Development 137, 2139-2146 (2010) doi:10.1242/dev.046672

Drosophila Tey represses transcription of the repulsive cue Toll and generates neuromuscular target specificity

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SUMMARY

Little is known about the genetic program that generates synaptic specificity. Here we show that a putative transcription factor, Teyrha-Meyhra (Tey), controls target specificity, in part by repressing the expression of a repulsive cue, Toll. We focused on two neighboring muscles, M12 and M13, which are innervated by distinct motoneurons in *Drosophila*. We found that *Toll*, which encodes a transmembrane protein with leucine-rich repeats, was preferentially expressed in M13. In *Toll* mutants, motoneurons that normally innervate M12 (MN12s) formed smaller synapses on M12 and instead appeared to form ectopic nerve endings on M13. Conversely, ectopic expression of *Toll* in M12 inhibited synapse formation by MN12s. These results suggest that Toll functions in M13 to prevent synapse formation by MN12s. We identified Tey as a negative regulator of *Toll* expression in M12. In *tey* mutants, *Toll* was strongly upregulated in M12. Accordingly, synapse formation on M12 was inhibited. Conversely, ectopic expression of *tey* in M13 decreased the amount of *Toll* expression in M13 and changed the pattern of motor innervation to the one seen in *Toll* mutants. These results suggest that Tey determines target specificity by repressing the expression of Toll. These results reveal a mechanism for generating synaptic specificity that relies on the negative regulation of a repulsive target cue.

KEY WORDS: Target recognition, Transcriptional regulation, Neuromuscular connectivity, *Drosophila*, Toll, Tey, Muscle, Motoneuron, Synapse, Synaptic specificity

INTRODUCTION

A remarkable feature of the nervous system is the precision of its circuitry. A neural circuit develops through a series of neuronal recognition events. First, neurons find their path, turn at mid-way guideposts, and fasciculate or defasciculate before reaching their final target area (Tessier-Lavigne and Goodman, 1996). Then, neurons select and form synapses with specific target cells in the target region. The final matching of pre- and post-synapses is thought to be mediated by specific cues expressed on the target cells (Benson et al., 2001; Holt and Harris, 1998). However, the regulation and function of such cues remain poorly understood.

The process of neuromuscular targeting in *Drosophila* features highly stereotypic matchings between 37 motoneurons and 30 target muscle cells, providing a unique model system for the study of neuronal target recognition (Keshishian et al., 1996; Chiba, 1999). Several target cues, including Capricious, Netrin-B and Fasciclin 3 (Shishido et al., 1998; Mitchell et al., 1996; Chiba et al., 1995), have been identified that are expressed in specific target cells and mediate attractive interactions between the synaptic partners. It has recently been shown that target specificity is also regulated by repulsion from non-target cells. Wnt4, a member of the Wnt family of secreted glycoproteins, is expressed in muscle 13 (M13) and prevents synapse formation by motoneurons targeted to a neighboring muscle, M12 (Inaki et al., 2007). In the absence of Wnt4, motoneurons targeted to M12 form ectopic nerve endings on M13, indicating that Wnt4 repulsion on M13 is required for proper targeting of the motoneurons. In addition to Wnt4, Toll and Semaphorin II (Sema-2a – FlyBase) are known to function as negative regulators of synapse formation in this system. However, whether they have a role in target selection remains unknown (Winberg et al., 1998; Rose et al., 1997).

Another unsolved issue is how the expression of such attractive or repulsive target-recognition molecules is regulated. It is amazing that the expression of these molecules is so precisely regulated that they are present at the right time and place. It is likely that the expression of these molecules is determined as part of the differentiation program of the target cells. However, little is known about the molecules and mechanisms involved. Several transcription factors, such as S59 (Lethal of Seto 59 – FlyBase), Krüppel and Vestigial, have been identified as being expressed in subsets of muscle cells. They are expressed from the progenitor stage, and their loss-of-function (LOF) and gain-of-function (GOF) alter the specific characteristics of the individual muscles, such as their size, shape, orientation and attachment sites to the epidermis, indicating that they function as determinants of a particular muscle fate (Dohrmann et al., 1990; Ruiz-Gomez et al., 1997; Baylies et al., 1998). However, whether these transcription factors regulate the expression of target-recognition molecules and thus determine the innervation pattern is unknown.

We have previously conducted a comparative microarray analysis of two neighboring target muscles, M12 and M13, which are innervated by distinct motoneurons (Inaki et al., 2007). By comparing the expression profile of the two muscles, we tried to understand the molecular mechanisms that make these muscles distinct targets for the motoneurons. From this screening, we identified ~25 potential target-recognition molecules as preferentially expressed in either muscle cell. Among them was Wnt4, mentioned above. Here, we report the functional analyses of two additional genes that were identified in the screening: *Toll* and

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Accepted 25 April 2010

teyrha-meyrha (*tey*). *Toll* encodes a transmembrane protein with extracellular leucine-rich repeats, and has multiple functions in development (Hashimoto et al., 1988). Toll is expressed in subsets of muscles, including 6, 7 and 15-17 (Nose et al., 1992; Rose et al., 1997). Previous studies have shown that Toll inhibits synapse formation by RP3, a motoneuron targeted to muscles 6 and 7 (Rose et al., 1997). Here, we show that *Toll* is preferentially expressed in M13 over M12 and, like Wnt4, inhibits synapse formation by motoneurons targeted to M12. We also show that *tey*, a previously uncharacterized gene, regulates the expression of *Toll* in specific muscles. *tey* is expressed specifically in M12, where it negatively regulates *Toll* expression. In the absence of *tey*, *Toll* is ectopically expressed in M12 and innervation of M12 is inhibited. These results suggest that Tey regulates targeting by downregulation of the repulsive cue Toll specifically in M12. Based on these results, we propose a mechanism for the generation of synaptic specificity that relies on negative regulation of repulsive target cues.

