wingless ligand, which is thought to be secreted from presynaptic terminals, signals through Frizzled2 receptors located both pre- and postsynaptically to promote NMJ growth (Packard et al., 2002; Salinas, 2005). Recently, a neuropeptide signaling pathway that exerts positive regulation of NMJ growth via the cholecystokinin-like receptor, which is localized presynaptically, and its ligand drosulfakinin, which is released by neurosecretory cells in the brain, was characterized (Chen and Ganetzky, 2012). This unexpected discovery highlights our incomplete understanding of how the nervous system changes in response to altered activity, environment, and experience.

Dominant genetic interactions together with altered levels of phosphorylated c-Jun N-terminal kinase (JNK) and puc-lacZ expression revealed that Tollo signals through the JNK pathway at the NMJ. Genetic interactions also revealed that the neurotrophin Spätzle3 (Spz3) is a likely Tollo ligand. Spz3 expression in muscle and proteolytic activation via the Easter protease was necessary and sufficient to promote NMJ growth. These results demonstrate the existence of a novel neurotrophin signaling pathway that is required for synaptic development in Drosophila.
In *Drosophila*, Toll, the best characterized member of the TLR family, signals through nuclear factor-κB (NF-κB) transcription factors (Dor and Dif) upon binding the ligand Spätzle (Spz; Anderson et al., 1985a, b; Stathopoulos and Levine, 2002; Minakhina and Steward, 2006; Ganesan et al., 2011; Valanne et al., 2011). Spz and Spz-like ligands are cysteine-knot growth factors that are similar in structure to vertebrate neurotrophins (Parker et al., 2001; Zhu et al., 2008), which are members of a key family of growth factors that regulate synaptic development and function. These secreted signaling molecules are important for stem cell survival and differentiation, axon growth and guidance, as well as synapse formation, maturation, and refinement (Chao, 2003; Park and Poo, 2013).

Recent work in *Drosophila* has shed new light on neurotrophin-like factors in invertebrates (Zhu et al., 2008). In the developing embryo, Spz2 (also called *Drosophila* Neurotrophin 1 [DNT1]) promotes cell survival in the central nervous system (CNS) and influences motor axon targeting, similar to functions of vertebrate neurotrophins (Zhu et al., 2008). In addition, embryos...
mutant for Spz5 (also called DNT2) exhibit an increase in apoptosis and a mild axon-targeting defect (Zhu et al., 2008). Six Spz-like ligands have been identified in the Drosophila genome (Parker et al., 2001), of which Spz2/DNT1 and Spz5/DNT2 exhibit neurotrophin-like functions in the nervous system. Here, we investigate the regulation of NMJ growth by another TLR (Tollo) and its neurotrophin family member ligand (Spz3). During preparation of this manuscript, Sutcliffe et al. (2013) reported studies on the roles of DNT1, DNT2, and Spz in NMJ growth regulation and found that these neurotrophins affect NMJ growth in a cell-specific manner: Spz regulates growth of NMJ4 whereas DNT1 and DNT2 regulate growth of NMJ6/7 (Sutcliffe et al., 2013).

We demonstrate that Tollo is required presynaptically to positively regulate NMJ growth and development. Our evidence suggests that Tollo signaling at the NMJ occurs through JNK activation of the transcription factors Jun and Fos rather than via the canonical NF-κB pathway. Genetic evidence further suggests that Spz3, secreted from the muscle, is a likely Tollo ligand. These results demonstrate that as in vertebrates a neurotrophin-like pathway in Drosophila plays an important role in regulating synapse growth and development at the larval NMJ.

Results

Tollo promotes NMJ growth

In the course of mapping a temperature-sensitive paralytic mutant generated in our laboratory, we tested various nearby candidate genes on chromosome arm 3L for failure of complementation. Fortuitously, we discovered that mutations of Tollo have an NMJ growth defect even though they complement the paralytic mutation we started with. Because of the strength of the phenotype and the inherent interest in Tollo, we pursued the analysis of this mutant. We found that loss-of-function mutations of Tollo cause a reduction in mean bouton number as well as a decrease in mean total branch length at NMJ3 (Fig. 1 and Fig. S2), without any associated reduction in muscle volume (Fig. S1). At NMJ3, the number of boutons in Tollo mutants is reduced by over 30% (Fig. 1 N) and the mean total branch length is shortened by at least 20% (Fig. S2) compared with wild-type or heterozygous (Tollo/+) controls. Although we focus on the analysis of NMJ3 in this study (see the following analysis of Tollo expression), we observe comparable undergrowth phenotypes for other NMJs, including NMJ4 (Fig. S2). These results identify Tollo as a positive regulator of NMJ growth.

Tollo functions presynaptically to regulate NMJ growth

To determine where Tollo expression is required to promote NMJ growth, we analyzed the expression pattern of a Gal4 enhancer trap of Tollo by expressing a membrane-targeted GFP construct (Tollo-Gal4>UAS-mCD8-GFP, Fig. 2 A). This Tollo-Gal4 recapitulates the endogenous expression pattern of Tollo in the imaginal wing disc (Ayyar et al., 2007). We observe strong GFP expression in motoneuron 3 (MN3) in wandering third instar larvae. GFP expression colocalizes with α-HRP labeling, which is specific for neuronal membranes (Fig. 2 A), indicating that Tollo...
is expressed presynaptically in MNs innervating muscle 3. We also observe GFP expression in other MNs, such as those innervating muscle 4 and muscles 6/7. However, this expression was variable and weak compared with MNs that innervate more dorsally located muscles (e.g., MN3). Thus, we concentrated on the analysis of NMJ3 to investigate the role of Tollo in NMJ growth. The undergrowth phenotype in Tollo mutants is consistent in abdominal segments A2–A4, although the phenotype is slightly stronger in A4 compared with more anterior segments (Fig. S2).

