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Genetic Analysis of the Mechanisms Controlling Target Selection: Complementary and Combinatorial Functions of Netrins, Semaphorins, and IgCAMs

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Summary

The molecular mechanisms controlling the ability of motor axons to recognize their appropriate muscle targets were dissected using *Drosophila* genetics to add or subtract Netrin A, Netrin B, Semaphorin II, and Fasciclin II alone or in combination. Fas II and Sema II are expressed by all muscles where they promote (Fas II) or inhibit (Sema II) promiscuous synaptogenesis. NetB is expressed by a subset of muscles where it attracts some axons and repels others. However, growth cones in this system apparently do not rely solely on single molecular labels on individual targets. Rather, these growth cones assess the relative balance of attractive and repulsive forces and select their targets based on the combinatorial and simultaneous input of multiple cues.

Introduction

Neuronal growth cones make pathfinding decisions apparently based on their ability to measure and respond to the relative balance of attractive and repulsive forces impinging on them (Tessier-Lavigne and Goodman, 1996). Is the molecular logic of how guidance signals are deployed and deciphered during target selection similar to or different from the mechanisms used during axon pathfinding? Discrete target selection might be specified in a point-to-point fashion such that each motor axon and its appropriate target have unique and complementary molecular labels. Alternatively, specificity might emerge from a dynamic and comparative process in which growth cones respond to qualitative and quantitative molecular differences on neighboring targets and make their decisions based on the relative balance of attractive and repulsive forces.

To distinguish between a lock-and-key versus a relative balance model, we used genetic analysis in *Drosophila melanogaster* to dissect the mechanisms controlling the ability of motor axons to select their appropriate muscle targets. In each abdominal hemisegment in the *Drosophila* embryo, ~40 motor axons select their specific targets from among 30 potential muscles, each consisting of a single, large multinucleate muscle fiber (Figures 1A and 1B). Muscle ablation and duplication experiments (Ball et al., 1985; Sink and Whitington, 1991; Cash et al., 1992; Chiba et al., 1993) indicate that individual motor axons select their appropriate muscle targets with great precision. Correct pathfinding decisions bring these growth cones to their appropriate target regions (Van Vactor et al., 1993). Motor growth cones then probe many neighboring muscles, withdraw most of these contacts, and form synapses with one or at most a few muscles.

Several kinds of evidence suggest that target selection in this system is not specified in a point-to-point fashion by unique molecular labels. For example, motoneurons can synapse on inappropriate muscles when either their normal muscle targets are absent (Cash et al., 1992), the motoneurons are misrouted into abnormal target regions (Lin and Goodman, 1994; Sink and Goodman, 1994; Fambrough and Goodman, 1996), or the inappropriate muscle is not properly innervated (Keshishian et al., 1993; Halfon et al., 1995; Kopczynski et al., 1996).

Further evidence comes from the genetic analysis of candidate target recognition molecules. At present, the best examples in this system are the Netrins (Netrin A and Netrin B, encoded by two tandem genes), which are expressed by 4 of the 30 muscles (Figure 1A). Embryos carrying a deletion of both Netrin genes (Mitchell et al., 1996) as well as embryos mutant in the *frazzled* gene, which encodes a DCC/UNC-40–like Netrin receptor (Kolodziej et al., 1996), show only partially penetrant defects in the projections of motor axons that normally innervate the four Netrin-expressing muscles. Thus, although the Netrins function as part of the targeting system for the axons that innervate the Netrin-expressing muscles, additional molecules must participate in specifying these connections. Genetic analysis of other candidate recognition molecules reveals even less penetrant loss-of-function phenotypes (Nose et al., 1994; Chiba et al., 1995). These results suggest that discrete target selection in this system is not controlled at the level of "one molecule, one synapse."

The dynamic and malleable nature of target selection in this system was revealed by the genetic analysis of the cell adhesion molecule Fasciclin II (Fas II) (Davis et al., 1997). Prior to synapse formation, Fas II is expressed at low levels by all muscles. During synapse formation, Fas II concentrates at the synapse and disappears from the rest of the muscle (Schuster et al., 1996; Zito et al., 1997); this dynamic change in Fas II levels influences the pattern of synapse formation (Davis et al., 1997). A transient increase in muscle Fas II stabilizes growth cone contacts and leads to novel stable synapses. Changing the relative levels of Fas II on neighboring muscles leads to dramatic changes in target selection. Thus far, the genetic tests of this relative balance model for target selection have relied solely on changing the expression of candidate molecules one at a time. But if target selection is based on a balance of multiple cues, then gaining a deeper understanding into the molecular logic of this process will require changing the expression of genes pair-wise or more. In the present study, we tested this model by altering the levels of Netrin A, Netrin B, Semaphorin II (Sema II), and Fas II on neighboring muscles alone and in combination, using mutations and transgenes that either add or subtract gene functions

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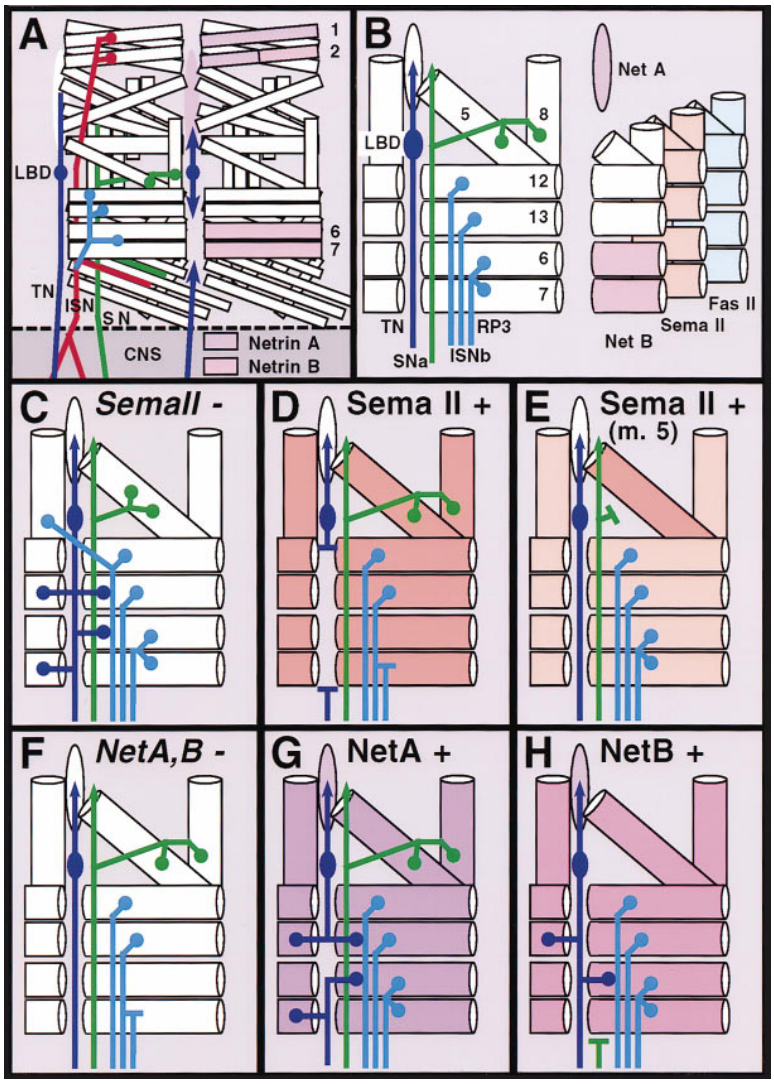

Figure 1. Motor Innervation Phenotypes in *SemaII* and *Netrin* Mutants

Schematic diagram showing wild-type pattern of innervation of muscles 7, 6, 13, 12, 5, and 8 and abnormal patterns of innervation in various *SemaII* and *Netrin* loss-of-function and gain-of-function mutant conditions. −, null loss-of-function mutant; +, overexpression gain-of-function condition. Anterior is left, and dorsal is at top.

