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Genetic Analysis of *Netrin* Genes in Drosophila: Netrins Guide CNS Commissural Axons and Peripheral Motor Axons

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Summary

Two tandem *Netrin* genes in Drosophila are expressed at the midline of the developing CNS and in different subsets of neurons, muscles, and epidermal patches. In embryos carrying a small deficiency that deletes both genes, CNS axon commissures are partially missing or thinner. This phenotype is rescued by expressing either *Netrin* gene at the midline. Pan-neural expression of either gene causes disruption of commissural and longitudinal tracts, indicating that the pattern of Netrin expression is crucial and that Netrins function as instructive cues. The double mutant also shows defects in motor axon projections. Expression of either *Netrin* gene in all muscles also results in aberrant motor projections. Thus, Drosophila Netrins are required for the guidance of commissural axons at the midline, and of motor axons to their target muscles.

Introduction

Neurons extend growth cones along stereotyped pathways to find and synapse on their correct targets. In recent years, significant progress has been made in identifying some of the guidance molecules to which growth cones respond and the receptors that they use to distinguish between them (reviewed by Goodman, 1996). These discoveries have been made using classical and reverse genetics, and by biochemical purification in a variety of organisms from worms and fruit flies to birds and mammals. In the process, a remarkable evolutionary conservation has been discovered in the molecules that are thought to guide growing axons including, for example, cell adhesion molecules, semaphorins, and netrins.

The netrins are a family of secreted proteins that are capable of guiding growth cones. The first netrin characterized, UNC-6 in the nematode, was identified as the product of a gene (*unc-6*) that when mutated leads to defects in circumferential cell migration and axon guidance (Hedgecock et al., 1990; Ishii et al., 1992; McIntire et al., 1992). A very different approach led to the identification of the first two vertebrate netrins. In vitro experiments had shown that the floor plate at the ventral midline of the developing vertebrate spinal cord provides long-range chemotropic guidance signals for commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990). To identify this signal, embryonic chick brain proteins were tested in vitro for their ability to promote axon outgrowth from explants of dorsal spinal cord. Biochemical fractionation led to the purification of two proteins, netrin-1 and netrin-2, both of which can function as chemoattractants for commissural axons and are expressed in the developing spinal cord (Serafini et al., 1994; Kennedy et al., 1994). The vertebrate netrins have a high degree of sequence similarity (~50%) to the nematode netrin UNC-6.

Netrins are secreted proteins of ~600 amino acids. The N-termini of the netrins (~450 amino acids) are related to the N-termini of laminin subunits including domain VI and the three epidermal growth factor (EGF)-like repeats in domain V (in netrins called domains V-1, V-2, and V-3). The C-terminal ~150 amino acid domain diverges from laminins.

Netrins are thought to function as secreted proteins that can form a gradient in the extracellular environment. In chick, netrin-1 is expressed by the floor plate and could form a ventral to dorsal gradient in the developing spinal cord (Serafini et al., 1994; Kennedy et al., 1994). In the mouse, netrin-1 is expressed by the floor plate and ventral spinal cord, and genetic analysis shows that it is required for commissural axons to project correctly to the ventral midline (T. S., unpublished data). In the nematode, UNC-6 is expressed by a number of different types of ventralmost neuroglia and neurons (Wadsworth et al., 1996), and is believed to provide both long-range ventral-dorsal information and more local cues important for subsequent guidance decisions. Experimental analysis suggests that netrins are bifunctional guidance molecules in both nematodes and vertebrates, playing roles as chemoattractants for axons extending toward the ventral midline and chemorepellents for axons growing away from it (Hamelin et al., 1993; Colamarino and Tessier-Lavigne, 1995; Wadsworth et al., 1996; reviewed by Culotti and Kolodkin, 1996; reviewed by Goodman, 1996).

The Drosophila CNS, like the vertebrate CNS, is bilaterally symmetric about the midline. Some axons appear to be attracted toward the midline, others repelled away from it, and others extend right along its boundary. Special midline cells, both midline glia and VUM neurons, are essential for formation of the axon commissures and appear to provide guidance signals (Klämbt et al., 1991). A large-scale genetic screen yielded many mutants that partially or completely disrupt commissural axon pathways (Seeger et al., 1993). The conservation of netrins from nematode to vertebrates led us to wonder whether a *netrin* gene or genes also existed in Drosophila, and whether such netrin(s) played a similar role in midline guidance. If a *netrin* gene(s) does exist in fly, what is its relationship to the genes uncovered in our screen for defective commissures?