To test whether Tollo is required presynaptically to regulate NMJ growth, we reduced Tollo expression using the neuronal-specific elav\textsuperscript{155}–Gal4 driver and RNAi directed against Tollo transcripts. We observe a significant decrease in bouton number in c155\textgreater UAS-Tollo-RNAi larvae compared with controls (Fig. 2, B–E and J). Moreover, c155\textgreater UAS-Tollo-RNAi larvae heterozygous for a Tollo mutation (c155/+: UAS-Tollo-RNAi/+; Tollo\textsuperscript{142}/+) display a further reduction in bouton number (Fig. 2, F and J). In contrast, we did not observe any significant reduction in bouton number when Tollo mRNA is reduced postsympaptically using the muscle-specific 24B–Gal4 even when Tollo expression was further reduced by heterozygosity for a Tollo mutation (Fig. 2, B, D, and G–J).

Conversely, presynaptic expression of Tollo in an otherwise wild-type larva (c155\textgreater UAS-Tollo) leads to a significant increase in bouton number compared with control larvae (Fig. 3, A–D and L), whereas, postsynaptic expression of Tollo (24B\textgreater UAS-Tollo) does not affect bouton number (Fig. 3 L). Moreover, c155\textgreater UAS-Tollo expression in Tollo mutants fully rescues bouton number to wild-type levels (Fig. 3, E–L). However, 24B\textgreater UAS-Tollo expression does not alter bouton number in Tollo mutants (Fig. 3 L). Together, these data demonstrate that Tollo expression in neurons is necessary and sufficient to promote NMJ growth.

### Tollo is not required for normal synaptic function

Because Tollo function is required in MNs for normal NMJ growth during development, loss of Tollo could also result in altered synaptic function at the NMJ. Thus, we examined the amplitude of evoked excitatory junction potentials (EJPs) and spontaneous miniature EJPs (mEJPs) at muscle 3 in Tollo mutant larvae. At 1 and 0.4 mM Ca\textsuperscript{2+}, EJP amplitude is normal (Fig. 4, G–K; and Fig. S3). At 1 mM Ca\textsuperscript{2+}, the frequency and amplitude of mEJPs as well as quantal content of evoked neurotransmitter release were all normal in Tollo mutant larvae (Fig. 4, A–F and L). These data indicate that despite the reduction in bouton number in Tollo mutants, basic aspects of synaptic transmission at the NMJ are unimpaired. We also examined synaptic structure at a finer level in Tollo mutants and found no differences from controls in the apposition of presynaptic (Bruchpilot [Brp]) and postsynaptic (GluRIII) components of active zones (Kittel et al., 2006; Wagh et al., 2006) or in the ratio of GluRIIA/GluRIIB levels in postsynaptic receptor fields (Marrus et al., 2004; Fig. S4).

Because synaptic function and organization are normal in Tollo mutants, whereas bouton number is decreased, one possible compensatory mechanism would be an increase in the number of active zones in each bouton. To examine this possibility, we quantified the mean number of BRP puncta in Tollo mutants. Compared with control larvae, Tollo mutants display an increase in the mean number of BRP puncta per bouton (Fig. 5, A–E). In contrast, the volume of boutons in Tollo mutants does not differ from controls (Fig. S1). Thus, the density of BRP puncta is increased in Tollo mutant boutons relative to controls (Fig. 5 G).
The proportionate increase in active zones per bouton in Tollo mutants is almost the same as the proportionate reduction in bouton number per NMJ. Thus, despite NMJ undergrowth in Tollo mutants, the total number of active zones per NMJ remains close to normal (Fig. 5 F).

**Tollo signals through the JNK pathway to regulate NMJ growth**

In both *Drosophila* and vertebrates, Toll and TLRs signal through highly conserved downstream components (Belvin and Anderson, 1996; O’Neill and Greene, 1998; Imler and Zheng, 2004). Receptor activation leads to activation of the interleukin-1 receptor-associated kinase, which, in turn, leads to phosphorylation and degradation of inhibitor κB (Cactus in *Drosophila*). Loss of inhibitor κB relieves cytoplasmic sequestration of the transcription factor NF-κB (Dorsal in *Drosophila*). NF-κB then enters the nucleus to regulate transcription of target genes (Belvin and Anderson, 1996; O’Neill and Greene, 1998; Imler and Zheng, 2004). To examine whether Tollo signals through this canonical pathway, we tested whether there were any dose-dependent dominant interactions between Tollo and dorsal mutations. We observed no change in bouton number in larvae doubly heterozygous for Tollo and dorsal (i.e., Tollo+/+ Dorsal; Fig. S5). If Cactus functions in MNs to inhibit growth-promoting functions of Tollo signaling, we would expect to observe a decrease in bouton number when cactus is overexpressed with c155-Gal4. However, overexpression of cactus in neurons had no effect on bouton number (Fig. S5). Moreover, Dorsal and Cactus are localized postsynaptically in muscle and regulate postsynaptic glutamate receptor localization via a nontranscriptional mechanism (Cantera et al., 1999; Heckscher et al., 2007), whereas, as described earlier, we do not observe any alteration in glutamate receptor levels or localization in Tollo mutants (Fig. S4). These data suggest that Tollo is not signaling through the canonical Toll pathway at the NMJ.