MATERIALS AND METHODS

Fly strains and phenotypic analysis

We used a transheteroallelic combination of *Df(3R)Tl9QRX* and *Df(3R)TlroXB3* as *Toll*-null mutants (Rose et al., 1997). For double mutants of *Toll* and *Wnt4*, we used the same combination of *Toll* alleles and $Wnt4^{P23}/Wnt4^{EMS23}$ (Sato et al., 2006; Inaki et al., 2007). For GOF analyses of *Toll*, we used *Mhc-Toll*, in which *Toll* is induced in all muscle cells (Rose et al., 1997), and *UAS-Toll* (EP1051) (Rørth, 1996) crossed with *5053A-Gal4* (see below). A *lacZ* insertion in the *Toll* locus, AK80 (Nose et al., 1992), was used as a reporter of *Toll* expression. *UAS-myristylated-GFP* (*mGFP*) was used for labeling M12 (Ritzenthaler et al., 2000). *5053A* and *H94* are Gal4 drivers that were used to induce gene expression in M12 and M13, respectively (Ritzenthaler et al., 2000; Davis et al., 1997). *5053A* is also a putative null allele of *tey* (see below), and *Df(3L)Exel6135* is a deficiency of the *tey* locus. *UAS-tey* was generated by inserting a *tey* EST, RE59010, into the pUAST P-element transformation vector using *Kpn*I and *Not*I sites present within the EST clone. Sequencing of RE59010 confirmed that it encodes a full-length protein of 717 amino acids (which differs from RE33994 used for a previous UAS-construct that contains a single nucleotide deletion, leading to a truncated protein of 313 amino acids) (Jacobson et al., 2006).

Phenotypic analysis of *tey*

tey had been identified in our previous microarray analysis (Inaki et al., 2007). *tey* showed an average 13.2-fold enrichment in M12 compared with M13 in the microarray analysis. In quantitative RT-PCR, *tey* was detected only in M12 and not in M13 (data not shown). In situ hybridization (Lehmann and Tautz, 1994) also confirmed M12-specific expression. tey^{5053A} is a Gal4 line in which a P element is inserted in the *tey* locus. We cloned the region in the vicinity of the insertion site by inverse PCR and found that the P element was inserted in the first exon of the *tey* gene.

Generation of an antibody against Tey

The open reading frame of *tey* was cloned as an *Eco*RI (introduced at the initiator ATG)/*Not*I (within the 3′UTR) fragment into the pET-30a expression vector (Novagen). The bacterially expressed and Ni-agarose-purified protein was used for immunization of guinea pigs.

Immunohistochemistry and quantification of the phenotype

Immunohistochemical staining of dissected embryos was performed as described previously (Nose et al., 1997). Antibodies used were monoclonal 1D4 (anti-Fasciclin 2; 1:10) (Nose et al., 1997), monoclonal nc82 (anti-Bruchpilot; 1:100) (Wagh et al., 2006), rabbit anti-Toll (1:50) (Nose et al., 1992), guinea pig anti-Tey (1:500), anti-HRP (1:4000; Jackson, West Grove, PA, USA) (Jan and Jan, 1982), and anti-β-galactosidase (1:2000; Cappel, Aurora, OH, USA). Confocal images were acquired with an LSM 510 (Zeiss, Oberkochen, Germany) or FV1000 (Olympus, Tokyo, Japan) microscope. To quantify the terminal size on M12 and M13 in LOF and GOF experiments, we measured the total area of axonal arborization on each muscle with IPlab software (Scanalytics, Fairfax, VA, USA) and normalized it to that of the muscle. The intensity of *Toll-lacZ* expression was defined as the total intensity of the area with signals above background intensity. The number of active zones in the nascent synapse was defined as the number of anti-Bruchpilot-staining puncta within the area of pre-synaptic varicosity (visualized by anti-HRP), which is surrounded by the post-synaptic membrane of M12 (visualized by mGFP).

RESULTS

Toll is required for proper targeting of M12 and M13

In the *Drosophila* neuromuscular system, 37 motoneurons innervate 30 muscles in a highly stereotypic manner in each abdominal hemi-segment. We focused on two neighboring target muscles, M12 and M13, which are innervated by distinct motoneurons. M12 is innervated by the V and RP5 motoneurons (collectively called MN12s), whereas M13 is innervated by RP1 and RP4 (MN13s) (Fig. 1A). These motoneurons extend their axons through the intersegmental nerve b (ISNb) pathway, make transient contacts with surrounding muscles and finally form separate endings on their own target. In wild-type embryos, MN12s and MN13s nerve terminals form as arborizations along the ventral edge of M12 and M13, respectively [see Fig. 1C; all motor axons are labeled with anti-Fasciclin 2 (Fas2) antibody].

Toll encodes a transmembrane protein with extracellular leucine-rich repeats. As described above, Toll is known to be expressed in subsets of muscles including ventral muscles 6, 7 and 15-17 (Nose et al., 1992; Halfon et al., 1995; Rose et al., 1997). We identified *Toll* in our previous microarray screening as being preferentially expressed in M13 compared with M12 (Inaki et al., 2007). M13 preference was also verified by quantitative RT-PCR (Inaki et al., 2007). We further confirmed the differential expression by studying *lacZ* expression in an enhancer-trap line of *Toll*, AK80 (Nose et al., 1992). The level of *lacZ* expression was indeed 5-fold higher in M13 than in M12 (Fig. 1B). We noted, however, that the level of expression in M13 was relatively low compared with other Toll-positive muscles (e.g. muscles 15 and 16).

Since Toll is known to inhibit synapse formation of RP3 motoneurons (targeted to muscles 6 and 7) (Rose et al., 1997), we asked whether differential expression of Toll in M12 and M13 might regulate the targeting of these muscles by local inhibition. To this end, we studied the M12/M13 targeting in *Toll* mutants. In *Toll* mutants, muscles and major motor nerves showed largely normal development. However, targeting of ISNb was specifically altered. As previously reported, the nerve endings at the cleft between muscles 6 and 7 were reduced in size (Fig. 1D) (Rose et al., 1997). In addition, we found that the nerve terminals synapsed to M12 were greatly reduced (Fig. 1D). Furthermore, the nerve terminals synapsed to M13 were expanded. These phenotypes are very similar to those observed in *Wnt4* mutants. We previously used single-cell labeling in *Wnt4* mutants to show that expansion of M13 terminals is caused by the formation of ectopic endings by MN12s (Inaki et al., 2007). The similarity of the phenotypes suggests that *Toll* mutant phenotypes also result from mistargeting of MN12s. Thus, Toll may function in M13 to prevent inappropriate innervation by MN12s. We quantified the phenotypes by measuring the average area of the nerve terminals along the muscles (Fig. 1F): M12 terminals were 57.0±4.6 (n=54) in *Toll* mutants, compared with 100±4.1 (n=40) in the control, whereas M13 terminals were 92.4±4.5 (n=54) in *Toll* mutants, compared with 67.7±4.3 (n=40) in the control (normalized to the size of M12 terminals in controls; P<0.001, Student's t-test). The quantitative