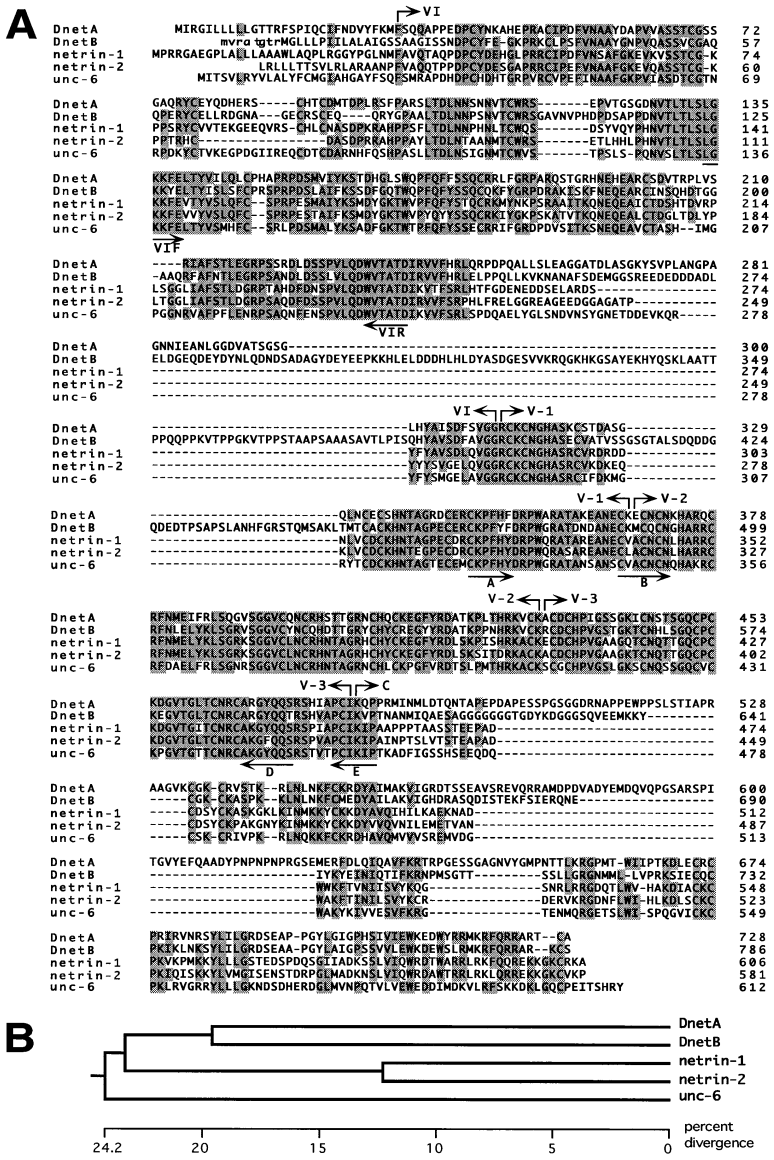

Figure 1. Alignment and Phylogeny of *Netrin* Sequences

(A) The predicted amino acid sequences of Drosophila *NetA* and *NetB* are aligned with those of C. elegans *unc-6* and chick netrin-1 and netrin-2 using the CLUSTALV algorithm (LaserGene software package). Amino acids present in three or more of the sequences are shaded. Sequence from an alternative initiation methionine for *NetB* is given in lower case. Bent arrows denote the boundaries of protein domains homologous to laminin (VI and V: EGF repeats -1, -2, and -3), and the unique C-terminus (C). Straight arrows show the positions of primers used to amplify Drosophila *Netrin* sequences (see Experimental Procedures).

(B) A phylogenetic tree was derived from an alignment that excluded regions of highly variable length. Percent divergence was calculated from this alignment using LaserGene software.

In this paper, we report on the identification and genetic analysis of two *Netrin* genes in Drosophila. Through a combination of loss-of-function analysis, rescue, and ectopic expression, we show that these two *Netrin* genes are indeed involved in guidance near the midline, and appear to play largely redundant roles in commissural axon guidance: either gene alone, when expressed at the midline, can rescue the double mutant phenotype. The observation that ectopic pan-neural CNS expression of either gene causes disruption of commissural and longitudinal tracts shows that the pattern of Netrin expression is indeed crucial. Moreover, it indicates that Netrins function as instructive, rather than simply permissive, guidance cues. We have also discovered that *Netrin* genes play a role in the peripheral guidance of motor axons. *Netrin-A* and *Netrin-B* are expressed by different subsets of muscles and epidermal patches. Loss-of-function analysis and ectopic expression phenotypes show that *Netrins* also control the targeting of specific motoneurons to particular muscles.

Results

Cloning of Two Drosophila *Netrin* Genes

We cloned two Drosophila *Netrin* genes using polymerase chain reaction (PCR) primers designed against regions of high conservation amongst chick netrin-1 and netrin-2 and Caenorhabditis elegans UNC-6 (see Experimental Procedures). An alignment of the two Drosophila genes and the chick and nematode genes is shown in Figure 1. The two fly genes are more similar to each other than either is to *netrin* genes from the other species. As this is also true of netrin-1 and netrin-2 in chick, this suggests that separate gene duplication events occurred in the vertebrate and insect lineages (see Figure 1B, phylogenetic tree). For this reason, we name the Drosophila genes *Netrin-A* (*NetA*) and *Netrin-B* (*NetB*) in order to avoid the implication that they are homologs of netrin-1 and netrin-2, respectively.

The level of homology among all family members is highest in the domains homologous to laminin, and low-

