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, neuronsobiles 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...
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)T9QRX and Df(3R)TroXB3 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[2P5/Wntα2NS] (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, ACK80 (Nose et al., 1992), was used as a reporter of Toll expression. UAS-myrstylated-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 Kmnl and Notl 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 other Toll-expressing 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

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).

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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 Toll-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 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 Gal4, disrupts the tey locus and used it to study the role of the gene (Fig. 3A). tey appears to be a null allele of tey for the following reasons. First, tey transcripts were undetectable by in situ hybridization in tey embryos (Fig. 3B,C). Second, tey homozygotes and transheterozygotes of tey over a deficiency showed similar phenotypes (see below). The presence of a Gal4 insertion allowed visualization of M12, a normally 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...
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 M12 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
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/tey;3L) homozygotes or tey/tey;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 ID4 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).
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; Bejsvec, 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.
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 Toll 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.

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Competing interests statement
The authors declare no competing financial interests.

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