(A) Schematic diagram showing abdominal body wall muscles in the *Drosophila* embryo. The second segment shows Netrin B expression by muscles 2, 6, and 7, Netrin A expression by muscles 1 and 2, and a dorsolateral stripe in the epidermis.

(B) Wild-type innervation by ISNb includes the projection of RP3 to muscles 6 and 7, and other ISNb neurons to muscles 13 and 12. The lateral branch of the SNa projects to muscles 5 and 8, while the dorsal branch continues to more distal targets. TN axons do not innervate any of the muscles depicted. Right, patterns of expression of NetA, NetB, Sema II, and Fas II.

(C) *SemaII* loss-of-function mutant shows ectopic innervation by the ISNb to muscle 8 and by the TN to muscles 7, 6, and 13. The SNa projects exuberantly to muscle 5 and sometimes fails to innervate muscle 8.

(D) Uniform overexpression of Sema II inhibits TN axons (from the LBD neurons and TN motoneurons) from fasciculating and inhibits RP3 from innervating muscles 7 and 6.

(E) Differential overexpression of Sema II on muscle 5 repels the lateral branch of the SNa.

(F) In the *NetA, NetB* loss-of-function mutant, RP3 often fails to innervate muscles 6 and 7.

(G) Expressing NetA by all muscles elicits ectopic TN innervation of muscles 7, 6, and 13 and sometimes leads to a failure of the TN to form.

(H) Overexpressing NetB by all muscles causes SN stalling as well as ectopic TN contacts onto muscles 7, 6, and 13.

in all or specific subsets of muscles. We find that by shifting the levels of guidance cues up or down, we can alter target selection. Motor growth cones in this system assess the relative balance of attractive and repulsive forces provided by NetA, NetB, Sema II, Fas II, and other unidentified signals and select their targets based on the complementary, combinatorial, and simultaneous input of these multiple cues.

Results

Sema II Prevents Promiscuous and Ectopic Synapse Formation

Drosophila Sema II is a secreted member of the semaphorin family (Kolodkin et al., 1993). Previous studies showed that *SemaII* mRNA is strongly expressed by one ventral muscle in thoracic segment T3 and more weakly by all body wall embryonic muscles (Kolodkin et al., 1993). We confirmed the pan-muscle expression of Sema II protein using a specific monoclonal antibody. A previous study used a transgenic construct (*Toll-SemaII*) to transiently increase Sema II levels on a subset of ventral muscles (Matthes et al., 1995) and showed that Sema II can inhibit the formation of the synaptic arbors of the RP3 motoneuron onto muscles 7 and 6.

In the present study, we examined *SemaII* loss-of-function mutants with particular attention to the function of the pan-muscle expression of Sema II. We focused our analysis on the well understood and accessible innervation of ventral longitudinal muscles 7, 6, 13, and 12 and neighboring muscles 5 and 8. In embryos lacking Sema II, the muscles appear normal, the overall pattern of motor projections appears normal, and many details of motor innervation are normal, including, for example, the innervation of muscles 7 and 6 by the RP3 axon.

Nevertheless, specific targeting errors occur. One frequent class of errors involves ectopic contacts, in which neurons make synaptic contact with their normal targets and promiscuous contacts with neighboring inappropriate muscles (Figures 1C and 2B). For example, the intersegmental nerve b (ISNb) normally innervates ventral longitudinal muscles 7, 6, 13, and 12 and does not project further dorsally. However, in *SemaII* mutant embryos, the ISNb makes its normal contact with muscle

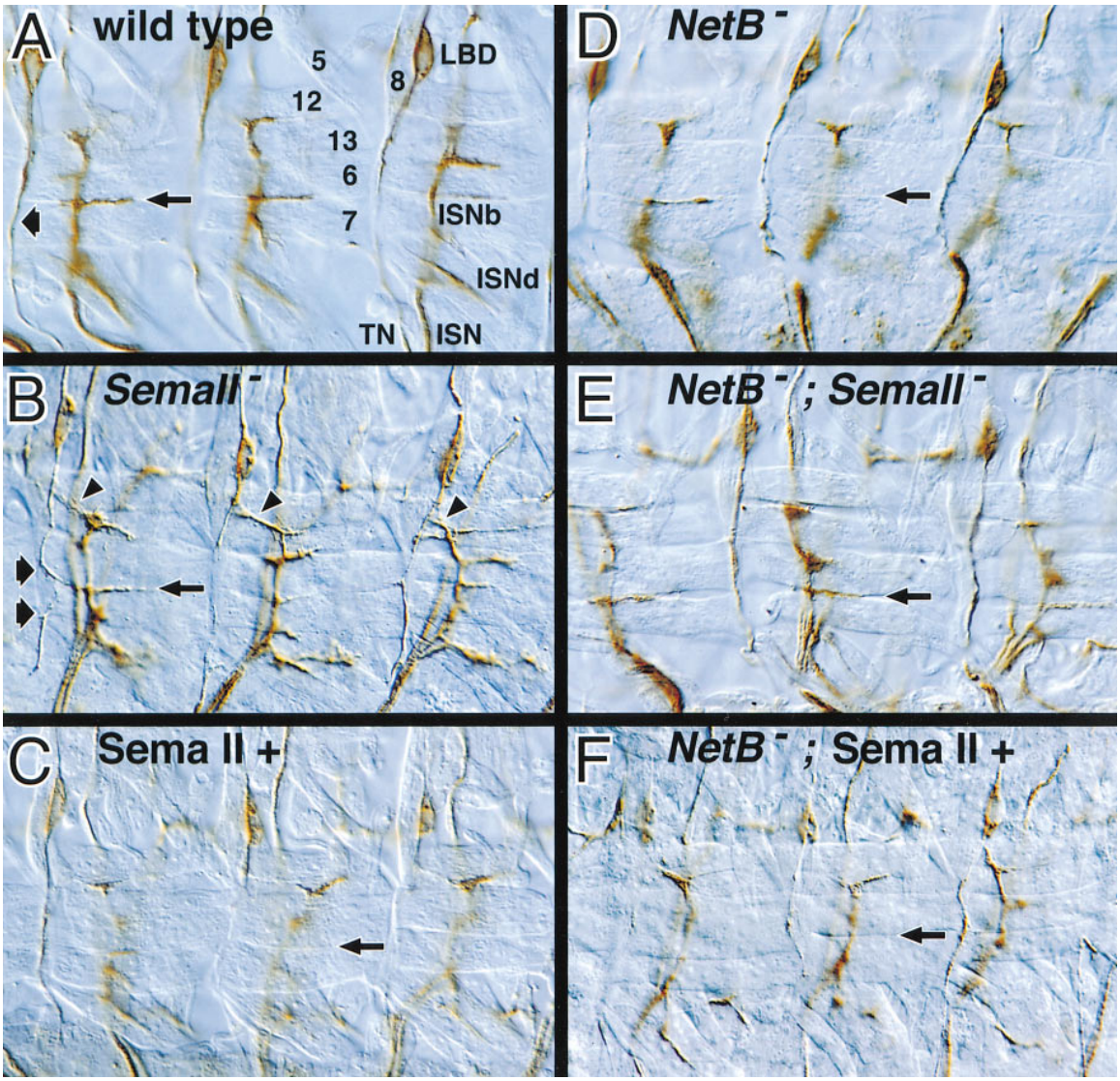

Figure 2. Abnormal ISN Motor Projections in *SemaII* and *Netrin* Loss- and Gain-of-Function Mutants