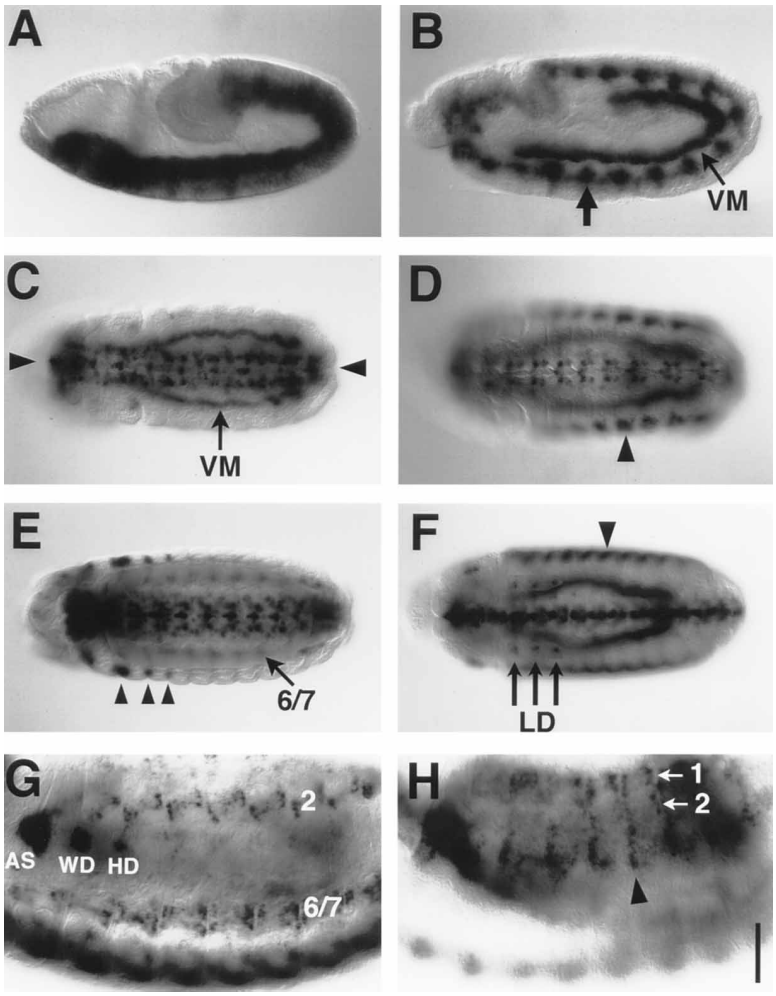

Figure 2. Embryonic Expression of *NetA* and *NetB*

In situ hybridization was performed with *NetA* or *NetB* antisense probes and developed with alkaline phosphatase. Anterior is to the left. In lateral views, dorsal is up. (E)–(G) have been assembled from multiple focal planes.

(A) *NetB*, stage 8; lateral view. The mesoderm is stained and can be seen curling around the embryo as the germband extends (*NetA* staining is similar at this stage).

(B) *NetB*, stage 11; lateral view. Much of the mesodermal staining has faded, but staining remains in the visceral mesoderm (VM). *NetB* is also expressed in small patches (thick arrow) that may be muscle precursors.

(C) *NetB*, stage 12/3; ventral view. The ventral midline of the CNS is marked by black arrowheads (this view is the same as that in [D], [E], and [F]). Midline staining is evident at this stage, as is expression in many neurons lying lateral to the midline. VM, visceral mesoderm.

(D) *NetA*, stage 12/3; ventral view (as in [C]). Weak staining is apparent in midline cells at this stage, in addition to fewer neurons than those that express *NetB*. *NetA* is also expressed in lateral epidermal patches (arrowhead).

(E) *NetB*, stage 14; ventral view. *NetB* is expressed in midline glia, and many lateral neurons in the CNS. Staining in muscles 6 and 7 is marked (slightly out of focus). In addition, at this stage, *NetB* is expressed in three epidermal patches in the thoracic segments (arrowheads), which we tentatively identify by their positions as the anterior spiracle, wing disc, and haltere disc (see [G]).

(F) *NetA*, stage 14; ventral view. *NetA* is expressed by more cells at the midline than *NetB* (also see Figure 4), and by fewer lateral neurons. The visceral mesoderm continues to express *NetA* very strongly, unlike *NetB*. In addition to the dorsolateral epidermal patches (arrowhead), *NetA* is also transiently expressed by small patches in the thoracic segments, which we believe are the leg discs (LD).

(G) *NetB*, stage 14; lateral view. Expression is observed in muscles 6 and 7 ventrally (directly apposed to each other) and muscle 2 dorsally. The CNS can be seen at the bottom. AS, anterior spiracle; WD, wing disc; HD, haltere disc.

(H) *NetA*, stage 14; lateral view. Dorsal muscles 1 and 2 are labeled. The epidermal stripe (arrowhead) extends along the anterior edge of each segment. The CNS is at the bottom, out of focus.

Scale bar, 100 μm (A–F); 50 μm (G and H).

est in the C-terminal domain. There are also several stretches of variable length and low sequence conservation in all the genes. For example, *NetB* has a unique stretch of 37 amino acids within the first EGF repeat of domain V. However, we do not know whether this alters the structure or function of this repeat.

Expression of *NetA* and *NetB*

To determine the embryonic expression of the two *Netrin* genes, we performed in situ hybridizations to embryos with antisense *NetA* or *NetB* probes. The expression of the two genes is initially similar but begins to diverge after stage 11. The first expression is observed at the cellular blastoderm stage and is restricted to the presumptive mesoderm (Figure 2A). This expression persists through gastrulation and then fades. However, *NetA* continues to be expressed very strongly, and *NetB* weakly, by the visceral mesoderm. Expression of *NetB* also remains in small patches within the somatic mesoderm layer (Figure 2B). At this stage (12/5), expression of both genes begins in the neurectoderm in subsets of cells of unknown identity (Figures 2C and 2D).