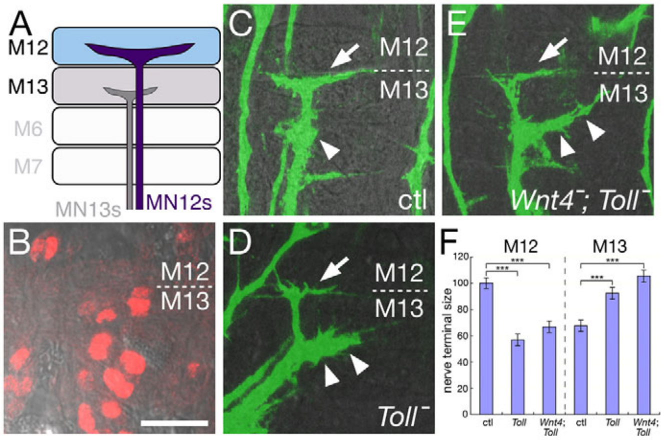

Fig. 1. Toll is required for precise targeting of M12 and M13. (**A**) Schematic of neuronal targeting of M12 and M13 by MN12s and MN13s, respectively, along the ventral edge of the muscle. (**B-E**) Fillet preparations of late stage 16 *Drosophila* embryos. (B) *Toll-lacZ* is highly expressed in M13, but at very low levels in M12. (C-E) Anti-Fas2 antibody staining (green) to visualize all nerve terminals. (C) *yw* control (ctl). In *Toll* single (D) and *Toll Wnt4* double (E) mutants, the nerve endings on M12 are smaller (arrows) whereas those on M13 are enlarged (arrowheads). (**F**) Quantification of the phenotypes by the average size of the nerve endings on M12 and M13. ***, *P*<0.001 by Student's *t*-test. Data represent mean ± s.e.m. Scale bar: 10 µm.

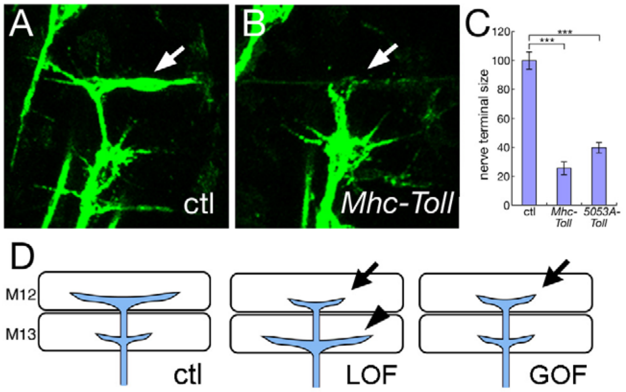

Fig. 2. Ectopic expression of *Toll* inhibits terminal formation in M12. (**A**,**B**) Innervation pattern of M12 in control (*yw*, A) and *Mhc-Toll* (B) *Drosophila* embryos, visualized with anti-Fas2 antibody. In *Mhc-Toll* embryos, the size of nerve endings on M12 was reduced (arrow). (**C**) Quantification of the reduction of nerve ending size on M12: 25.6±4.4 (*n*=43) in *Mhc-Toll* and 39.7±3.5 (*n*=25) in *5053A-Toll*, compared with 100±6.0 (*n*=42) in control; ***, *P*<0.001 by Student's *t*-test. Data represent mean ± s.e.m. (**D**) Schematic of loss-of-function (LOF) and gain-of-function (GOF) phenotypes of *Toll* versus control. Arrows, M12 terminals; arrowheads, M13 terminals.

analyses show that *Toll* LOF affects the size of M12 and M13 terminals to similar degrees as *Wnt4* LOF (Inaki et al., 2007). Simultaneous knockout of *Toll* and *Wnt4* did not significantly enhance the phenotypes (Fig. 1E,F) [M12 terminals, 66.8±4.4 (*n*=44); M13 terminals, 105.6±4.6 (*n*=44)]. Thus, Wnt4 and Toll may function in the same signaling pathway to regulate targeting of the muscles. Additional molecules might also be involved in the targeting of the muscles (see Discussion).

Toll misexpression inhibits targeting of M12

If Toll functions in M13 to inhibit synapse formation by MN12s, one would expect that ectopic expression of Toll in M12 would inhibit synapse formation on this muscle. We examined motoneuron targeting in *Mhc-Toll* embryos, which strongly express *Toll* in all muscles (Rose et al., 1997). As expected, the nerve terminals along M12 were greatly reduced in size in *Mhc-Toll* embryos (Fig. 2A-C). As described previously (Rose et al., 1997), misexpression of *Toll* in all muscles also affects the innervation of subsets of other ventral muscles including 6, 7, 15 and 16. However, the projection of other motor nerves (e.g. ISN and SNa) appeared normal (see Fig. S1 in the supplementary material). The reduction in M12 terminals was also observed when *Toll* was specifically misexpressed in M12 using the Gal4-UAS system (Fig. 2C). These results indicate that Toll inhibits synapse formation by MN12s. Taken together with the LOF phenotypes (as summarized in Fig. 2D), these results suggest that Toll, like Wnt4, functions as a repulsive cue on M13 to prevent targeting by MN12s.