Photomicrographs of stage 17 embryos stained with mAb 1D4 (anti-Fas II) to show motor projections. Anterior is left, and dorsal is up.
(A) Wild type shows normal ISNb and TN projections. Long arrow, RP3 innervation in the cleft between muscles 6 and 7; large arrowhead, the TN.
(B) $SemaII^{P1}/SemaII^{P2}$ loss-of-function mutant, showing ectopic innervation of muscle 8 by the ISNb (three arrowheads), ectopic TN contacts onto ventral muscles (large arrowheads), and normal innervation of muscles 7 and 6 by RP3 (long arrow).
(C) Using *Toll-SemaII* to overexpress Sema II on ventral muscles prevents RP3 innervation to muscles 7 and 6 (long arrow).
(D–F) Three panels showing embryos carrying the *Netrin* deficiency *T9-B118* in (D) an otherwise wild-type genetic background, (E) a $SemaII^{ex59}/+$ loss-of-function heterozygote, and (F) Sema II overexpression background as driven by the transgene *Toll-SemaII*.
(D) *Netrin* mutants often lack innervation at the muscles 7/6 cleft (long arrow).
(E) Reducing Sema II restores normal RP3 innervation in *Netrin* deficiency mutants.
(F) Increasing Sema II on muscles enhances the lack of innervation to muscles 6 and 7 in *Netrin* deficiency mutants.

12 and then extends ectopic connections dorsally and laterally onto muscle 8 in 12.6% of segments (n = 222) compared to 1.9% of controls (n = 206).

Ectopic projections were also observed for the transverse nerve (TN). In wild-type embryos, a peripheral neuron, the lateral bipolar dendritic (LBD) cell (Bodmer and Jan, 1987), located next to muscle 8, projects one axon distally toward the alary muscle and another axon proximally toward the CNS. The LBD's proximal projection is along the surface of a long thin mesodermal cell, the dorsal median (DM) cell (Chiang et al., 1994; Gorczyca et al., 1994). Two neurons located in the CNS, the TMNs (Thor and Thomas, 1997), send their axons out distally along the DM cell process. Early in stage 16, the proximally projecting LBD axon meets and fasciculates with one of the distally projecting TMN axons to form the TN. This occurs near the segment border just adjacent to muscle 7.

Normally, although the LBD and TMN axons contact the ventral longitudinal muscles, they do not synapse

on these muscles. In contrast, in *SemaII* mutants, the axons in the TN make ectopic projections onto ventral muscles 7, 6, or 13 in 13.5% of late stage 16 segments (n = 222) compared to 3.1% in controls (n = 190) (Figures 1C and 2B); these muscles still receive their normal innervation. In most segments, these axons both make these ectopic contacts and also fasciculate with one another to form the TN, whereas in some segments, they make ectopic contacts but fail to fasciculate. Thus, the low level of Sema II expressed by ventral muscles provides repulsion to help prevent TN axons from innervating these muscles.

A related kind of ectopic innervation phenotype is observed for the segmental nerve branch a (SNa). The lateral branch of the SNa normally grows past muscle 5 and then bifurcates, with one branch extending back to synapse on muscle 5 and the other branch extending further laterally to synapse on muscle 8. In *SemaII* mutant embryos, the SNa stops abruptly at muscle 5, does not innervate muscle 8, but rather makes an increased contact with muscle 5 in 9.2% of segments (n = 229) compared to 0.7% in controls (n = 153) (Figures 1C and 3E). Apparently the normal low level of Sema II on muscle 5 is sufficient to repel the axon branch that normally innervates muscle 8, but it does not overcome the attraction of the axon branch that normally does innervate muscle 5. In the absence of Sema II, both can innervate muscle 5.

We also observed two new *SemaII* gain-of-function overexpression phenotypes in addition to the previously described effect on RP3 innervation (Matthes et al., 1995) with the GAL4 system (Brand and Perrimon, 1993) (Figures 1D and 2C). A *UAS-SemaII* transgenic strain was crossed to the *24B-Gal4* enhancer trap line (Luo et al., 1994) to generate strong pan-muscle Sema II expression. The levels of Sema II from this combination are higher than with *Toll-SemaII* and result in failure of the TN to form. Both branches (the LBD and the TMN axons) stall and do not enter the region of the ventral muscles (Figures 1D, 2C, and 3A). These neurons, like RP3, are repelled by Sema II.

Additionally, we found defects in SNa innervation. When Sema II is uniformly overexpressed using the pan-muscle *24B-Gal4*, muscles 5 and 8 are innervated normally. However, if *A51-Gal4* is used to increase local Sema II expression by lateral muscle 5 and a few cells near muscle 8, then the lateral branch of the SNa is specifically repelled in 45% of segments (n = 94) (Figures 1E and 3F). (*A51-Gal4* also drives Sema II expression by dorsal muscles 1 and 2, and here, too, we observe a local disruption of targeting decisions, phenotypes not seen when we drive pan-muscle Sema II expression [data not shown].) This shows that for specific targeting decisions, the differential level of Sema II expression on neighboring muscles can be more important than the absolute level.

Relative Balance of Sema II and Fas II Controls Synaptogenesis

To test the model that Sema II prevents promiscuous and ectopic synapse formation, we examined the genetic interactions of *SemaII* and *FasII*. Increased expression of the IgCAM Fas II drives a dramatic increase in ectopic innervation of ventral muscles (Davis et al., 1997). If the model is correct, then increasing or decreasing the levels of Sema II should respectively suppress or enhance the effects of increased Fas II. We used the GAL4 enhancer trap line H94 to drive the *UAS-FasII* transgene specifically in muscles 6, 13, 4, and to a lesser degree in 12 (Figure 4A). As predicted, removing Sema II leads to a significant enhancement of the Fas II overexpression phenotype as measured by an increase in ectopic TN contacts. Conversely, simultaneously overexpressing Sema II along with Fas II reduces the number of ectopic contacts.

Netrins Function as Short-Range Target Recognition Molecules for Specific Motoneurons

The two *Drosophila* Netrin genes, *NetA* and *NetB*, are differentially expressed by subsets of muscles (Mitchell et al., 1996). *NetA* is expressed by dorsal muscles 1 and 2 and also in a dorsolateral stripe of epidermal cells along the segmental border (near the alary muscle). *NetB* is expressed by muscle 2 and also by ventral muscles 6 and 7 (Figures 1A and 1B). The two Netrin genes are encoded by adjacent loci and can be simultaneously removed using a very small synthetic deficiency, *T9-B118*. Embryos carrying this deficiency display defects in commissure formation, with fewer axons crossing the midline than normal. They also exhibit frequent errors in the dorsal projections of the ISN and of the ventral projections of ISNb. Specifically, the RP3 growth cone fails to innervate muscles 7 and 6 in 35% of hemisegments (Figures 1F, 2D, and 5A). Projections of the SN are unaffected. The same phenotypes are observed at comparable frequencies in embryos mutant in the gene encoding the DCC/UNC-40–like Netrin receptor Frazzled (Kolodziej et al., 1996).