In response to immune and stress signals, vertebrate Toll/Interleukin-1 receptors can signal through noncanonical pathways such as a MAPK cascade (O’Neill and Greene, 1998; Ruse and Knaus, 2006; Chen et al., 2007). To investigate whether Tollo regulates NMJ growth through a MAPK pathway, we examined bouton number in larvae heterozygous for mutations of both Tollo and basket (bsk), which encodes a JNK that is activated by MAPK-dependent phosphorylation. Whereas bsk+/+ or Tollo+/+ heterozygotes alone do not affect NMJ growth, bsk+/+; Tollo+/+ double heterozygotes exhibit a significant decrease in bouton number (Fig. 6, A–H and Q). This dominant genetic interaction between Tollo and bsk suggests that Tollo is signaling through a JNK pathway to regulate NMJ growth.

The idea that Tollo signals through a JNK pathway predicts a decrease in levels of phosphorylated JNK (pJNK) in Tollo mutants. To examine this prediction, we used an antibody specific for the phosphorylated form of JNK to analyze levels of pJNK at normal (Fig. S1). Consequently, to quantify pJNK levels we measured the fluorescence intensity ratio of anti-pJNK to anti-HRP (see Materials and methods) in Tollo mutants and control larvae (Fig. 7, A–E). Tollo mutant NMJs exhibit a significant decrease in normalized pJNK fluorescence intensity as expected if Tollo signals through a JNK pathway. Moreover, the fact that we observe a decrease in pJNK levels in synaptic boutons suggests that Tollo may function locally in presynaptic terminals to initiate a positive NMJ growth signal, although we cannot rule out the possibility that some Tollo signaling...
occurs back in the MN cell body. In either case, the positive growth signal ultimately is likely to require transcriptional activation of target genes in the nucleus.

If Tollo signaling activates the JNK pathway, we predict that an increase in Tollo expression should increase JNK activity. To test this, we used the puc-lacZ reporter, an enhancer trap of the JNK phosphatase encoded by puckered (puc), whose expression is activated by JNK (Martín-Blanco et al., 1998). We examined expression of puc-lacZ in Tollo-Gal4 expressing cells in the ventral region of the abdominal ventral nerve cord (VNC) where MN3 cell bodies are located (Landgraf et al., 1997). In control larvae, puc-lacZ expression levels are low (Fig. 7, F and I), and it is detected in relatively few Tollo-Gal4 expressing cells (Fig. 7, F and H). When Tollo is overexpressed using the Tollo-Gal4 driver (Tollo-Gal4>UAS-Tollo), we observe an increase in the number of puc-lacZ expressing nuclei (Fig. 7, G and H) as well as an increase in puc-lacZ fluorescence intensity in those cells expressing Tollo-Gal4 (Fig. 7, G and I). Together, the decrease in presynaptic levels of pJNK in Tollo mutants and the increase in puc-lacZ expression when Tollo is overexpressed, strongly support the conclusion that Tollo activates the JNK signaling cascade in MNs.

**Tollo genetically interacts with the Jun and Fos transcription factors**

The major downstream effectors of the JNK pathway are the transcription factors Jun and Fos, which function both individually and as a heterodimeric (AP1) complex to regulate expression of various target genes. Both Jun and Fos have previously been implicated in regulating NMJ growth and plasticity (Sanyal et al., 2002, 2003; Collins et al., 2006). Thus, one or both of these transcription factors are likely candidates for mediating...
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or mutations of Spz3 dominantly interact with Tollo (Fig. 8). We then tested deletions that uncover several other Spz family genes for similar dominant interactions with Tollo. Only a few of the family members exhibit a dominant interaction with Tollo, of which Spz3 and Spz5 exhibit the most robust interactions. We subsequently focused on the analysis of Spz3 because it exhibited the strongest dominant interaction with Tollo. Larvae hemizygous for Spz3 and heterozygous for Tollo display a reduction in bouton number compared with controls (Fig. 8, A–C, E, G, I, M, and Q). Error bars indicate SEM. ***, P < 0.001. n ≥ 30. Bar, 20 µm.

the effect of Tollo signaling on NMJ growth. We again used a genetic approach to examine this possibility by testing whether Tollo also exhibits dominant interactions in combination with mutations of Jun (encoded by Jra) and Fos (encoded by kayak; Fig. 6). Larvae doubly heterozygous for both Tollo and Jra mutations (Jra+/+; Tollo+/+) display a significant decrease in bouton number (Fig. 6, A–J and P). We observe a comparable dominant interaction between Tollo and kayak (Fig. 6, K–P). These results support the conclusion that Tollo positively regulates NMJ growth by stimulating Jun and Fos activity via activation of JNK.