Characterization of the *tey* locus

The results of *Toll* misexpression indicate that downregulation of *Toll* in M12 is crucial for proper targeting of this muscle. We next asked how the expression of *Toll* is regulated. Since *Toll* is expressed in almost all ventral muscles except for M12 (Fig. 1B), we hypothesized that *Toll* expression is specifically repressed in M12. As candidates that might be involved in the repression, we studied the function of M12-enriched genes identified in our microarray analyses. We found that one such gene, which we named *teyrha-meyrha* (*tey*), is involved in the regulation of *Toll* expression. *tey* encodes a nuclear protein with no homology to any proteins outside of the insect clade (see below). We confirmed preferential M12 expression of *tey* by quantitative RT-PCR and by in situ hybridization. In situ hybridization showed that *tey* expression is highly specific, being expressed in M12 but not in any other muscles in the body wall (Fig. 3B). Staining with an antibody against Tey (see Materials and methods) showed that the protein is specifically localized in the nuclei of M12 (Fig. 3D). M12-specific expression was further verified by expressing GFP from a *Gal4* insertion in the *tey* locus (*tey*^{5053A}-*Gal4*) (Fig. 3E). *tey* was also expressed in subsets of interneurons in the ventral nerve cord, but not in motoneurons (see Fig. S2 in the supplementary material).

We found that this *Gal4* insertion line, *tey*^{5053A}-*Gal4*, disrupts the *tey* locus and used it to study the role of the gene (Fig. 3A). *tey*^{5053A} appears to be a null allele of *tey* for the following reasons. First, *tey* transcripts were undetectable by in situ hybridization in *tey*^{5053A} embryos (Fig. 3B,C). Second, *tey*^{5053A} homozygotes and transheterozygotes of *tey*^{5053A} over a deficiency showed similar phenotypes (see below). The presence of a *Gal4* insertion allowed visualization of M12, a normally *tey*-expressing muscle, in the mutants. We thus used this convenient marker to identify and examine M12 in the mutants. In *tey* mutant embryos, M12 appeared to differentiate into a muscle fiber of normal size. However, the positions of the attachment sites were specifically altered (Fig. 3E-H). Normally, M12 is the most distal among the ventral muscles. In *tey* mutants, the position of M12 was shifted

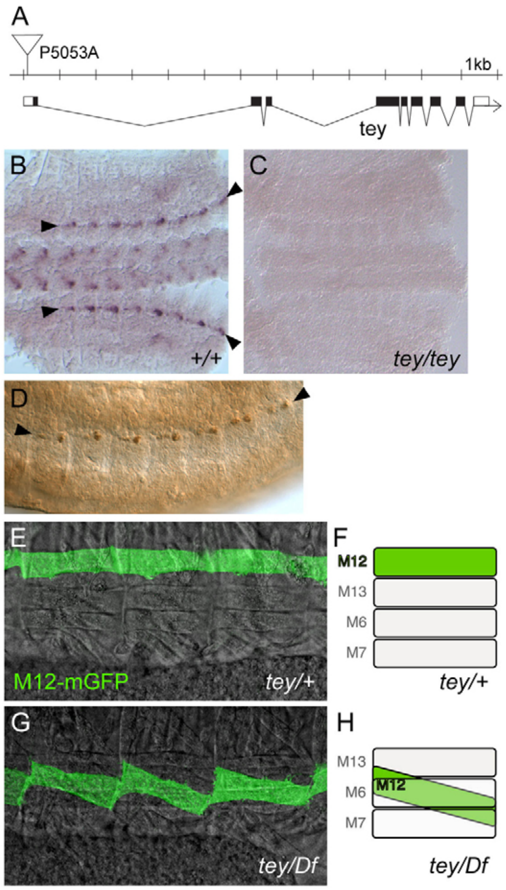

Fig. 3. *tey* is specifically expressed in M12. (**A**) Genomic structure of the *Drosophila tey* locus. In *5053A*, a P element is inserted in the 5′ untranslated region of the *tey* gene. (**B**,**C**) In situ hybridization of *tey*. Filleted preparation of wild-type (B) and *tey*^{5053A} homozygous (C) embryos. *tey* is expressed exclusively in M12 (arrowheads). The *tey* transcripts are not detected in the *tey* mutant. (**D**) A stage 14 embryo stained with anti-Tey antibodies. Tey is expressed in the nuclei of M12 (arrowheads). (**E-H**) mGFP expression in M12 driven by *5053A-Gal4* (E,G) and schematic interpretations thereof (F,H). (E,F) In heterozygotes, M12 was longitudinally aligned in the most dorsal position of the ventral muscle group. (G,H) In the *tey* mutant, attachment sites of M12 were specifically altered. M12 became oblique and was located ventral to M13, underneath (external to) muscles 6 and 7.

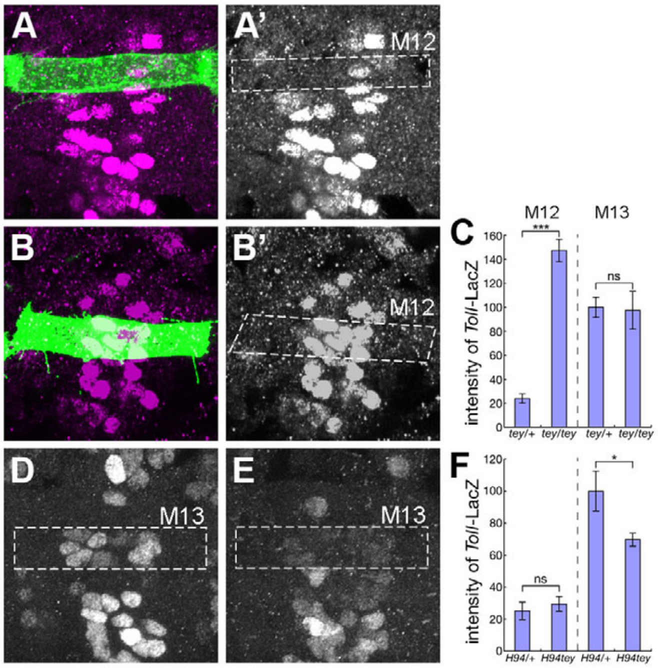

Fig. 4. *Toll* is negatively regulated by *tey*. (**A-B′**) *Toll-lacZ* (magenta) is highly expressed in M13 and at low level in M12 (boxed) of *tey*^{5053A}/+ *Drosophila* embryos (A,A′). *Toll-lacZ* is strongly upregulated in M12 of *tey*^{5053A} homozygous embryos (B,B′). Nuclear expression of *Toll-lacZ* in M12 was distinguished from that in neighboring muscles by localization within the membrane of M12 expressing mGFP (green). The *Toll-lacZ* channel is shown alone in A′,B′. (**D**,**E**) Conversely, *Toll-lacZ* was downregulated in M13 (boxed) when *tey* was driven in M13 using *H94-Gal4* (E), as compared with *H94-Gal4*/+ control (D). (**C**,**F**) Quantification of the LOF (C) and GOF (F) phenotypes by the intensity of *Toll-lacZ* staining. ***, $P<0.001$; *, $P<0.05$; ns, not significant; Student's *t*-test. Data represent mean ± s.e.m.

towards the ventral nerve cord and was situated ventral to M13 and underneath (external to) muscle 6/7. Furthermore, the normally longitudinal orientation of the muscles became oblique because the ventral shift of the muscle attachment was more severe for the posterior than anterior attachment site. Thus, *tey* is required for proper formation of the muscle attachment sites of M12.