Although these data suggest that NetB muscle expression functions as an attractive targeting cue for the RP3 axon, an alternative explanation is that errors in the projections of these axons across the CNS midline indirectly lead to targeting errors in the periphery. However, the embryonic phenotype can be rescued by transgenically reintroducing either NetA or NetB expression by both ventral muscles and midline cells (for NetA, 94% of segments, and for NetB, 95% of segments show normal ISNb innervation; n = 68 and 59, respectively), while restoring Netrin expression only to the midline and not the ventral muscles is not sufficient to restore muscle innervation (60% and 68% for NetA and NetB, n = 74 and 139, respectively).

We draw three conclusions from this analysis. First, the function of Netrins in muscle targeting is independent of their function in midline guidance. Second, Netrin expression is required by muscles 7 and 6 to help recruit RP3 innervation of these muscles. Third, although only NetB is normally expressed by muscles 7 and 6, either Netrin will suffice.

Several observations suggest that the RP3 growth cone requires NetB as a short-range targeting signal rather than as a long-range chemoattractant for guidance. First, in *Netrin* or *frazzled* mutant embryos, the RP3 growth cone makes its normal guidance decisions in a timely fashion, exiting the CNS, leaving the ISN with

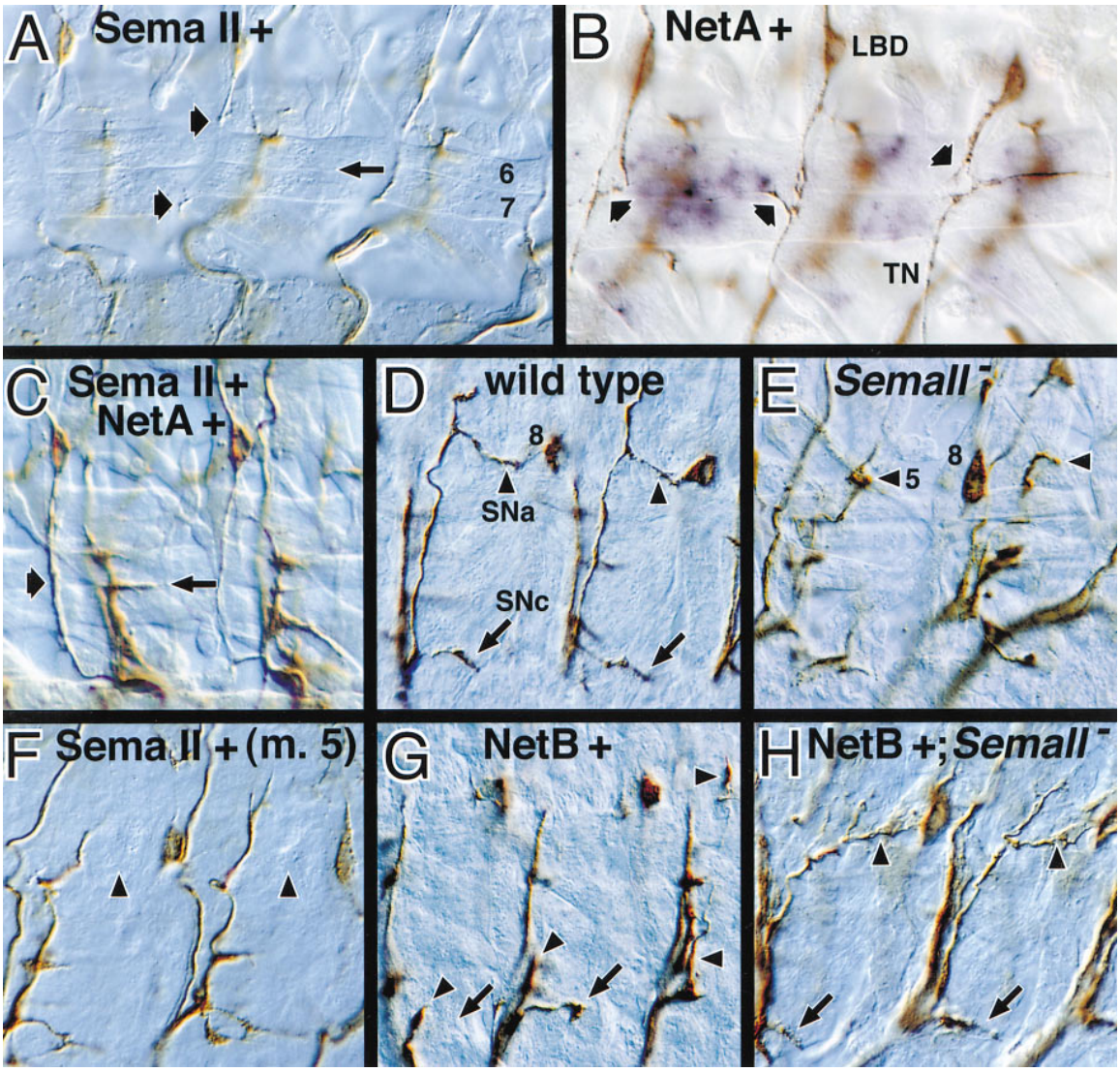

Figure 3. Abnormal TN and SN Motor Projections in *SemaII* and *Netrin* Loss- and Gain-of-Function Mutants

Photomicrographs of stage 17 embryos stained with mAb 1D4 (anti-Fas II) and in (B) NetA mRNA in situ hybridization. (E) and (H) are also stained with anti-Sema II mAb 19C2 to confirm genotype.

(A) *24B-Gal4* driving *UAS-SemaII* by all muscles prevents innervation by the ISNb to the muscle 6/7 cleft (long arrow) and frequently inhibits formation of the TN by preventing the axons that pioneer it from extending past ventral muscles (large arrowheads).

(B) Ectopic NetA on muscles 6 and 7 as driven by *F63-Gal4* elicits inappropriate innervation by the TN (arrowheads).

(C) *24B-Gal4* driving both *UAS-SemaII* and *UAS-NetA* by all muscles; the TN (large arrowhead) is normal, and RP3 innervates muscles 7 and 6 (long arrow).

(D–H) Montages of two or three focal planes to show the SN branches.

(D) Wild type. The SNc (long arrow) and the lateral branch of the SNa (small arrowheads) are indicated. Muscle 8 is marked just next to the Fas II–positive LBD neuron.

(E) In a *SemaII* null mutant, the SNa extensively contacts and fails to extend past muscle 5 (small arrowheads); muscle 8 is not innervated.

(F) *A51-Gal4* driving *UAS-SemaII* in muscle 5 specifically disrupts the lateral SNa branch; muscles 5 and 8 are not innervated (small arrowheads).

(G) *24B-Gal4* driving *UAS-NetB* by all muscles: the entire SN sometimes stalls as a clump of growth cones either within the CNS or in the ventral muscle region (small arrowheads). The SNc often extends for only a short distance and then ends in a clump (long arrow at right) or fails altogether (long arrow at left). When the SN reaches the lateral muscle domain, the SNa often fails to branch (small arrowhead in upper right).