Tollo signaling is likely activated by the putative neurotrophin Spz3

To identify other components of the Tollo signaling pathway, we screened deletions to uncover genes that interact with Tollo in a dose-dependent manner to reduce bouton number in Df/+; Tollo/+ larvae. From this screen, we discovered that deletions or mutations of Spz3 dominantly interact with Tollo (Fig. 8). We then tested deletions that uncover several other Spz family genes for similar dominant interactions with Tollo. Only a few of the family members exhibit a dominant interaction with Tollo, of which Spz3 and Spz5 exhibit the most robust interactions. We subsequently focused on the analysis of Spz3 because it exhibited the strongest dominant interaction with Tollo. Larvae hemizygous for Spz3 and heterozygous for Tollo (i.e., Spz3-Df/+; Tollo+/+) display a significant reduction in bouton number compared with controls (Fig. 8, A–F and U). We observe a comparable but somewhat weaker interaction in Spz3+/+; Tollo145/+ larvae, where Spz3 is a hypomorphic P-element insertion allele of Spz3 (Fig. 8, G, I, J, and U). Larvae mutant for Spz3 (Spz3-Df/Spz3) also display a reduction in bouton number compared with controls (Fig. 8, A, B, G, H, and U). Moreover, in larvae hemizygous for a Tollo mutation or carrying a heteroallelic combination of Tollo mutations, the decrease in bouton number is attenuated (Fig. 8, L–P). These results suggest that Tollo signaling is likely activated by the putative neurotrophin Spz3.

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Figure 6. Tollo interacts genetically with components of the JNK pathway to promote NMJ growth. [A–P] Representative confocal images of NMJ3 in larvae of indicated genotypes labeled with FITC–anti-HRP. [Q] Quantification of bouton numbers at NMJ3 in larvae of genotypes shown. Larvae heterozygous for both Tollo and bsk (D, F, and Q), Tollo and Jra (=DJun; J, K, and Q), or Tollo and kay (=DFos; N, O, and Q) or hemizygous for Tollo and heterozygous for bsk, Jra, or kay (H, I, P, and Q) display a reduction in bouton number at NMJ3 compared with control larvae (A–C, E, G, I, M, and Q). Error bars indicate SEM. ***, P < 0.001. n ≥ 30. Bar, 20 µm.
number (Fig. 8, K, M, and U) is further enhanced by a partial reduction of Spz levels (Spz3+/; Tollo145/; Fig. 8, L, N, and U). Conversely, the NMJ overgrowth associated with presynaptic expression of Tollo (c155+/; UAS-Tollo/+; Fig. 3 and Fig. 8, O and U) is suppressed back to wild-type levels by partial reduction of Spz3 (e.g., c155+/; UAS-Tollo/Spz3P; Fig. 3 and Fig. 8, O–Q and U). These results demonstrate that Spz3 is a positive regulator of NMJ growth and most likely functions as a ligand that activates the Tollo receptor. Consistent with this idea, we found that Spz3-Df/Spz3P larvae display a significant reduction in pJNK levels compared with control larvae (Fig. 9), as expected if Spz3 signals through Tollo to promote the phosphorylation of JNK.

During embryonic patterning, Spz is cleaved by Easter, a serine protease, to generate an active Toll ligand (DeLotto and DeLotto, 1998). Thus, it was of interest to determine whether easter (ea) also plays a role in regulating NMJ growth. We observe that ea1+/+ Tollo145 larvae exhibit NMJ undergrowth comparable to that of Spz3+/; Tollo145+/+ larvae (Fig. 8, S and U). NMJs in larvae doubly heterozygous for Spz3 and ea (Spz3+/+; ea+/+) are similarly undergrown (Fig. 8, T and U). Thus, Easter is required for normal NMJ growth, presumably by cleavage and activation of the Spz3 ligand.

Spz3 expression is required postsynaptically to regulate NMJ growth

If Spz3 is a Toll ligand that promotes NMJ growth, what is the source of this signal? To address this question, we examined the effect of tissue-specific reduction in Spz3 expression on NMJ growth. Using the pan-neuronal c155-Gal4 driver to express UAS-Spz3-RNAi (c155>UAS-Spz3-RNAi), we do not observe any effect on NMJ growth (Fig. 10, A–D and R). Moreover, neuronal-specific reduction of Spz3 does not affect NMJ growth even in a sensitized Tollo/+ background (c155/+; UAS-Spz3-RNAi/+; Tollo145+/+; Fig. 10, E, F, and R). In contrast, when the 24B-Gal4 driver was used to reduce Spz3 expression in muscle (24B-Gal4>UAS-Spz3-RNAi), we observe a significant decrease in bouton number (Fig. 10, A, C, G, H, and R). We observe a comparable decrease in NMJ growth when easter expression is reduced in muscle (24B-Gal4>UAS-ea-RNAi; Fig. 10, P–R). Moreover,
boutron number is further reduced in 24B-Gal4>UAS-Spz3-RNAi larvae that are hemizygous for Spz3 (24B-Gal4>UAS-Spz3-RNAi/Spz3-Df) or heterozygous for Tollo (UAS-Spz3-RNAi/+; 24B-Gal4/Tollo145; Fig. 10, J, K, and L).

To further examine the spatial requirement for Spz3 expression, we assessed the phenotypic effects on NMJ growth when Spz3 was overexpressed in different tissues in an otherwise wild-type background. Consistent with our aforementioned results, pan-neuronal expression of Spz3 (c155-Gal4>UAS-Spz3) has no effect on NMJ growth (Fig. 10, M and R), whereas expression in muscle (24B-Gal4>UAS-Spz3) enhances NMJ growth (Fig. 10, N and R). This increase in NMJ growth is mediated through the Tollo pathway because it is fully suppressed by heterozygosity for Tollo (Fig. 10, O and R). Together, these data demonstrate that Spz3, produced and potentially cleaved in muscle, is sufficient to promote NMJ growth by activation of the Tollo receptor at the presynaptic terminal.

**Discussion**

Using a phenotype-based approach, we discovered a novel signaling pathway mediated by the TLR, Tollo, and its presumptive ligand Spz3, a neurotrophin-like molecule that positively regulates NMJ growth in Drosophila larvae via activation of a JNK cascade. These studies define new roles both for TLRs and neurotrophin-like ligands in the regulation of synaptic growth in Drosophila.