Toll is negatively regulated by Tey

In addition to defects in muscle attachments, we found dramatic upregulation of *Toll* expression in M12 of *tey* mutants, as assessed by *Toll-lacZ* reporter expression. As described above, in wild-type embryos, *Toll* is only weakly expressed in M12. In the *tey*^{5053A} mutant, *Toll-lacZ* was dramatically upregulated in M12, where *tey* is normally expressed (Fig. 4A,B). The results suggest that *tey* normally suppresses expression of *Toll* in this muscle. Quantification showed that the level of *Toll-lacZ* expression in M12 in the mutants was increased to a level comparable to that of normally *Toll*-positive muscles (e.g. muscles 15 and 16) (Fig. 4C): the intensity of *Toll-lacZ* expression in M12 was 147.3±9.4 (n=21) in the *tey* mutant as compared with 24.0±3.8 (n=23) in the control (normalized to the intensity in M13 of control; $P<0.001$, Student's *t*-test). No change in *Toll-lacZ* expression was observed in other muscles in the mutants: the intensity of *Toll-lacZ* in M13 was 97.6±15.7 (n=16) in the *tey* mutant versus 100.0±8.3 (n=11) in the control. Similarly, the expression of Toll protein in M12 was upregulated in *tey* mutants (see Fig. S3 in the supplementary material). Thus, *tey* is required to suppress *Toll* expression specifically in M12.

To further study the role of *tey* in *Toll* expression, we examined the effects of ectopically expressing *tey* in M13, a *Toll*-positive muscle. Expression of *tey* in M13 using *H94-Gal4* caused a significant reduction in expression of the *Toll-lacZ* reporter in M13 (Fig. 4D-F): the intensity of *Toll-lacZ* in M13 was 69.7±4.4 (n=14) in *H94-tey*, compared with 100±12.5 (n=12) in the control ($P<0.05$, Student's *t*-test). Again, no change in *Toll-lacZ* expression was observed in other muscles: the intensity of *Toll-lacZ* in M12 was 25.1±5.6 (n=14) in *H94-tey*, as compared with 29.4±4.9 (n=12) in the control. Unlike in LOF mutants, no defects were seen in the formation of attachment sites or in other morphological aspects of muscle development in *H94-tey* embryos. The results further

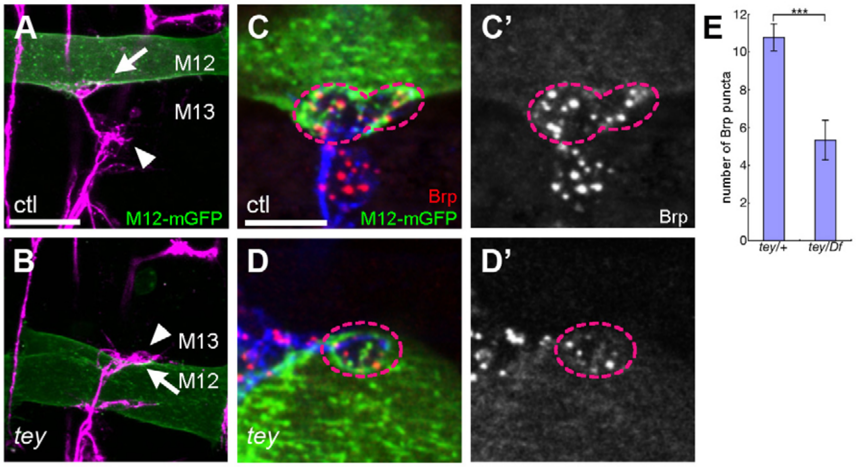

Fig. 5. Reduced synaptic sites of M12 in the *tey* mutant. (**A**,**B**) Fas2 staining to visualize all synaptic endings (magenta). M12 was marked by mGFP (green). In control *Drosophila* embryos (*tey*^{5053A}/+, A), discrete endings along M12 and M13 are observed, whereas in the *tey* mutant (*tey*^{5053A}/*Df(3L)Exel6135*, B) only a single ending between M12 and M13 can be seen. Arrows, M12 terminals; arrowheads, M13 terminals. (**C-D′**) At 18 hours after egg laying, embryos were stained with anti-Brp (red), anti-HRP (to visualize pre-synapses, blue) and marked by mGFP expression (green). The anti-Brp channel is shown alone in C′,D′. The number of anti-Brp-staining puncta within the area of pre-synaptic varicosity for M12 (circles) was reduced in the *tey* mutant (D,D′) as compared with the control (C,C′). (**E**) Quantification of the reduction in the number of Brp-staining puncta: 5.3±1.0 (*n*=18) in *tey/Df* compared with 10.8±0.7 (*n*=13) in *tey/+* control; ***, $P<0.001$ by Student's *t*-test. Data represent mean ± s.e.m. Scale bars: 10 μm in A; 5 μm in C.

support the notion that *tey* negatively regulates *Toll* expression. Taken together, these results indicate that M12-specific expression of Tey is crucial for the downregulation of *Toll* in M12.