(H) Ectopic NetB in a *SemaII* loss-of-function mutant background: both the SNa (small arrowheads) and SNc (long arrows) again project normally.

the other ISNb motor axons, and entering the appropriate ventral muscle domain. However, while the other ISNb growth cones innervate their muscle targets, the RP3 growth cone often does not. The RP3 growth cone can sometimes be visualized in stage 17 embryos in segments that lack innervation at muscles 7 and 6; we observe the RP3 growth cone in the correct neighborhood and within filopodial grasp of muscles 7 and 6 (Figure 6). Furthermore, in segments in which muscles 7 and 6 are innervated, we sometimes observe that this

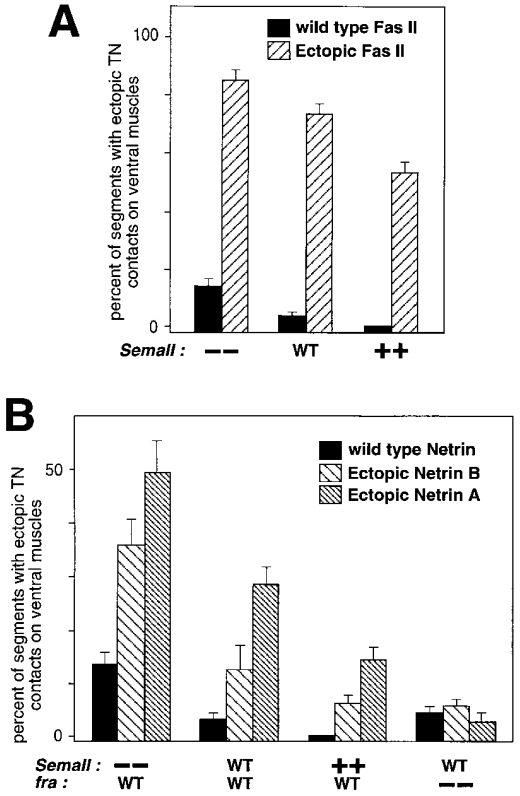

Figure 4. Ectopic TN Innervation of Ventral Muscles as Driven by Fas II or Netrins

(A) The ability of ectopic Fas II to promote ectopic TN connections (*H94-Gal4* driving *UAS-FasII*) depends upon the level of Sema II. WT, wild-type Sema II levels; − −, null loss-of-function mutant; ++, strong Sema II overexpression. Hemisegments were assigned scores of either 0 (no ectopic contacts) or 1 (1 or more ectopic TN contacts present). Mean ± SEM: (from left to right) genotype (n), $SemaII^{P2}/SemaII^{P2}$ (222, 96); wild type (190, 152); *UAS-SemaII/+; H94-Gal4/+* (84, 162).

(B) Netrin-mediated attraction of ectopic TN connections depends upon the level of Sema II expression and requires the DCC-like Netrin receptor Frazzled. *UAS-NetA*, *UAS-NetB*, and *UAS-SemaII* constructs were driven by *24B-Gal4*, giving strong expression by all muscles. Left to right, genotype (n): $SemaII^{P2}/SemaII^{P2}$ (222, 93, 69); wild type (190, 56, 196); *UAS-SemaII/+; 24B-Gal4/+* (134, 46, 224); *fra/fra* (91, 70, 138).

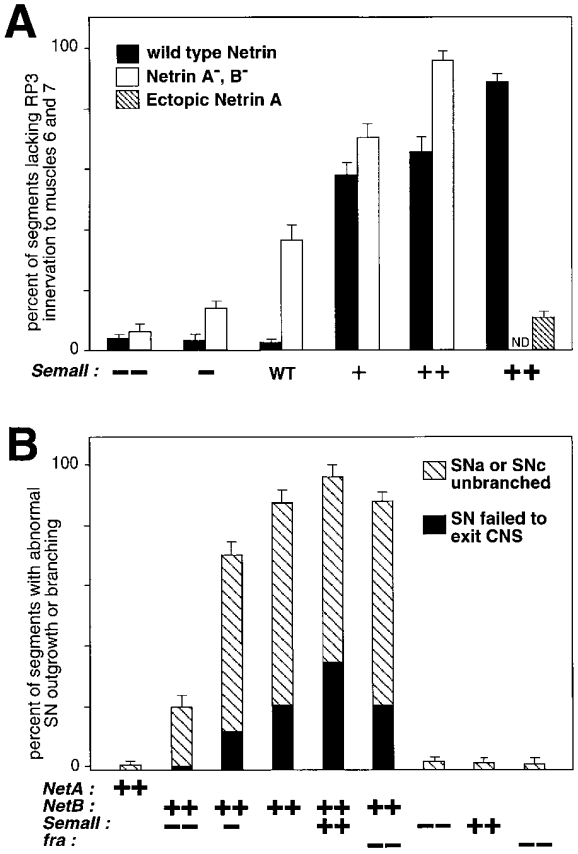

Figure 5. Abnormal Innervation and Branching of Motor Nerves as Driven by Netrins and Sema II

(A) RP3 innervation of muscles 6 and 7 depends on the expression of Netrins relative to Sema II. Embryos with or without Netrin, lacking or overexpressing Sema II, were examined for RP3 innervation. Hemisegments were scored only when other branches of the ISNb were intact and were assigned scores of 0 (innervation missing or greatly reduced) or 1 (normal). Data were pooled for two null alleles of Sema II. Left to right, genotype (n): *SemaII/SemaII* (237, 81); *SemaII/+* (68, 173); wild type (194, 89); *Toll-SemaII/+* (113, 99); *Toll-SemaII/Toll-SemaII* (104, 46); *UAS-SemaII/+*; *24B-Gal4/+* (137, 0, 262).

(B) Overexpression of NetB but not NetA leads to abnormal outgrowth or branching of the SN. The ability of NetB to repel SN axons depends upon the level of Sema II expression but does not require Frazzled. Left to right, genotype (n): *UAS-NetA/24B-Gal4* (212); $SemaII^{P2}/SemaII^{P2}$; *UAS-NetB/24B-Gal4* (96); $SemaII^{P2}/+$; *UAS-NetB/24B-Gal4* (113); *UAS-NetB/24B-Gal4* (128); *UAS-SemaII/+; UAS-NetB/24B-Gal4* (108); fra^3/fra^3; *UAS-NetB/24B-Gal4* (142); $SemaII^{P2}/SemaII^{P2}$ (229); *UAS-SemaII/+; 24B-Gal4/+* (172); fra^3/fra^3 (52). Gene expression levels: − −, homozygous loss-of-function mutant; −, heterozygous loss-of-function mutant; ++, moderate overexpression; and ++ (bold), strong overexpression.

innervation does not follow the normal route. Rather than projecting straight into the cleft between the two muscles, the axon extends dorsally past but adjacent to these two muscles but then reaches back to its two targets (Figure 6). Together, these phenotypes suggest that in the absence of Netrins or Frazzled, the RP3 growth cone makes its normal guidance decisions and reaches the vicinity of its target muscles, but it then fails to recognize its target muscles 7 and 6 in the normal robust fashion. These local targeting phenotypes are reminiscent of those observed for the dorsal ISN in embryos lacking Netrins (Mitchell et al., 1996) or Frazzled (Kolodziej et al., 1996): the motor axons make correct pathfinding decisions and successfully reach the area of their dorsal targets, but then they branch abnormally and wander among these muscles.