Both *NetA* and *NetB* are strongly expressed by midline cells during the initial period of commissure formation and axonogenesis in the ventral nerve cord. *NetA* is initially expressed at stages 12 and 13 by the two anterior pair of midline glia (MGA and MGM) and by the VUM neurons (Figure 3A). During this period, both the anterior and posterior commissures are being pioneered by axons that project directly toward, and make intimate contact with, these midline glia and the growth cones of the VUM neurons (Klämbt et al., 1991). The expression in the VUM cluster subsequently fades, while the anterior and middle pairs of midline glia continue to express *NetA* strongly throughout embryogenesis. In addition, a large pair of cells located posterior to the posterior

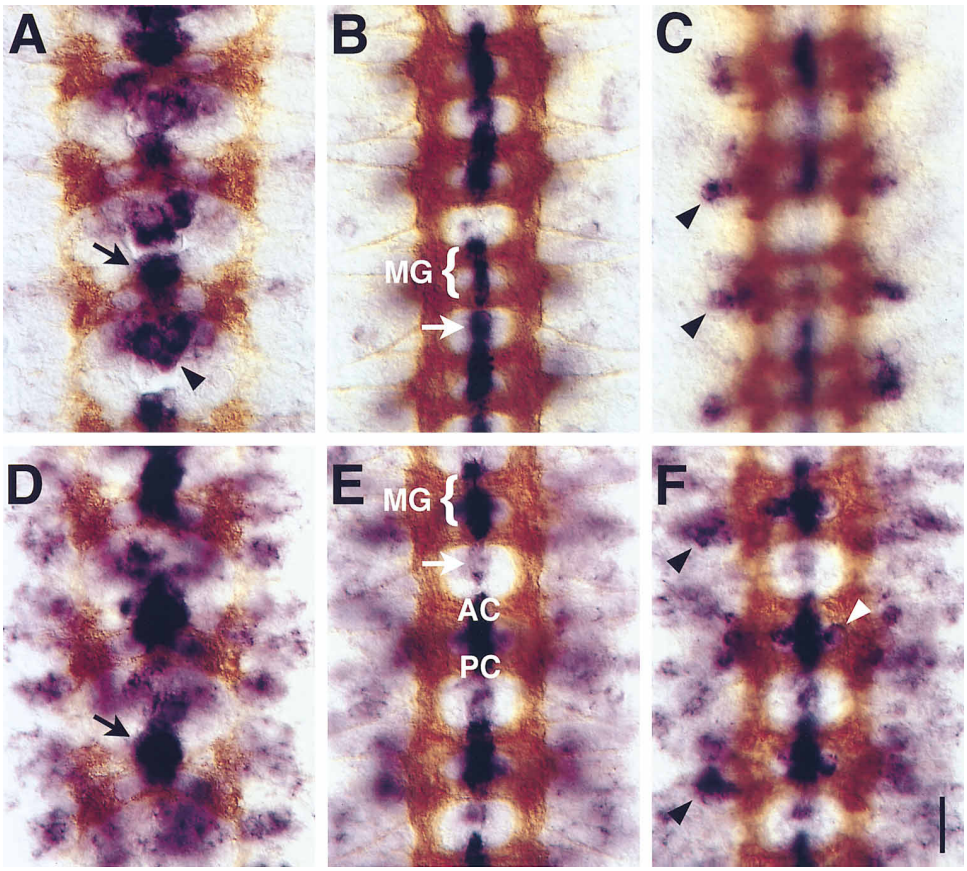

Figure 3. Embryonic CNS Expression of *NetA* and *NetB*

Embryos were double-stained with *NetA* (A–C) or *NetB* (D–F) in situ probes (purple) and antibody BP102, which stains all CNS axons (brown). They were dissected to expose the inner surface of the CNS. Each panel shows three to four abdominal segments of the CNS, with the midline in the center. Anterior is up.

(A) *NetA*, stage 13. The commissures are just being pioneered and are not yet fully separated into anterior and posterior commissures, forming a bow-tie shaped structure. *NetA* is expressed in midline glia (arrow) and in cells in the VUM cluster (arrowhead), in addition to weaker staining in a small number of lateral neurons.

(B) *NetA*, stage 14. By this stage the commissures have formed and separated completely, and the longitudinal connectives are also well formed. Expression is visible in the midline glia (MG), and in large cells posterior to the posterior commissure (arrow).

(C) Same embryo as in (B), shown in a more ventral (outer) focal plane. Staining in ventral clusters of cells, which are probably neurons, is marked with arrowheads.

(D) *NetB*, stage 13. Expression at the midline is largely restricted to the midline glia (arrow) with no obvious staining in the VUM cluster (compare with [A]). There is more staining in other cells, however, throughout the CNS.

(E) *NetB*, stage 14. Expression is evident in midline glia (MG), but is much weaker than *NetA* in the large posterior cells (arrow). AC and PC mark the anterior and posterior commissures, respectively.

(F) Same embryo as in (E), focused more ventrally. The white arrowhead marks cells in the MP cluster. Black arrowheads mark neurons throughout the CNS.

Scale bar, 10 μm.

commissure also stains strongly with *NetA* at this stage (Figure 3B). These cells may be associated with the median neuroblast (MNB) (Bossing and Technau, 1994). In contrast with the wide expression of *NetA* at the midline, *NetB* is expressed in a more restricted pattern (compare Figures 2E and 2F; Figures 3B and 3E). The midline glia express *NetB* very strongly, but there is no evidence of strong expression in either the VUM cluster or the MNB cluster.

NetA and *NetB* are also expressed in dynamic patterns by different but possibly overlapping subsets of CNS neurons (see Figures 2C–2F; Figures 3C and 3F). *NetB* is expressed by many more neurons than *NetA*, and notably by some of the MP neurons, which lie on either side of the midline glia and which pioneer the well characterized MP1 longitudinal fascicle (Figure 3F). Given this pattern of expression, it is possible that Netrin proteins may be expressed on subsets of axons, labeling specific pathways. Such expression has been observed in nematodes, where expression of UNC-6 by various cells, including neurons, is believed to provide local cues that direct the migration of other cells or growth cones (Wadsworth et al., 1996).