Synapse formation of M12 is inhibited in *tey* mutants

Next we investigated what happens to motoneuron innervation in *tey* mutants or upon *tey* misexpression. As described above, Toll inhibits synapse formation by MN12s. Since *Toll* is ectopically expressed in M12 in *tey* mutants, synapse formation by MN12s might be inhibited. Similarly, as *Toll* is downregulated when *tey* is misexpressed in M13, MN12s may fail to innervate M12 properly, as observed in *Toll* mutants. We therefore examined motoneuron targeting in *tey* LOF mutants, an analysis complicated by the misplacement of M12. Instead of the two discrete endings formed on the proximal edges of M12 and M13, only one large ending was seen near the M13 terminal region in *tey* mutant embryos (*tey*^{5053A} homozygotes or *tey*^{5053A}/*Df(3L)Exel6135*; Fig. 5A,B). This phenotype is likely to result from the fact that the ventral shift of M12 causes the endings of M12 to form in the vicinity of those of M13 (Fig. 5A,B). The proximity of the two endings made it difficult to discriminate and characterize terminal formation by M12 and M13 motoneurons by motor axon staining (with the 1D4 antibody). We therefore used anti-Bruchpilot (Brp) staining to study synapse formation on M12. Anti-Brp visualizes active zones formed on the nascent synaptic sites in embryos at 18 hours after egg laying (Fig. 5C,D). We counted the number of Brp-positive puncta (putative active zones) localized on the M12 synaptic sites (as visualized with mGFP, see Materials and methods). The number was significantly decreased in *tey* mutants compared with controls (Fig. 5E). Thus, synapse formation on M12 is inhibited in *tey* mutants. It remains to be determined whether MN12s instead form ectopic endings on M13 or other muscles. However, this seems unlikely because no abnormality was seen in the number of Brp-positive clusters in any neighboring muscles (data not shown).

Ectopic *tey* alters M12 and M13 terminals in a similar manner as in *Toll* LOF

The decrease in synaptic sites on M12 is consistent with the hypothesis that suppression of *Toll* by *tey* is important for proper targeting by MN12s. However, it is also possible that mislocalization of M12 in the mutants affects neuronal targeting indirectly. To obtain further evidence for the role of *tey* in muscle targeting, we examined the effect of ectopic expression of *tey*. As described above, ectopic expression of *tey* in M13 by *H94-Gal4* downregulates *Toll* expression but does not affect other characteristics of M13, such as its position or orientation (see Fig. 4E). We hypothesized that decreased Toll repulsion on M13 might change the target preference of MN12s to M13, as observed in *Toll* LOF mutants. As expected, in *H94-tey* embryos, the arborizations on M13 were enlarged, and those on M12 were reduced in size (Fig. 6A-C): M12 terminals were 26.8±6.8 (*n*=28) in *H94-tey* as compared with 100±13.7 (*n*=13) in the control, whereas M13 terminals were 175.2±18.1 (*n*=28) in *H94-tey* versus 48.3±12.5 (*n*=13) in the control ($P<0.001$, Student's *t*-test). Furthermore, these phenotypes were reversed when *tey* was co-expressed with *Toll* (Fig. 6C): M12 terminals were 135.0±11.2 (*n*=22) in *H94-tey+Toll*, compared with 16.9±3.9 (*n*=32) in *H94-tey+GFP* (GFP was co-expressed in control embryos to normalize the number of UAS constructs), whereas M13 terminals were 75.3±11.1 (*n*=22) in *H94-tey+Toll* versus 165.3±28.5 (*n*=32) in *H94-tey+GFP* ($P<0.001$, Student's *t*-test). These results strongly support the notion that Tey mediates muscle targeting in part by suppressing *Toll* expression in specific muscles.

DISCUSSION

Multiple repulsive cues function in single muscle targeting

Several molecules have been identified to function as attractive target cues that determine synaptic target specificity (Shen et al., 2004; Shinza-Kameda et al., 2006; Yamagata and Sanes, 2008).

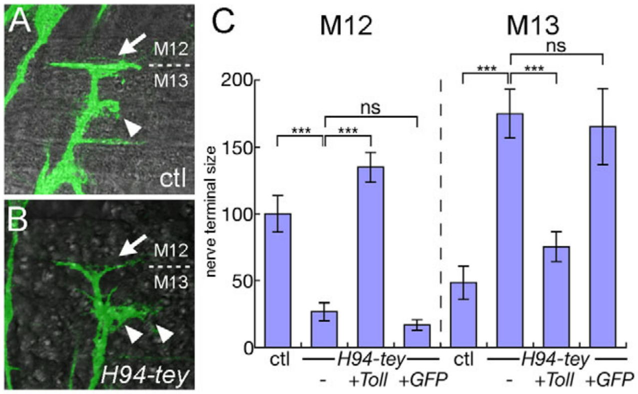

Fig. 6. Ectopic *tey* alters M12 and M13 terminals in a similar manner as in *Toll* LOF. (**A**,**B**) Nerve terminals visualized by anti-Fas2 staining in control *Drosophila* embryos (*H94-Gal4*/+, A) and with ectopic expression of *tey* in M13 (*H94-tey*, B). Upon ectopic expression of *tey*, M13 terminals were enlarged (arrowheads), whereas those of M12 were reduced in size (arrows) (B). (**C**) Quantification of the phenotype by nerve terminal size. The *tey* GOF phenotype was reversed by simultaneous expression of *Toll* but not *GFP*. ***, $P<0.001$; ns, not significant; Student's *t*-test. Data represent mean ± s.e.m.

However, less is known about the role of repulsion in target selection. We have previously shown that Wnt4, a secreted protein of the Wnt family, functions as a repulsive cue that regulates the targeting of M12 and M13. Here, we show that Toll, a transmembrane protein with leucine-rich repeats, may function in a similar manner to determine the target specificity of the same muscles. A previous study showed that Toll can function in other muscles (muscles 6 and 7) as an inhibitory cue for synapse formation (Rose et al., 1997). However, it was unknown whether Toll-mediated repulsion is required for the generation of synaptic specificity. Here, we show that *Toll* is preferentially expressed in M13 over M12. The size of M12 terminals was decreased in *Toll* mutants, with concomitant expansion of M13 terminals. This phenotype is very similar to that of *Wnt4* LOF and is likely to be caused by MN12s forming ectopic synapses with M13, although it remains possible that some of the ectopic nerve endings on M13 are formed by other motoneurons. Furthermore, we observed that the size of M12 terminals is reduced when *Toll* is misexpressed on the muscle. The LOF and GOF analyses suggest that Toll functions as a repulsive factor in M13 that is important for target selection by MN12s. Thus, Toll provides another example of a repulsive factor that is involved in target selection. How Toll mediates the repulsive signal to motoneurons is currently unknown. A model is that Toll functions as a ligand that is expressed in muscles and signals through receptor(s) expressed in motoneurons. However, no receptor has been identified for Toll. Toll has been shown to function as a receptor, not a ligand, in other systems, such as in dorsoventral patterning and innate immunity (Belvin and Anderson, 1996; Imler and Hoffmann, 2001). Another possibility is that Toll might mediate the modification or regulation of other targeting molecules, such as Wnt4.