Relative Balance of Sema II and Netrins Controls the Choice of a Specific Target

To test the hypothesis that NetB is required by RP3 to overcome general Sema II–mediated repulsion, we studied the effects of changing the gene dosage of *SemaII* in *Netrin* mutant backgrounds. Removing one or both copies of *SemaII* gives dosage-sensitive suppression of the Netrin-mediated RP3 phenotype (Figures 2E and 5A). Although embryos deficient for NetB often lack RP3 innervation, embryos lacking both NetB and Sema

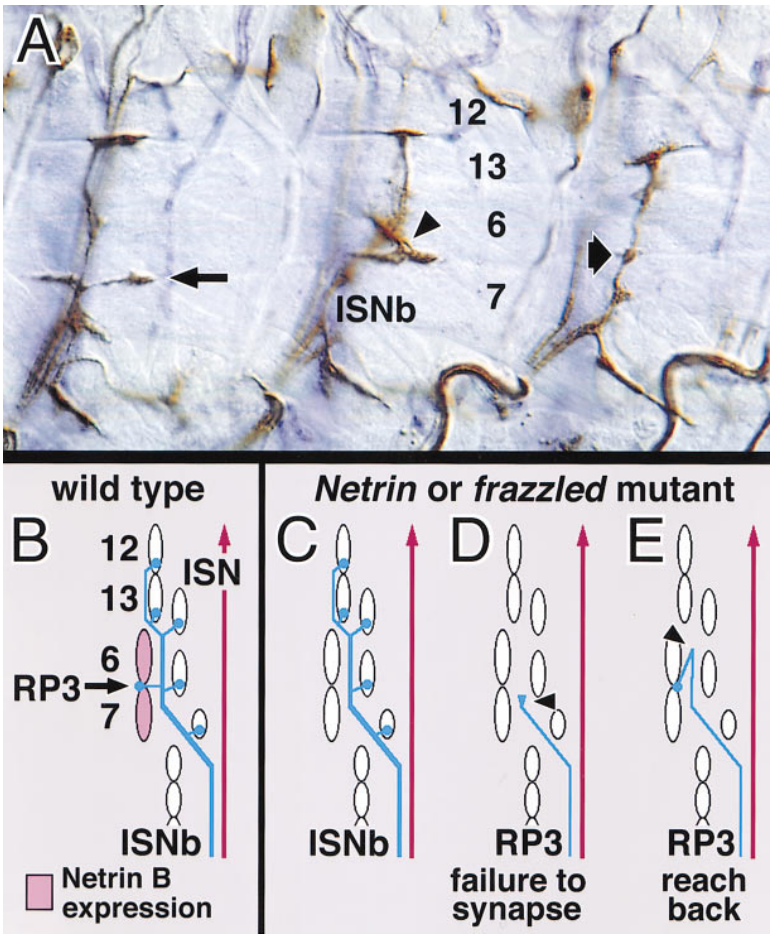

Figure 6. Behavior of RP3 Axon in *Netrin* or *frazzled* Mutant Embryos

(A) Photomicrograph of three adjacent segments showing three different behaviors of RP3 in a *frazzled* mutant embryo, stained with mAb 1D4 (anti-Fas II). In the left segment, the innervation in the cleft between muscles 7 and 6 appears normal (long arrow). In the middle segment, the RP3 axon has extended dorsally past and just adjacent to muscles 7 and 6 but then reached back (arrowhead) to innervate them. In the right segment, the RP3 growth cone has extended into its normal muscle domain (short arrow) but has failed to innervate muscles 7 and 6.

(B–D) Schematic diagrams showing the projection of motor axons to the ventral longitudinal muscles in wild type (B) and *Netrin* or *frazzled* mutant (C–E) embryos.

(C) In *Netrin* or *fra* mutant embryos, the ISNb leaves the ISN at its normal choice point and enters the ventral muscle domain. All of the other muscles are innervated as normal, but muscles 7 and 6 often lack innervation.

(D) In segments lacking normal innervation of muscles 7 and 6, the RP3 growth cone can be observed in the vicinity of and within filopodial grasp of muscles 7 and 6.

(E) In some segments in which muscles 7 and 6 are innervated by RP3, the axon has extended past these muscles and then reached back to innervate them.

II show approximately normal RP3 innervation of muscles 7 and 6. This result confirms that these two proteins have antagonistic activities with respect to RP3 target selection, and it underscores the action of other unknown target-derived molecules involved in RP3 targeting.

To test this interaction further, we examined *Netrin* mutant embryos carrying transgenes that lead to an increased level of muscle Sema II expression. A direct fusion of the *SemaII* cDNA with the *Toll* promoter drives moderate transient expression in the ventral muscles; this genetic strain partially inhibits RP3 innervation (Matthes et al., 1995). Introducing one or two copies of *Toll-SemaII* into *Netrin*-deficient embryos gives dosage-dependent enhancement of the RP3 phenotype, resulting in even fewer innervated segments (Figures 2F and 5A).

The converse experiment was also performed. A *UAS-SemaII* transgenic strain crossed to the *24B-Gal4* enhancer trap line generates robust pan-muscle Sema II expression, resulting in a very high incidence of RP3 innervation failures (Figure 5A). Simultaneously overexpressing NetA or NetB restores innervation to muscles 7 and 6 in most segments. Thus, the targeting defect caused by increased repulsion can be compensated for by simultaneously increasing attraction, just as the targeting defect caused by decreased attraction can be compensated for by simultaneously lowering repulsion. These results indicate that RP3 is sensitive to the relative rather than absolute levels of these attractants and repellents and that Netrins and Sema II act in an antagonistic fashion for this motoneuron.

Relative Balance of Sema II and Netrins Controls the Avoidance of a Potential Target

When NetA is ectopically expressed by ventral muscles, the TN axons extend ectopic projections that innervate muscles 7, 6, and occasionally 13 (Figures 1G, 3B, and 4B). In extreme cases the LBD and TMN axons do not fasciculate (and thus the TN does not form), but rather both axons turn toward and make inappropriate contacts with ventral muscles. These ectopic inputs from the TN occur even though the ISNb inputs to these muscles are normal. This TN ectopic projection phenotype is still evident but less penetrant when *UAS-NetB* is used instead of *UAS-NetA*, suggesting that NetB is less attractive to the TN axons than is NetA (Figure 4B). This is consistent with the fact that the TN axons normally do not innervate the NetB-expressing muscles 7 and 6, but they do grow distally toward the dorsal epithelial stripe expressing NetA near the alary muscle.

As described above, a decreased level of Sema II leads to an increase in ectopic TN innervation of muscles 7 and 6, while an increased level of Sema II leads to an

increase in the failure of the TN to form; both branches (the LBD and TMN axons) stall at the edges of the ventral muscle domain (Figures 1D and 3A). Given the reciprocal effects of NetA and Sema II on the TN, we asked whether the Sema II–mediated inhibition of TN formation could be suppressed by simultaneously increasing Netrin and similarly whether the NetA-mediated attraction of TN ectopic innervation could be suppressed by simultaneously increasing Sema II. Indeed, both predictions hold up. When both Sema II and NetA levels are increased, the TN forms normally, and the TN does not ectopically innervate muscles 7 and 6 (Figures 3C and 4B). Thus, the TN serves as a second example in which Netrin and Sema II function in an antagonistic fashion for a potential targeting decision.

Netrin B and Sema II Can Both Be Repulsive for Certain Motor Axons

In contrast to NetA, the overexpression of NetB on all muscles (using *24B-Gal4*) has its strongest effect on the SN. As this nerve extends dorsally away from the CNS, its first major branch, the SNc, extends laterally to innervate ventral oblique muscles (Figure 3C). The SNa extends further dorsally and then just past muscle 12 divides into two branches. One branch extends dorsally to innervate lateral transverse muscles, while the other extends laterally to innervate muscle 5 and then muscle 8.

Overexpressing NetB on all muscles has a dramatic effect on SN pathfinding (Mitchell et al., 1996). In many segments the entire nerve stalls, either still within the CNS or outside the CNS in the ventral muscle region (Figures 1H, 3G, and 5B). Overexpression of NetA does not generate this phenotype. Using NetB, the SNa sometimes succeeds in reaching the lateral domain but fails to branch. In many segments, even if the SNa extends dorsally, the SNc does not emerge as a separate projection. When the SN terminates early, it does not seek alternative targets, but rather the axons remain clumped and unbranched, suggesting that the SN axons are repelled by NetB.