In addition to expression within the CNS, both genes are also expressed in the periphery. *NetA* is expressed in lateral patches in the epidermis, which subsequently

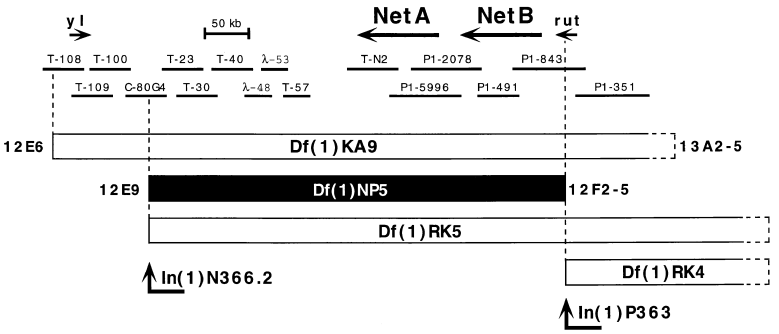

Figure 4. Genetic and Physical Map of the Region Surrounding the *Netrin* Genes

A rough map showing the overlaps of physical clones. "T" refers to cosmids from the Tamkun library, "C" to cosmids from the Crete library (Madueno et al., 1995), "P1" to P1 clones from the BDGP, and "λ" to λ-clones. The contig on the left around the *yolkless* gene (*yl*) was provided by C. Schonbaum. The positions and direction of transcription of genes are denoted by arrows above the contigs. The boxes show the extent of several deficiencies with respect to the physical map, and the rough cytological positions of their breakpoints. Bent arrows show the distal breakpoints of inversion chromosomes. The distal breakpoint of *Df(1)NP5* [and In(1)N366.2 and *Df(1)RK5*] was mapped by identifying a restriction length polymorphism by probing genomic digests with the cosmid C-80G4 (our data not shown; T. Raabe, personal communication). The proximal breakpoint of *Df(1)NP5* appears to be within the *rutabaga* gene *(rut),* as the breakpoint of In(1)P363 has been mapped cytologically to the same site as *rut* (12F2-5), and in situ hybridization to In(1)P363 embryos reveals that they have no *rut* transcript. This is also true of *Df(1)NP5* and *Df(1)RK4*. We do not believe that *Df(1)NP5* extends any farther than the *rut* gene, as the lethality is rescued by Dp(1;f)LJ9 (12A6-10; 13A2-5), unlike the lethality of *Df(1)RK5*.

elongate along the anterior edge of the segment border (see Figures 2D, 2F, and 2H; see Figure 6A). *NetA* is also strongly expressed by dorsal muscle 1, and weakly by dorsal muscle 2 (see Figure 2H). *NetB* is also expressed by dorsal muscle 2 (but not muscle 1), and by ventral muscles 6 and 7 (see Figures 2E and 2G).

Both genes are transiently expressed by the embryonic precursors of imaginal discs: *NetA* in the leg discs at stages 13 and 14 (see Figure 2F) and *NetB* in the wing and haltere discs (and anterior spiracle) at stages 13 and 14 (see Figures 2E and 2G). *NetB* is also weakly expressed in cardioblasts. Other tissues that express the *Netrin* genes include many cells in the brain and some epidermal structures in the head that may be imaginal discs.

The expression of the *Netrin* genes in the nervous system, especially at the midline, suggests that Drosophila Netrin proteins might be involved in guiding axons in the vicinity of the midline. In addition, the muscle and peripheral expression led us to speculate that they might also be involved in motoneuron pathfinding. We have therefore focused our analysis of Netrin function on axon guidance in these two regions.

Genomic Mapping of *NetA* and *NetB*

NetA maps to cytological position 12E10 on the X chromosome (Figure 4). We constructed a genomic walk of the region around the gene, from 12E9 to 13A1, using P1 clones obtained from the Berkeley Drosophila Genome Project and cosmids isolated from the Tamkun library (Tamkun et al., 1992). In addition, we obtained another walk that spans 12E6 to 12E9 (Schonbaum et al., 1995), which does not overlap our own, but which we used to map deficiency breakpoints (see Figure 4). We mapped the physical extent of the *NetA* gene by hybridization to clones within the walk, and subsequently discovered that *NetB* also hybridizes to this same region. The two genes are, in fact, very close to each other, occupying a region of ~150 kb in total, and are transcribed in the same direction.

Genetic Analysis of *Netrin* Gene Function

We obtained several deficiencies that delete part of the 12-13 region of the X chromosome (Figure 4) and stained hemizygous deficiency embryos (Df/Y) with antisense *Netrin* probes in order to determine whether they deleted the *Netrin* genes. We also stained these embryos with the antibody BP102, which labels CNS axons, to assess whether CNS development was normal. We found that *Df(1)KA9* (12E6; 13A2-5) and *Df(1)RK5* (12E9-11; 13B2-5) both delete *NetA* and *NetB*. Both of these deficiencies cause similar CNS phenotypes although those of *Df(1)KA9* embryos are more severe. The axon commissures are drastically thinner or missing, and the longitudinal tracts are highly disorganized and discontinuous. Because the phenotypes of these deficiencies may be confounded by loss of other genes besides the *Netrin* genes, we decided to generate a smaller deficiency, which should more accurately reflect the true *Netrin* loss-of-function phenotype.