**Role of TLRs in nervous system development**

TLRs are highly conserved between Drosophila and vertebrates (Imler and Zheng, 2004). Toll was first identified in Drosophila for its role in dorsoventral patterning in the embryo (Anderson et al., 1985a,b). Subsequent studies revealed that TLRs also play a key role in regulating the innate immune response in
the degradation of inhibitor kB (Cactus in Drosophila; Stathopoulos and Levine, 2002; Minakhina and Steward, 2006). However, for larval NMJ phenotypes, we do not observe any dominant genetic interactions in double mutant combinations of Tollo with dorsal, and overexpression of cactus in the MN does not affect NMJ growth. These results are consistent with previous findings that dorsal and cactus are expressed postsynaptically in muscle (Cantera et al., 1999) to regulate localization of glutamate receptors (Heckscher et al., 2007). In contrast, we do observe dominant phenotypic interactions of Tollo in combination with components of the JNK signaling pathway, including JNK (basket), Jun (Jra), and Fos (kayak). These results strongly suggest that Tollo signals presynaptically through a downstream JNK pathway to positively regulate NMJ growth. Consistent with this idea, we find that pJNK is reduced in boutons in Tollo mutants and expression of the puc-lacZ reporter is increased when Tollo is overexpressed. Although we have not confirmed that total levels of JNK in boutons remain unchanged in Tollo mutants because of technical difficulties associated with ubiquitous expression of JNK in other cell types such as muscle and glia, all of our results support the conclusion that Tollo is signaling through the JNK pathway to regulate NMJ growth. The most parsimonious hypothesis is that signaling through Tollo receptors occurs locally at motor terminals, but the absence of a Tollo antibody, we cannot rule out the possibility that some Tollo receptors are localized at the MN cell body. In any case, execution of the Tollo-dependent growth signal likely requires transcriptional activation of target genes back in the nucleus.

### Tolll signals through the JNK pathway to promote NMJ growth

RNAi expression, transgenic rescue, and expression analysis of a Tolll-Gal4 enhancer trap line demonstrate that Tolll functions presynaptically in MNs to promote NMJ growth, consistent with a previous study demonstrating that Tolll is expressed during formation of neural lineages (Brody et al., 2002). In addition, FlyAtlas analysis (Chintapalli et al., 2007) reveals that Tolll is expressed at high levels in the larval CNS. Recent studies have shown that other Drosophila TLRs are also expressed in the nervous system (McIlroy et al., 2013). Thus, it is very likely that Tolll expression is required within MNs to regulate NMJ growth.

In other developmental contexts in Drosophila and vertebrates, canonical Toll signaling is mediated by activation of NF-κB transcription factors (Dorsal and Dif in Drosophila) via
and Jun share common functions as components of AP1, but they also have distinct functions in certain cells and tissues at particular developmental stages (Riesgo-Escovar and Hafen, 1997). For example, mutations of *hiw* (*highwire*) cause striking NMJ overgrowth via Wind (Wallenda), a MAPKKK protein, which in turn regulates NMJ growth through Fos but not Jun (Collins et al., 2006). Thus, Jun and Fos can function separately in the regulation of NMJ growth. The dominant genetic interactions we observe in heterozygous double mutant combinations of *Tollo* with either Jun or Fos suggest that Tollo can signal through both components of AP1, but whether Jun and Fos are acting individually to regulate NMJ growth or jointly as the AP1 complex remains to be determined.

**Tollo does not impair synaptic transmission**

Despite a 30% reduction in bouton number at NMJ3 in *Tollo* mutant larvae, the basic parameters of synaptic function, such as mEJP size and frequency and quantal content, remain normal.
This uncoupling between NMJ size and function has been observed in many other instances. For example, hiv mutations cause a very striking increase in bouton number, but synaptic transmission is reduced. Moreover, wnd mutations suppress NMJ overgrowth in hiv but not the defect in synaptic transmission (Collins et al., 2006). Thus, there is no direct correlation between morphology and function, presumably because Hiw acts on different downstream targets in the regulation of these two processes.

The lack of correlation between bouton number and synaptic strength is readily explained by Peled and Isacoff (2011), who demonstrate that most presynaptic active zones have a very low release probability. At each NMJ, there are only a small number of high probability release sites and these tend to be located at distal boutons (Peled and Isacoff, 2011). Thus, because overall synaptic transmission relies on relatively few active zones, synaptic function in Tollo mutants may not be affected if the number of high probability release sites remains normal. In addition, some type of homeostatic mechanism may act to compensate for the decrease in bouton number in Tollo mutants based on our observation that the number of active zones per bouton is increased compared with controls. The net result is that the total number of active zones per NMJ is not altered in Tollo mutants. A previous study has shown that glutamate receptors preferentially cluster opposite larger and more active sites (Marrus and DiAntonio, 2004). As another compensatory mechanism to maintain synaptic function, it may be possible that active zones are larger in Tollo mutants and thus have greater synaptic strength.

The absence of a defect in synaptic function in our experiments contrasts with a previous study showing that AP1 overexpression that the number of active zones per bouton is increased compared with controls. The net result is that the total number of active zones per NMJ is not altered in Tollo mutants. A previous study has shown that glutamate receptors preferentially cluster opposite larger and more active sites (Marrus and DiAntonio, 2004). As another compensatory mechanism to maintain synaptic function, it may be possible that active zones are larger in Tollo mutants and thus have greater synaptic strength.