Ectopically expressing NetB on only a subset of muscles further supports this interpretation. Muscles 5 and 8 are normally innervated by the lateral branch of the SNa. As seen above with *UAS-SemaII*, using *A51-Gal4* to drive *UAS-NetB* in muscle 5 specifically repels the lateral branch of the SNa in 51% of segments ($n = 200$). It often appears to stay fasciculated with the dorsal branch and sometimes takes a circuitous path to muscle 8 without innervating muscle 5.

Removing one or two copies of *SemaII* while simultaneously overexpressing NetB substantially restores both SNa and SNc outgrowth and branching, giving a nearly normal SN innervation (Figures 3H and 5B). Furthermore, simultaneous overexpression of both Sema II and NetB results in an even greater incidence of stalling (Figure 5B, black bars). These results suggest that SN growth cones are repelled by both Sema II and NetB and that they respond to the sum of the repulsion from both cues.

Frazzled Is Required for Netrin-Mediated Attraction but Not Repulsion

The *frazzled* (*fra*) gene has been proposed to encode a conserved component of a Netrin receptor (Hedgecock et al., 1990; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Fazeli et al., 1997). To test whether Fra is required for Netrin-mediated attraction and/or Netrin-mediated repulsion, we examined the effect of removing Fra while ectopically expressing NetA or NetB on all muscles. When we overexpress either NetA or NetB by ventral muscles, the axons of the TN ectopically innervate muscles 7 and 6. We crossed the *fra* mutation into these genetic backgrounds and observed very few segments with ectopic TN contacts onto muscles 7 and 6 (Figure 4B), demonstrating that the NetA- or NetB-mediated TN ectopic innervation phenotype requires Fra. This genetic suppression is strong evidence supporting the role of Frazzled as a receptor mediating the attractive activity of *Drosophila* Netrins.

In contrast, removing Fra has no effect on the NetB-mediated repulsion of SN axons (Figure 5B). Although these axons normally express Fra (Kolodziej et al., 1996), both the SNa and SNc remain highly abnormal in its absence. Thus, NetB-mediated repulsion of SN axons must act through another receptor that is independent of Fra.

Table 1. Attractive and Repulsive Guidance Forces for Motor Axons

	RP3	TN	SN
NetA	++	++	0
NetA, *fra*$^-$	0	0	0
NetB	++	+	−
NetB, *fra*$^-$	0	0	−
Sema II	−	−	−
Fas II	+	+	+

+, attractive; ++, more strongly attractive; −, repulsive; 0, no effect.

Discussion

In this study, we have used genetic analysis to dissect the molecular mechanisms controlling the ability of motor axons to recognize their appropriate muscle targets in *Drosophila*. By adding or subtracting Netrin A, Netrin B, Semaphorin II, and Fasciclin II alone or in combination, we have found that motor growth cones in this system appear to assess the relative balance of attractive and repulsive forces (Table 1, Figure 7) and to select their targets based on the complementary, combinatorial, and simultaneous input of multiple cues. This targeting system appears to be constructed out of both broadly and specifically expressed and functionally overlapping components that work together to guide growth cones toward their appropriate targets.

Fas II Promotes While Sema II Inhibits Promiscuous Synaptogenesis

Target recognition and synapse formation involves positive and negative regulation. A low level of pan-neural attraction helps promote synaptogenesis (Fas II) (Davis et al., 1997), while a low level of pan-muscle repulsion helps inhibit it (Sema II) (this study). The modest level of Sema II, while not enough to stop growth cones from exploring their environment, nevertheless provides a

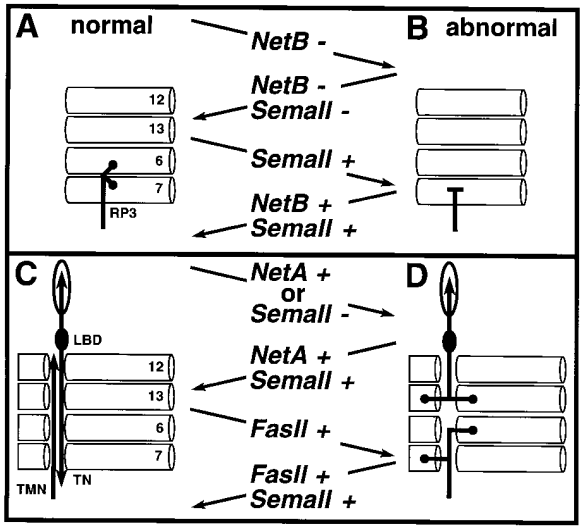

Figure 7. The Relative Balance of Guidance Cues Controls Target Selection

(A and B) Normally, the RP3 motor axon innervates muscles 7 and 6. Reduction of muscle-derived NetB leads to a lack of innervation; this abnormal phenotype can be restored to normal by simultaneously reducing Sema II levels. Similarly, increasing muscle-derived Sema II leads to a lack of innervation; this abnormal phenotype can be restored by simultaneously increasing NetB or NetA.

(C and D) Normally, the proximally projecting LBD axon meets and fasciculates with the distally projecting TMN axon around muscle 7, and they form the TN. Increasing muscle expression of either NetA, NetB, or Fas II or decreasing muscle expression of Sema II causes the TN axons to innervate muscles 7, 6, or 13 abnormally. This abnormal phenotype can be restored to normal by simultaneously increasing NetA or NetB and Sema II, or Fas II and Sema II.

threshold that specific attractive signals must overcome in order to permit synapse formation.

Decreasing Sema II leads to an increase in innervation. In the absence of Sema II, we observe targeting errors, usually in the form of additional ectopic connections to neighboring muscles, although in some cases we observe the absence of the normal connection or inappropriate choice point decisions as well. Increasing Sema II leads to a decrease in innervation.

The modest and dynamic level of Fas II helps adjust the threshold for innervation. Prior to synapse formation, Fas II is expressed at a low level across the entire surface of the muscle, making it permissive for growth cone exploration and synapse formation. As the first synapse forms on a muscle, the Fas II level dramatically plummets over the muscle surface while Fas II clusters under the developing synapse (Davis et al., 1997; Zito et al., 1997). The first successful synapse leads to a rapid reduction in this general attractant, thereby shifting the relative balance in favor of Sema II–mediated repulsion and thus raising the hurdle over which attractive signals must pass in order to promote further synapse formation. In this way, the innervated muscle becomes more refractory to further innervation. Fas II, as a modulator of the balance of attraction and repulsion, becomes a temporal measure of the muscle's synaptic history.

Sema II Helps Pattern Specific Connections

While Sema II generally prevents exuberant synapse formation, it can also play an important role in patterning connections. For example, the two axons that pioneer the TN normally meet and fasciculate near muscle 7. In the absence of Sema II, these axons often innervate muscles 7 and 6, and sometimes fail to fasciculate with one another. In this case, Sema II provides a repulsive force (from muscles 7 and 6) at a specific choice point, and in its absence, the TN growth cones make a different decision.

Similarly, as the lateral branch of the SNa extends posteriorly, one axon branch innervates muscle 5 while another continues posteriorly to innervate muscle 8. In the absence of Sema II, both sometimes stop and innervate muscle 5. In this case, Sema II provides a key repulsive force (from muscle 5) at a specific choice point, and in its absence, the growth cone that usually innervates muscle 8 instead makes a different decision. Both examples show how Sema II can do more than simply sharpen the pattern of innervation; Sema II can also influence specific targeting decisions in a dosage-dependent fashion.