We isolated such a deficiency by allowing recombination to occur between two inversion chromosomes that break in this region and screening recombinants for X-linked lethality (see Experimental Procedures; Figure 4). This resulted in two independent recombinant chromosomes (NP3 and NP5), both of which carry a deficiency of the region from 12E9-11 to 12F2-5. These two lines have the same phenotypic properties and deficiency breakpoints (see Experimental Procedures; Figure 4), and we refer below to only one, *Df(1)NP5*.

Loss-of-Function CNS Phenotype

Df(1)NP5 is significantly smaller than *Df(1)KA9* but still deletes both *NetA* and *NetB* (Figure 4). The phenotype of *Df(1)NP5* embryos is less severe than that of *Df(1)KA9* embryos and should more closely reflect the true functions of these genes. In general, the commissures are much thinner than normal and sometimes completely absent, and there are occasional breaks in the longitudinal tracts (2.5 per embryo, $n = 24$) (Figures 5A–5C). The posterior commissure is more severely affected than the anterior commissure: only 32% of posterior commissures appear of wild-type or near wild-type thickness in mutant embryos, while 74% of anterior commissures remain relatively normal (Figures 5A–5C; Table 1).

There are no obvious morphological defects in *Df(1)NP5* embryos, no segmentation defects, and there

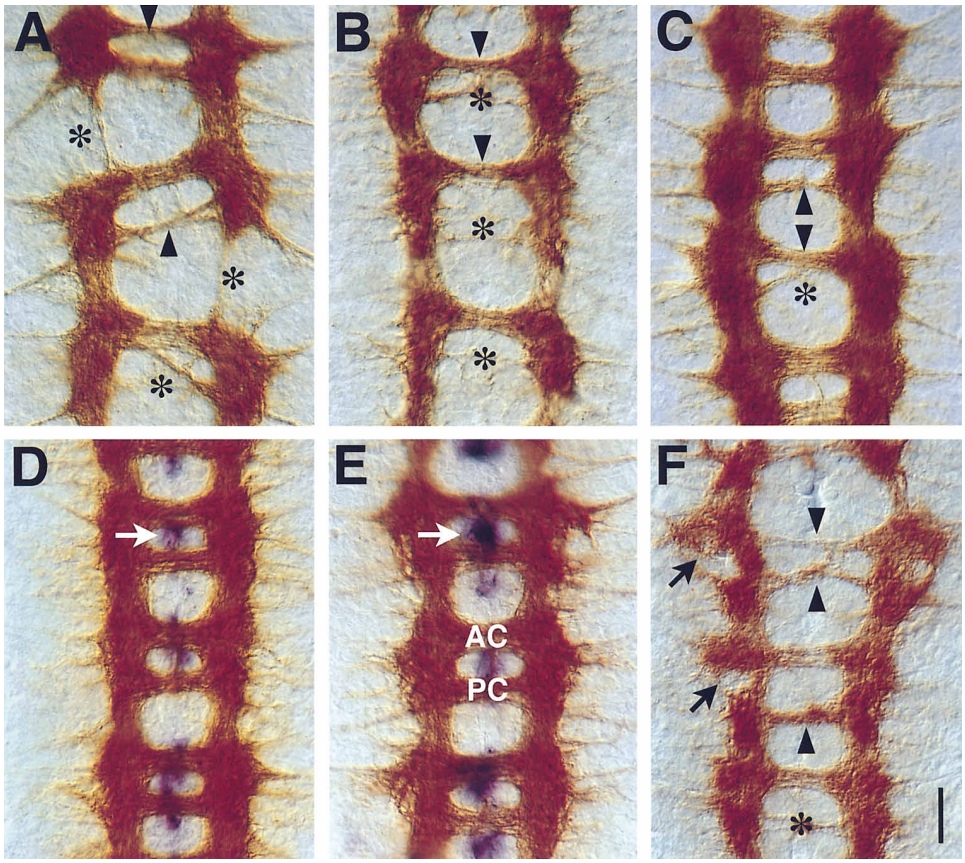

Figure 5. CNS Mutant Phenotypes and Rescue of *Netrin* Genes

Embryos were double-stained for *Netrin* mRNA (purple) and with MAb BP102 (brown), as in Figure 3. (A)–(C) show examples of *Df(1)NP5*/Y embryos, which lack both *Netrin* mRNAs. (D)–(E) show *Df(1)NP5*/Y embryos with midline expression restored using slit–*Netrin* transgenes.

(A) Severe *Df(1)NP5* phenotype. The commissures are often thin (arrowheads) and sometimes absent (asterisk marks missing posterior commissure). In addition, the longitudinal connectives are sometimes broken between segments (asterisks).

(B) Moderate phenotype. The general organization of the nerve cord appears better than in (A), although the anterior commissure is thin in two segments (arrowheads), and the posterior commissure is nearly absent in three segments (asterisks).

(C) Weak phenotype. Many of the commissures appear relatively normal, although several are thin (arrowheads) or missing (asterisk).

(D) *Df(1)NP5*/Y; slit–*NetA*/+ rescue embryo. Midline expression of *NetA* has been restored at the midline (arrow) using the *slit* enhancer/promoter. This leads to a remarkably normal looking CNS axon scaffold: the commissures are of wild-type thickness, and the CNS seems better organized (see Table 1).

(E) *Df(1)NP5*/Y; slit–*NetB*/+ rescue embryo. One copy of the slit–*NetB* construct drives expression at the midline (arrow) and rescues most of the commissural defects (see Table 1). AC, anterior commissure; PC, posterior commissure.

(F) *Scabrous*–GAL4/+; UAS–*NetB*/+ ectopic expression embryo. This embryo expresses *NetB* in all neurons throughout the CNS. The commissures are thinner than normal (arrowheads) or nearly absent (asterisk). In addition, bundles of axons can be seen projecting inappropriately laterally toward the edge of the CNS, leaving gaps in the longitudinal tracts (arrows).