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The identification of Spz3 as a likely Tollo ligand is of particular interest because Spz and the Spz-like ligands are cysteine knot growth factors proposed to be Drosophila neurotrophins based on their structural homology with vertebrate neurotrophins (in particular brain-derived neurotrophic factor; DeLotto and DeLotto, 1998; Parker et al., 2001; Zhu et al., 2008). In the developing Drosophila embryo, Spz2 (renamed DNT1) functions similarly to vertebrate neurotrophins by promoting cell survival in the CNS and affecting motor axon targeting (Zhu et al., 2008). Mutations in Spz5 (DNT2) influence neuronal survival with modest effects on axon targeting (Zhu et al., 2008). In addition to our findings with Spz3, Sutcliffe et al. (2013) recently found that Spz, DNT1, and DNT2 also regulate NMJ growth in a cell-specific manner. Thus, Spz affects NMJ4, whereas DNT1 and DNT2 affect NMJ6/7. Although the precise effect on NMJ growth varies with different Spz/DNT ligands and specific motor terminal affected, our studies together with those of Sutcliffe et al. (2013) demonstrate that Spz-like ligands play important and previously unrecognized roles in regulating NMJ development.

Spz3, a neurotrophin-like ligand, signals through Tollo at the NMJ

In an unbiased screen for genes that interact with Tollo at the NMJ, we found that Spz3 genetically interacts with Tollo to promote NMJ growth. Similar to Tollo mutants, Spz3 mutants display a decrease in bouton number, indicating that Spz3 positively regulates NMJ growth. Our genetic analyses strongly suggest that Spz3 is expressed in abdominal muscles and is a ligand for the Tollo receptor at the NMJ. First, Spz3 and Tollo interact in a dominant, dose-dependent manner to cause NMJ undergrowth. Second, a partial reduction in Spz3 levels suppresses NMJ overgrowth associated with overexpression of Tollo in MNs. Third, RNAi knockdown of Spz3 in muscles causes NMJ undergrowth that becomes enhanced in a heterozygous Tollo mutant background. Fourth, heterozygosy for a Tollo mutation completely suppresses the NMJ overgrowth associated with overexpression of Spz3 in muscles. This interpretation is consistent with a recently published parallel study showing that other Spz-like ligands are expressed in larval abdominal muscles (Sutcliffe et al., 2013) and with FlyAtlas (Chintapalli et al., 2007) data indicating high levels of Spz3 expression in larval carcasses, which are enriched for abdominal muscles. Together, these genetic data imply that Spz3 functions through the Tollo receptor to promote NMJ growth, but direct biochemical evidence of Spz3 as the Tollo ligand awaits confirmation.

Tollo genetically interacts with components of the JNK pathway to promote NMJ growth, and loss of Tollo leads to a decrease in the levels of pJNK at the NMJ. Because our data indicate that Spz3 signals through Tollo at the NMJ, we examined the levels of pJNK at the NMJ and found a significant decrease in Spz3 mutant larvae relative to controls. Thus, the data suggest a signaling pathway at the NMJ whereby Spz3 activates the Tollo receptor, which then activates the JNK signaling cascade.

The identification of Spz3 as a likely Tollo ligand is of particular interest because Spz and the Spz-like ligands are cysteine knot growth factors proposed to be Drosophila neurotrophins based on their structural homology with vertebrate neurotrophins (in particular brain-derived neurotrophic factor; DeLotto and DeLotto, 1998; Parker et al., 2001; Zhu et al., 2008). In the developing Drosophila embryo, Spz2 (renamed DNT1) functions similarly to vertebrate neurotrophins by promoting cell survival in the CNS and affecting motor axon targeting (Zhu et al., 2008). Mutations in Spz5 (DNT2) influence neuronal survival with modest effects on axon targeting (Zhu et al., 2008). In addition to our findings with Spz3, Sutcliffe et al. (2013) recently found that Spz, DNT1, and DNT2 also regulate NMJ growth in a cell-specific manner. Thus, Spz affects NMJ4, whereas DNT1 and DNT2 affect NMJ6/7. Although the precise effect on NMJ growth varies with different Spz/DNT ligands and specific motor terminal affected, our studies together with those of Sutcliffe et al. (2013) demonstrate that Spz-like ligands play important and previously unrecognized roles in regulating NMJ development.