The Sema II experiments show that the pattern of expression (i.e., the differential levels on neighboring muscles) can be more important than the absolute level. Simply increasing Sema II on all muscles has little influence on the SNa. But increasing Sema II expression on muscle 5 and not its neighboring muscles does influence the SNa axons, presumably because it presents these axons with a sharp repulsive boundary. This differential expression prevents the lateral branch of the SNa from extending towards muscles 5 and 8.

Netrins Function as Target Recognition Molecules

The netrins were initially discovered as long-range chemoattractants that are secreted by midline cells and that attract commissural growth cones toward the midline (Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). We previously suggested that netrins might have another function (Mitchell et al., 1996), and here we have presented strong evidence supporting that notion. In addition to their CNS midline expression and function in axon guidance, NetA and NetB are also expressed by distinct subsets of muscles where they function as short-range target recognition molecules. Genetic analysis suggests that both types of Netrin-mediated attractive responses (i.e., pathfinding and targeting) require Frazzled, the DCC/UNC-40–like Netrin receptor (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). In contrast, Fra is not required for NetB-mediated repulsion of the SN.

Netrins Do Not Function Alone in Specifying the Target

Even though they are expressed by distinct subsets of muscles and function as target recognition molecules, the two netrins, NetA and NetB, do not act alone in specifying any one of these muscle targets. NetB is expressed by muscles 7 and 6, but NetB is not the sole attractant used by RP3 to innervate these muscles. In the absence of NetB, in 35% of segments RP3 makes the correct pathfinding decisions in the periphery but fails to innervate muscles 7 and 6 properly. However, in the other 65% of segments it does innervate muscles

7 and 6. Clearly, other unknown cues must play a major role in this targeting decision. One potential candidate for an additional targeting cue is the Ig CAM Fasciclin III (Chiba et al., 1995; Kose et al., 1997). However, removal of *FasIII* does not alter the penetrance of the RP3 phenotype of *Netrin* or *frazzled* mutants (data not shown).

In addition, NetB functions within the context of the relative balance of general attractants and repellents such as Fas II and Sema II. For example, since the TN axons are attracted by NetB, and muscles 7 and 6 express NetB, why do the TN axons not synapse on muscles 7 and 6? Evidently, they are sufficiently repelled by Sema II to prevent inappropriate synapse formation. Either increasing the level of NetA or NetB or decreasing the level of Sema II leads to ectopic TN synapses. The choice of synaptic partner by TN axons is controlled by the balance of NetB in relation to Sema II and Fas II.

Distinct Classes of Motor Axons Respond Differentially to NetA and NetB

While all motor axons in this system appear to be attracted by Fas II and repelled by Sema II, the different types of motor axons respond differently to NetA and NetB (Table 1). NetB is expressed by a subset of muscles (7 and 6) where it strongly attracts appropriate (RP3) axons, more weakly attracts certain inappropriate (TN) axons, and repels other inappropriate (SN) axons. RP3 and TN axons can also be strongly attracted by NetA, while SN axons are apparently indifferent to NetA. The TN axons display a stronger responsiveness to NetA than to NetB, as judged by the frequency of ectopic innervation of ventral muscles overexpressing either Netrin. This difference may make biological sense, as TN axons normally extend toward a dorsal stripe of epithelial cells expressing NetA but grow past NetB-expressing ventral muscles without innervating them.

Toward a Molecular Logic for Discrete Target Recognition

Although we do not yet know all of the molecular signals used for this targeting system, we do know four key components: the pan-muscle expression of Fas II and Sema II and the muscle-specific expression of NetA and NetB. Our analysis of these four genes shows that the signals they encode are potent, function as short-range signals in a dosage-dependent fashion, and work in combinations that either amplify or antagonize one another. Fas II and Sema II help control the fidelity and precision of the targeting system, while NetA and NetB provide muscle-specific targeting cues. The results presented here suggest that target selection in this system is not based on absolute attractants or repellents that either ensure or prevent synapse formation, but rather it is based on the balance of attractive and repulsive forces on any given target cell in relationship to its neighboring cells (Figure 7). Targeting molecules such as Netrins, Semaphorins, and IgCAMs sometimes function as antagonists and sometimes as collaborators. This model of target selection is very similar to how we currently view axon guidance in terms of a relative balance of attractive and repulsive forces (Tessier-Lavigne and Goodman, 1996).

Experimental Procedures

Immunohistochemistry

Embryos were prepared for mRNA in situ hybridization and antibody staining according to standard protocols. Genotypes were determined using a combination of antisense NetA or NetB probes, anti-NetA or anti-NetB antibodies (Harris et al., 1996), mAb 19C2 (anti-Sema II, see below), or anti-β-galactosidase (to detect balancer chromosomes). Motor projections were stained with mAb 1D4 (anti-Fas II) (Van Vactor et al., 1993). mAb 19C2 was made against a C-terminal Sema II peptide (M. L. W., A. Kolodkin, and C. S. G., unpublished data).

Genetics

Three alleles of *SemaII* were used. *SemaII*^{P1} and *SemaII*^{P2} are insertional mutants with *rosy*-containing P elements in the ORF and 5′ UTR, respectively (Kolodkin et al., 1993). *P2* was outcrossed to remove background mutations, resulting in a semiviable line that was subsequently used to generate excision alleles, including *ex59*, a genomic deletion. The same embryonic and adult phenotypes are exhibited by *P1/P2* or *P2/ex59* transheterozygotes as by *P2/P2* homozygotes. mAb 19C2 shows no staining in *P2* or *ex59* embryos and vastly reduced staining in *P1* embryos.

The *Netrin* allele *T9-B118* is a synthetic deficiency generated by combining two X;Y translocation chromosomes, *T9* and *B118* (Stewart and Merriam, 1973). It removes both the *NetA* and *NetB* transcription units but not the *rutabaga* gene. Rescue experiments were performed with *slit-Netrin* constructs, expressed in the CNS midline, or with *F63-Gal4/UAS-Netrin* combinations.

Two null alleles of *frazzled*, *fra*^{3} and *fra*^{4}, were used (Kolodziej et al., 1996). The *24B-Gal4*, *Toll-SemaII*, *UAS-Netrin*, and *slit-Netrin* stocks have been previously described (Luo et al., 1994; Matthes et al., 1995; Mitchell et al., 1996). *UAS-SemaII*, a homozygous viable line, was a gift from A. Kolodkin. *UAS-FasII* is a homozygous viable strain carrying the PEST+ transmembrane isoform (D. Lin, unpublished data). The Gal4 enhancer trap lines *A51*, *F63*, and *H94* were generated in a screen for inserts showing CNS or muscle expression (Lin and Goodman, 1994). Embryonic transgene expression patterns were confirmed by mRNA in situs to *NetA*, *NetB* or *SemaII*, or monoclonal antibodies to Sema II or Fas II. *A51-Gal4* is expressed in muscle fibers 1, 2, 5, 18, and 27, and unidentified cells near muscle 8. *F63-Gal4* is expressed in the midline glia and in muscles 1, 2, 6, 7, and 16. *H94-Gal4* is expressed in muscles 4, 6, 13, and more weakly by 12. The expression is graded from segment to segment, with a peak in A3; care was taken to represent equally all the abdominal segments A2 through A7.

Results using *UAS-NetA*, *UAS-NetB*, or *Toll-SemaII* transgenic inserts were replicated for two or more insertion lines for each genotype.

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