Scale bar, 10 μm.

do not appear to be any gross changes in fate or patterning of midline cells or CNS neurons, as determined by the pattern of staining with several antibodies that stain subsets of cells (e.g., anti-eve, 22C10, 1D4). Using a variety of cell markers, we also see no signs of an increase in cell death. This suggests that the axonal phenotypes observed are not secondary to general abnormal CNS development, but are more likely directly attributable to defects in axonal outgrowth and guidance. Although the reduction in the number of axons in the commissures suggests a failure in the attraction of growth cones toward the midline, we cannot rule out the possibility that some CNS axons are normally repelled by Netrin and that some of the axons that do cross the midline in *Df(1)NP5* embryos are doing so inappropriately.

Either *Netrin* Gene Can Rescue the Commissural Phenotype

In addition to deleting *NetA* and *NetB*, *Df(1)NP5* also probably deletes several other genes. To determine whether the commissural defects we observe in NP5 embryos are due to lack of *Netrin* expression, and whether both *Netrins* play similar or redundant roles in commissure formation, we restored expression of *NetA* or *NetB* at the midline. Transgenic flies were created that carry either the *NetA* or *NetB* cDNA under the control of an enhancer/promoter construct from the *slit* gene

Table 1. Penetrance and Rescue of Commissural Defects

Genotype	% of Anterior Commissures: Absent[a]	Thin	Wild-Type[b]	(n)[c]	% of Posterior Commissures: Absent	Thin	Wild-Type	(n)[c]
Df(1)NP5/Y	2	24	74	(191)	29	39	32	(188)
Df(1)NP5/Y; slit:A[d]/+	0	4	96	(80)	1	8	91	(80)
Df(1)NP5/Y; slit:B[e]/+	0	6	94	(135)	11	24	65	(130)
Df(1)NP5/Y; slit:B/slit:B	0	0	100	(91)	3	4	93	(88)
Df(1)KA9/Y	52	29	19	(90)	74	17	9	(91)
Df(1)KA9/Y; slit:A/+	2	14	84	(84)	11	22	67	(84)
Df(1)KA9/Y; slit:B/+	10	25	65	(91)	17	29	54	(90)

[a] Absent or nearly absent
[b] Wild-type or nearly wild-type
[c] n = the number of commissures scored, usually including those in segments A1-A7 of a given embryo
[d] *NetA* driven by the *slit* promoter
[e] *NetB* driven by the *slit* promoter

(Wharton and Crews, 1993). This construct drives expression at early stages in all midline cells, but becomes restricted by stage 13 to just the midline glia. We crossed flies carrying these constructs to *Df(1)NP5* flies and analyzed the phenotypes of embryos hemizygous for *Df(1)NP5* and carrying one copy of the slit–*NetA* or slit–*NetB* construct on an autosome (Figures 5D and 5E).

We found that restoration of midline expression of either *NetA* or *NetB* effectively rescues the commissural phenotype in these embryos (Figures 5D and 5E). We quantified the penetrance and degree of rescue of commissural defects in *Df(1)NP5* and rescued embryos by scoring the anterior and posterior commissures into three bins as follows: wild-type or nearly wild-type, thinner than normal (for example, see arrowheads in Figures 5A–5C), and absent or nearly absent (for example, see asterisks in Figures 5A–5C). These data are presented in Table 1. We obtained several transgenic lines carrying the slit–*NetA* construct and determined that two independent lines with inserts on different chromosomes both rescued, indicating that rescue was not dependent on insertion at a particular site. In addition, *Df(1)NP5* hemizygous sibling progeny from these crosses, which failed to inherit the transgene, showed the same phenotype as *Df(1)NP5* hemizygous embryos from the NP5 stock, indicating that rescue was not due to differences in genetic background.

We isolated only one transformant of the slit–*NetB* construct and found that, on average, it did not rescue as well as one copy of our strongest expressing slit–*NetA* construct (for example, 65% of posterior commissures relatively normal versus 91%). However, the expression from the slit–*NetB* construct was rather low, compared with the endogenous *NetB* midline expression, as indicated by intensity of in situ hybridization staining. We determined that increasing the copy number of this construct by making *Df(1)NP5* embryos homozygous for the slit–*NetB* chromosome rescued much more effectively (93% of posterior commissures relatively normal; Table 1). Thus, it appears that wild-type levels of either NETA or NETB can function to attract axons to the midline to form the commissures, and both must be absent to cause a substantial midline phenotype. Although the restoration of midline expression rescues commissure formation, it does not reduce the incidence of breaks in the longitudinal tracts in these embryos (see Discussion).

In contrast with the phenotype of the NP5 deficiency, the more severe phenotype of embryos hemizygous for the larger deficiency, KA9, is not as well rescued by midline expression of either *Netrin* gene (see Table 1). Netrin expression does improve commissure formation in KA9 to some degree. However, the rescued KA9 embryos do not have as completely normal appearing commissures as do the rescued NP5 embryos, suggesting that something else on the KA9 chromosome is affecting CNS development, independent of the lack of the two Netrins.

Ectopic Expression of Netrins in the Developing CNS

If axons extract guidance information from the precise localization of Netrin proteins secreted by particular cells, then by ectopically expressing *Netrin* genes we should be able to disrupt guidance decisions. This approach allows us to assess directly the contributions of one or the other of the two *Netrin* genes, and to look at the responses of axons to Netrin without the complication of possible effects from other genes.