Since their discovery in vertebrates, numerous studies have characterized the role of these growth factors in multiple different aspects of neuronal survival, growth, function, and plasticity (Chao, 2003; Park and Poo, 2013). However, only recently have neurotrophin-like proteins been found in invertebrates, and their identification as bona fide neurotrophins still engenders some controversy. A neurotrophin-like protein (ApNT) whose structural and functional properties are conserved with vertebrate neurotrophins was recently reported in Aplysia (Kassabov et al., 2013). Importantly, this study also identified a conserved Trk receptor (ApTrk) through which ApNT functions to regulate neurite...
outgrowth and long-term facilitation (Kassabov et al., 2013). Vertebrate neurotrophins signal either through Trk or p75 receptors, and identification of a corresponding receptor in *Aplysia* strongly argues that ApNT is a true invertebrate neurotrophin. Conversely, Kassabov et al. (2013) question the identity of Spz and Spz-like ligands as bona fide neurotrophins, emphasizing the fact that an orthogonal neurotrophin receptor has not been identified in *Drosophila*. In this context, it is of interest that the ligand binding domain of TLRs is similar to that of the ligand binding domain of Trk receptors (Bothwell, 2006). In addition, Toll and p75NTR activate NF-κB signaling through interleukin-1 receptor-associated kinase, MyD88, and TRAF proteins (O’Neill and Greene, 1998; Zapata et al., 2000; Mamidipudi et al., 2002; Bothwell, 2006; Valanne et al., 2011), and vertebrate p75NTR can bind *Drosophila* TRAF1 (Zapata et al., 2000). Similar to our findings with Tollo at the larval NMJ, the vertebrate p75NTR can also signal through the JNK cascade (Roux and Barker, 2002; Chao, 2003). Thus, the identity of Spz3 and other family members as true *Drosophila* neurotrophins cannot be readily dismissed. In any case, our study convincingly demonstrates that Spz3, a protein with neurotrophin-like features, functions in a neurotrophic-like manner to promote NMJ growth. We have focused on the interaction between Spz3 and Tollo. However, we note that our data do not exclude the possibility that other Spz-like ligands can signal through Tollo or that Spz3 can signal through other TLRs at the NMJ. Similar to vertebrate neurotrophins, Spz and its relatives including Spz3 require proteolytic cleavage for activation (DeLotto and DeLotto, 1998; Park and Poo, 2013). Spz is cleaved in the developing embryo by the secreted serine protease, Easter. Genetic interactions between *ea* and *Spz3* as well as between *ea* and *Tollo* suggest that Spz3 is also cleaved and activated by Easter in muscles during NMJ growth regulation.

In summary, we have uncovered a novel retrograde signaling pathway that promotes growth of the larval NMJ. We propose that Spz3, a neurotrophin-like ligand, is secreted by muscle and activates presynaptic Tollo receptors thereby triggering a downstream JNK cascade that culminates in activation of Jun and Fos and transcription of their target genes that ultimately dictate NMJ growth. This study provides important new information about regulation of NMJ development and also defines a novel role for Tollo receptors in synaptic development. It will be of interest to determine how this retrograde signaling pathway is integrated with other signaling pathways, such as BMP signaling, which also acts in a retrograde manner to stimulate NMJ growth.

**Materials and methods**

**Fly stocks**

*w1118* was used as a wild-type control for genetic background. Tollo1169, which is an imprecise P-element excision that removes the genomic sequence from −1,560 to −351 bp upstream of the translational start site, and Tollo11769, where P{lacW} is inserted 439 bp upstream of the translational start site (Kim et al., 2006), were provided by J. Yim (Seoul National University, Seoul, Korea). Tollo2, which is an imprecise P-element excision that removes 2,247 bp including part of the Tollo gene and the translational start site, and TolloGal4, which is a Gal4 enhancer trap of Tollo (Ayyar et al., 2007), were provided by P. Simpson (University of Cambridge, Cambridge, UK). cact511 (Govind et al., 1993), which is a hypomorphic allele of cactus generated by EMS mutagenesis, and UAS-cactus (Qiu et al., 1998) were provided by S. Govind (The City College of New York, New York, NY). puc-lacZ261 (Martin-Blanco et al., 1998), which is a lacZ enhancer trap of puc, was provided by D. Bohmann (University of Rochester, Rochester, NY). The following fly lines were obtained from the Bloomington Stock Center: Df(3R)BSC578 (Tollo deficiency), Df(2R)BSC234 (Spz3 deficiency), Spz32527 (Spz3-2), Spz32501 (Spz3-3), hsk13750, kay1, eya1, elav1-12G, 24B-Gal4, and UAS-Gal80ts. The following RNAi lines that target different *Drosophila* RNAi Center: UAS-Tollo-RNAi (13549), UAS-Spz3-RNAi (18949), and UAS-eya-RNAi (102357). For a complete list of all genotypes examined in this study and their genotype counts, see Table S1. All crosses were performed on cornmeal/molasses fly medium and maintained at 25°C.

**Immunohistochemistry**

Female larvae were dissected in Ca2+-free saline and fixed in 4% paraformaldehyde for 18–20 min or in cold methanol for 5 min (Glur antibodies). Larval body walls were incubated in primary and secondary antibodies overnight at 4°C. Body wall preparations were mounted in VectaShield (Vector Laboratories) for microscopy analysis. Larval brains were placed on polylysine–coated coverslips, dehydrated through ethanol series, cleared in xylens, and then mounted in DPX (Sigma-Aldrich). The following antibodies were used: FITC- or Cy3-conjugated anti-HRP (Jackson ImmunoResearch Laboratories, Inc.) at 1:100, rabbit or chicken anti-GFP (Invitrogen) at 1:1,000, rabbit anti-pJNK (Cell Signaling Technology) at 1:1,000, rabbit anti-βgal (MP Biomedicals) at 1:1,000, rat anti-ela2 (Developmental Studies Hybridoma Bank) at 1:100, mouse anti-nc82 (Epic Developmental Studies Hybridoma Bank) at 1:250, mouse anti-GlurA1 at 1:100 (Developmental Studies Hybridoma Bank), rabbit anti-GLRIBII (raised against ASSAKKKKTIRRIK in Marrus et al. [2004]) at 1:2,500, and rabbit anti-GLRIII (raised against GQSGSSSGSN-NAGGERKEARV in Marrus et al. [2004]) at 1:1,000 (A. DiAntonio, Washington University, St. Louis, MO). Species-specific Alexa 488, 568, or 647 secondary antibodies were used at 1:500 (Invitrogen).