This study showed that M13 expresses at least two repulsive cues, Wnt4 and Toll, which are important for the targeting of M12 and M13. It seems that these two molecules contribute to target specificity in a manner that is redundant with yet other molecules because in both single and double mutants of these genes, the connectivity is only partially disrupted. Previously, we identified other potential repulsive cues that are expressed in M13, including Beat-IIIc and Glutactin (Inaki et al., 2007). Ectopic expression of these molecules in M12 inhibits synapse formation by MN12s, as observed when Toll and Wnt4 are misexpressed. Although the precise roles of these molecules remain to be verified by LOF analyses, these results suggest the possibility that a single muscle, M13, expresses a number of repulsive cues that are involved in targeting of motoneurons. This is consistent with previous hypotheses that *Drosophila* neuromuscular connectivity is determined by highly redundant mechanisms (Winberg et al., 1998; Rose and Chiba, 1999). It will be important to determine how the signals from multiple cues are integrated to generate the precise pattern of synaptic connections. It will also be interesting to examine whether other muscles similarly express repulsive cues to prevent inappropriate innervation. The phenotypes of *Wnt4 Toll* double mutants were of similar severity to those of the single mutants. This might be due to the presence of other targeting molecules, as described above. Toll and Wnt4 might also function in the same signaling pathway. For example, Toll may be involved in the regulation of Wnt4 activity through influencing its secretion, localization or protein modification (Dhoot et al., 2001; Ciani and Salinas, 2005; Bejsovec, 2005). Toll and Wnt4 might also act as repellents for distinct MN12s.

Transcriptional regulation of target-recognition molecules

Here, we have shown that a novel nuclear protein, Tey, regulates the expression of *Toll* and is important for the determination of target specificity. We also showed that *tey* regulates the position, orientation and attachment sites of M12. Thus, Tey seems to act as a determinant of several important properties of M12, regulating both the differentiation of the muscle itself and the specificity of nerve innervation. Expression of *tey* is remarkably specific, being limited within the somatic musculature to a single muscle, M12. Other, known muscle-determinant genes were expressed in broader subsets of muscles.

We showed that Tey negatively regulates the expression of *Toll* in M12. In *tey* mutants, *Toll* expression is strongly upregulated in M12. This indicates that *tey* is required in this muscle to specifically suppress *Toll* expression. Consistent with this, ectopic expression of *tey* in M13 partially suppressed *Toll* expression. *Toll* is normally expressed in most of the other ventral muscles, including muscles 6, 7, 13-17, but not in M12, suggesting that some positive transcriptional regulator(s) higher up in the hierarchy activate *Toll* expression in this group of muscles and that negative regulation by Tey is required to suppress *Toll* expression only in M12. The regulation of *Toll* by Tey should be at the transcriptional level because the expression of the exogenously introduced *Toll* enhancer-trap *lacZ* reporter is affected in *tey* mutants or when *tey* is misexpressed. It remains to be determined whether Tey binds directly to the regulatory region of the *Toll* gene or regulates *Toll* transcription in an indirect manner (e.g. by regulating other transcription factors). Tey contains no known transcription factor motifs. The expression of another M13-enriched gene, *Wnt4*, was not affected in *tey* mutants or when *tey* was misexpressed (data not shown). Unlike *Toll*, *Wnt4* is expressed in only two ventral muscles: 13 and 30. Thus, expression of *Wnt4* might be regulated in a different manner to *Toll*, possibly by positive transcription factors that are specifically expressed in these muscles. It will be interesting to determine how the expression of target-recognition molecules is precisely regulated by the combinatorial action of positive and negative transcription factors.

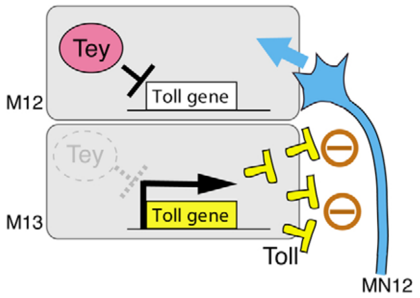

Fig. 7. A model of Toll and Tey function in muscle-specific innervation in *Drosophila*. The transmembrane protein Toll has a repulsive function against MN12s. With the exception of M12, Toll is expressed in all ventral muscles, including M13, and prevents MN12s from making synapses with them. In M12, *Toll* is repressed by Tey, which allows MN12s to form nerve terminals on the target muscle.

In *tey* mutants or when *tey* is misexpressed, neuromuscular connectivity was also altered in a manner consistent with the misregulation of *Toll* expression. The inappropriate presence of Toll repulsion in *tey* LOF mutants suppressed synapse formation on M12. Conversely, suppression of Toll expression in M13 in *tey* GOF mutants led to changes in the innervations of M12 and M13, similar to those observed in *Toll* mutants. Furthermore, the effects of *tey* GOF were dramatically reversed when *Toll* was co-expressed with *tey*, suggesting that *Toll* is the major target of *tey* in causing the GOF phenotypes. These results suggest that Tey regulates neuromuscular connectivity by specifically repressing Toll expression in M12. As noted above, Toll is normally expressed in a number of ventral muscles, but not in M12. Furthermore, Toll is expressed in M12 in the absence of Tey suppression in *tey* mutants. This suggests that the default state is for *Toll* to be expressed in all ventral muscles, possibly by the action of positive transcription factor(s) expressed in these muscles. Tey might therefore generate target specificity by suppressing the expression of Toll in one amongst a group of muscle cells expressing the repulsive cue (Fig. 7). Our data thus suggest a mechanism of transcriptional control of target specificity, namely, the negative regulation of repulsive cues.