**Imaging and quantification**

Quantification of bouton number was performed at NMJ3 because of the expression pattern of Tollo-Gal4 (Ayyar et al., 2007) and its relative specificity. However, comparable phenotypes were observed at other NMJs where Tollo-Gal4 is expressed. Segments A2–A4 were analyzed for bouton number and branch length. For quantification, we defined a bouton as a discrete synaptic swelling labeled with the presynaptic marker eHRP. The muscle size was similar in all larvae examined. At least 10 NMJs of each genotype were analyzed, and all genotypes were examined simultaneously for each experiment. Confocal images were obtained at 21–23°C on a confocal microscope (LSM 510; Carl Zeiss) with a Plan Apochromat 63× NA 1.4 oil differential interference contrast objective and accompanying software. Images were processed in ImageJ (National Institutes of Health) and Photoshop (Adobe) software. Branch length was determined using ImageJ, where arbors of primary and secondary branches were measured starting at the first bouton or branch point after defasciculation [which occurred first]. pJNK and puc-lacZ fluorescence intensities were measured using methods described previously (Mosca and Schwartman, 2010a,b). For puc-lacZ analysis, confocal z stacks of distal and proximal boutons were imported into ImageJ and converted into multichannel composite images. A region of interest was drawn around the terminal bouton as well as an internal bouton based on the HRP fluorescence channel. The mean fluorescence intensity was determined for the HRP channel and the pJNK channel. For puc-lacZ analysis, 2 µm of confocal slices of the ventral region of the abdominal VNC were imported into ImageJ and converted into multichannel composite images. A region of interest was taken of abdominal segments that contained puc-lacZ-positive nuclei that also expressed Tollo-Gal4. The mean fluorescence intensity was determined for the anti-eHHRP channel and anti-βgal channel. All samples were processed under the same conditions and imaged using the same confocal parameters. The ratio of pJNK to HRP or puc-lacZ to Elav fluorescence was then determined. The fluorescence intensity for an internal and a terminal bouton was measured for 10 NMJs or for six abdominal ganglia. To determine muscle or bouton volume, confocal stacks were imported into Amira software (Visualization Sciences Group). Muscles from segment A3 (n = 10) or individual boutons (n ≥ 89) were reconstructed by selecting and assigning pixels throughout the series of images in the confocal stack. Muscle or bouton volume was measured using the approximate voxel dimensions (in micrometers).

**Molecular cloning**

UAS-Tollo comprises the full-length Tollo sequence cloned into pUAST-attB. cDNA constructs were generated by amplification of the Tollo coding sequence from a full insert cDNA clone (Berkeley Drosophila Genome Project LD33590). UAS-Spz3 comprises a full-length Spz3 sequence cloned into
pUAST-atb. cDNA constructs were generated by amplification of the Spz3 sequence from a full insert cDNA clone (Berkeley Drosophila Genome Project RE22741). After germline transformation, UAS-Tollo and UAS-Spz3 were inserted into attP40 and attP2, respectively, in a w1118 background. To facilitate directional cloning into pUAST-atb, primers were created with unique restriction sites that flank the open reading frames. The sequences of primers used are provided in Table S2. All constructs were sequenced for accuracy.

Electrophysiology

Electrophysiology was performed on muscle 3 in segments A2–A5 of wandering third instar larvae using standard techniques (Jan and Jan, 1976). Dorsal-ventral recordings were performed in HL3.1 containing 0.0 mM Ca2+, and intracellular recordings were performed in HL3.1 containing the indicated Ca2+ concentration (Feng et al., 2004). Recording electrodes [resistance: 15–20 MΩ] were filled with 3M KCl and stimulating electrodes with saline. Recordings were acquired using pClamp10 software (Molecular Devices). Mean EJP amplitudes were calculated from 40 consecutive traces (20–60 of 60 stimulations). Mean mEJP amplitude and frequency were determined using Mini Analysis Software (v. 6.0.7, Synaptosoft). Quantal content was determined by dividing the mean EJP amplitude of a synapse by the mean mEJP amplitude from the same synapse. For this calculation, EJP amplitudes were corrected for nonlinear summation according to McPälltan and Martin (1981).

Statistical analyses

Error bars represent SEM. We performed one-way analysis of variance followed by post-hoc tests with Tukey-Kramer correction for multiple comparisons. We report the significance values to be <0.05, 0.01, or 0.001 denoted by one, two, or three asterisks, respectively.

Online supplemental material

Fig. S1 shows that muscle volume, bouton volume, and HRP levels are unaltered in Tollo mutants. Fig. S2 shows that branch length at NMJ3, bouton number at NMJ3, and bouton number at NMJ3 in segments in abdominal segment A2, A3, or A4 are significantly reduced in Tollo mutants compared with control larvae. Fig. S3 shows that synaptic function is not altered in Tollo mutants at NMJ3 in 0.4 mM Ca2+. Recording electrodes [resistance: 15–20 MΩ] were filled with 3M KCl and stimulating electrodes with saline. Recordings were acquired using pClamp10 software (Molecular Devices). Mean EJP amplitudes were calculated from 40 consecutive traces (20–60 of 60 stimulations). Mean mEJP amplitude and frequency were determined using Mini Analysis Software (v. 6.0.7, Synaptosoft). Quantal content was determined by dividing the mean EJP amplitude of a synapse by the mean mEJP amplitude from the same synapse. For this calculation, EJP amplitudes were corrected for nonlinear summation according to McPältlan and Martin (1981).

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