Acknowledgements

We thank A. Chiba, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for fly stocks and reagents; E. Suzuki and members of the A.N. laboratory for helpful discussion; Y. Fujioka, H. Kato and M. Sotodate for technical assistance; S. Israeli for sequencing the *tey* EST; and A. Cliffe for critical reading of the manuscript. This study was supported by a Grant-in-Aid for Scientific Research B and for Scientific Research on Priority Areas Molecular Brain Science from MEXT and a grant from Uehara Memorial Foundation to A.N. and by NIH grants from NIDDK and NICHD to M.F. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.046672/-/DC1

References

Baylies, M. K., Bate, M. and Ruiz Gomez, M. (1998). Myogenesis: a view from Drosophila. *Cell* **93**, 921-927.

Bejsovec, A. (2005). Wnt pathway activation: new relations and locations. *Cell* **120**, 11-14.

Belvin, M. P. and Anderson, K. V. (1996). A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu. Rev. Cell Dev. Biol.* **12**, 393-416.

Benson, D. L., Colman, D. R. and Huntley, G. W. (2001). Molecules, maps and synapse specificity. *Nat. Rev. Neurosci.* **2**, 899-909.

Chiba, A. (1999). Early development of the Drosophila neuromuscular junction: a model for studying neuronal networks in development. *Int. Rev. Neurobiol.* **43**, 1-24.

Chiba, A., Snow, P., Keshishian, H. and Hotta, Y. (1995). Fasciclin III as a synaptic target recognition molecule in Drosophila. *Nature* **374**, 166-168.

Ciani, L. and Salinas, P. C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat. Rev. Neurosci.* **6**, 351-362.

Davis, G. W., Schuster, C. M. and Goodman, C. S. (1997). Genetic analysis of the mechanisms controlling target selection: target-derived Fasciclin II regulates the pattern of synapse formation. *Neuron* **19**, 561-573.

Dhoot, G. K., Gustafsson, M. K., Ai, X., Sun, W., Standiford, D. M. and Emerson, C. P., Jr (2001). Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science* **293**, 1663-1666.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new Drosophila homeobox gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.

Halfon, M. S., Hashimoto, C. and Keshishian, H. (1995). The Drosophila toll gene functions zygotically and is necessary for proper motoneuron and muscle development. *Dev. Biol.* **169**, 151-167.

Hashimoto, C., Hudson, K. L. and Anderson, K. V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269-279.

Holt, C. E. and Harris, W. A. (1998). Target selection: invasion, mapping and cell choice. *Curr. Opin. Neurobiol.* **8**, 98-105.

Imler, J. L. and Hoffmann, J. A. (2001). Toll receptors in innate immunity. *Trends Cell Biol.* **11**, 304-311.

Inaki, M., Yoshikawa, S., Thomas, J. B., Aburatani, H. and Nose, A. (2007). Wnt4 is a local repulsive cue that determines synaptic target specificity. *Curr. Biol.* **17**, 1574-1579.

Jacobsen, T. L., Cain, D., Paul, L., Justiniano, S., Alli, A., Mullins, J. S., Wang, C. P., Butchar, J. P. and Simcox, A. (2006). Functional analysis of genes differentially expressed in the Drosophila wing disc: role of transcripts enriched in the wing region. *Genetics* **174**, 1973-1982.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Keshishian, H., Broadie, K., Chiba, A. and Bate, M. (1996). The drosophila neuromuscular junction: a model system for studying synaptic development and function. *Annu. Rev. Neurosci.* **19**, 545-575.

Lehmann, R. and Tautz, D. (1994). In situ hybridization to RNA. *Methods Cell Biol.* **44**, 575-598.

Mitchell, K. J., Doyle, J. L., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., Goodman, C. S. and Dickson, B. J. (1996). Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* **17**, 203-215.

Nose, A., Mahajan, V. B. and Goodman, C. S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila. *Cell* **70**, 553-567.

Nose, A., Umeda, T. and Takeichi, M. (1997). Neuromuscular target recognition by a homophilic interaction of connectin cell adhesion molecules in Drosophila. *Development* **124**, 1433-1441.

Ritzenthaler, S., Suzuki, E. and Chiba, A. (2000). Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. *Nat. Neurosci.* **3**, 1012-1017.

Rørth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93**, 12418-12422.

Rose, D. and Chiba, A. (1999). A single growth cone is capable of integrating simultaneously presented and functionally distinct molecular cues during target recognition. *J. Neurosci.* **19**, 4899-4906.

Rose, D., Zhu, X., Kose, H., Hoang, B., Cho, J. and Chiba, A. (1997). Toll, a muscle cell surface molecule, locally inhibits synaptic initiation of the RP3 motoneuron growth cone in Drosophila. *Development* **124**, 1561-1571.

Ruiz-Gomez, M., Romani, S., Hartmann, C., Jackle, H. and Bate, M. (1997). Specific muscle identities are regulated by Kruppel during Drosophila embryogenesis. *Development* **124**, 3407-3414.

Sato, M., Umetsu, D., Murakami, S., Yasugi, T. and Tabata, T. (2006). DWnt4 regulates the dorsoventral specificity of retinal projections in the Drosophila melanogaster visual system. *Nat. Neurosci.* **9**, 67-75.

Shen, K., Fetter, R. D. and Bargmann, C. I., (2004). Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell* **116**, 869-881.

Shinza-Kameda, M., Takasu, E., Sakurai, K., Hayashi, S. and Nose, A. (2006). Regulation of layer-specific targeting by reciprocal expression of a cell adhesion molecule, capricious. *Neuron* **49**, 205-213.

Shishido, E., Takeichi, M. and Nose, A. (1998). Drosophila synapse formation: regulation by transmembrane protein with Leu-rich repeats, CAPRICIOUS. *Science* **280**, 2118-2121.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Wagh, D. A., Rasse, T. M., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., Buchner, S., Dabauvalle, M. C., Schmidt, M., Qin, G. et al. (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. *Neuron* **49**, 833-844.

Winberg, M. L., Mitchell, K. J. and Goodman, C. S. (1998). Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell* **93**, 581-591.

Yamagata, M. and Sanes, J. R. (2008). Dscam and Sidekick proteins direct lamina-specific synaptic connections in vertebrate retina. *Nature* **451**, 465-469.