We have taken advantage of the versatile GAL4–UAS system (Brand and Perrimon, 1993) to express *NetA* and *NetB* in various tissues. This system utilizes enhancer trap lines or transgenic constructs that express the transcription factor GAL4 in a particular pattern. We created constructs that fused the yeast upstream activating sequence (UAS) to either the *NetA* or *NetB* cDNA, and established transgenic lines carrying these constructs. When these lines are crossed to the GAL4 enhancer trap lines, the *Netrin* construct will now be expressed in the cells where GAL4 is being made.

By crossing a *Scabrous*–GAL4 construct line (Klaes et al., 1994) to one carrying UAS–*NetA* or UAS–*NetB*, we created flies that express either *Netrin* gene throughout the developing CNS. An example of an embryo carrying *Sca*–GAL4 and UAS–*NetB* is shown in Figure 5F. The commissures are thinner than normal or entirely absent, similar to the phenotype observed in *Netrin* deficiency embryos. This suggests that commissural axons are now either indifferent to the midline *Netrin* source

owing to uniform levels throughout the CNS, or are instead now attracted to axons or cell bodies lateral to the midline. In addition, the longitudinal tracts appear somewhat ragged, and we frequently observe bundles of BP102-stained axons projecting inappropriately laterally toward the nerve roots, as if repelled by *Netrin* in the CNS.

We observe qualitatively similar phenotypes when we cross *Sca*–GAL4 lines to UAS–*NetA*, although they are less severe and less penetrant. We also see a range of severity depending on the particular insert line of UAS–*NetA* that we use, suggesting that different expression levels from different inserts yield varying levels of effect. This is also observed when comparing the strongly driving pan-neural *Sca*–GAL4 with the more weakly driving pan-neural *elav*–GAL4 construct (Luo et al., 1994), which causes less severe, but qualitatively similar, phenotypes when crossed to either UAS–*NetA* or UAS–*NetB*. These data suggest that the differences we observe in the effects of UAS–*NetA* and UAS–*NetB* may result from a lower level of expression from the UAS–*NetA* line. However, it is also possible that the activities of the two proteins are somehow different.

These experiments confirm that axons rely on differential midline *Netrin* cues to correctly form commissures, and also suggest that a subset of CNS axons may be repelled by *Netrin*.

Netrins Guide Motoneuron Growth Cones in the Periphery

As described above, *NetA* and *NetB* are also expressed by discrete subsets of muscles and epidermal patches. The abdominal segments have an array of 30 muscles along the body wall. *NetA* is expressed in dorsal muscles 1 and 2 and in a patch of epidermal cells that extends along the anterior border of each segment in a dorsolateral position. *NetB* is expressed by muscle 2, but not muscle 1, and in ventral muscles 6 and 7. These patterns of expression suggest that *Netrins* might be involved in guiding motoneurons in the periphery. To investigate this, we analyzed the projections of motoneurons in *Df(1)NP5* embryos, which lack both *Netrin* genes, and in embryos in which we ectopically expressed NETB on all muscles.

Motor projections can be visualized with a monoclonal antibody against Fasciclin II, 1D4 (Van Vactor et al., 1993) (Figure 6). Motoneurons exit the CNS in one of two main nerves. The main branch of the intersegmental nerve (ISN) projects dorsally past the epidermal stripe of *NetA* expression (just posterior and internal to it) to innervate the dorsal muscles, some of which express *NetA* and *NetB*. (Figures 6A and 6B). The segmental nerve b (SNb) is actually a branch of the ISN (Krueger et al., 1996) that leaves the ISN at a specific choice point to project to the ventral muscles, including muscles 6 and 7, which express *NetB*, and others (e.g., muscles 12 and 13) that do not. The true segmental nerve (SN) splits into several branches, including SNa, which projects to the lateral muscles, which do not express either *Netrin* gene.

In *Df(1)NP5* embryos, we observe occasional errors in the projection patterns of the motor nerves. These often include lack of innervation of muscles 6 and 7 (39% of 143 segments). This particular phenotype, however, is difficult to interpret, since in the *Netrin* deficiency mutant there are also defects in midline guidance of the motoneuron that normally innervates these muscles (RP3), suggesting that the muscle innervation defects could be secondary to errors in guidance within the CNS. Although lack of innervation of muscles 6 and 7 is still observed at similar frequencies in embryos where we have rescued commissure formation in general, we cannot be certain that we have restored midline crossing of the motor axons in question (i.e., RP3) in every segment.

However, in the case of the ISN, it appears that the early projections of the motoneurons that comprise this nerve (e.g., aCC) are made correctly and that the nerve exits the CNS normally. In older embryos, however, when the ISN has reached the dorsal region of the body wall, the axons appear to explore and wander more than normal. The ISN often (36% of 230 segments) branches inappropriately, stalls, or projects past its dorsal target muscles, and sometimes (16% of 230 segments) crosses segment borders to fasciculate with the ISN in the adjacent segment (Figure 6C) or sends collaterals into adjacent segments (Figure 6D). These dramatic crossover phenotypes may reflect instances where excessive exploration has led to stabilization of chance contacts with axons in neighboring segments.

Several lines of evidence suggest that the ISN defects are direct consequences of lack of *Netrin* expression in the periphery. During normal development, the ISN travels along various substrates, including branches of the tracheae and ingrowing sensory axons, before innervating the dorsal muscles. The projection of sensory axons into the CNS in *Df(1)NP5* embryos appears almost completely normal, with very occasional instances of the dorsal cluster axons crossing into the adjacent segment (data not shown). These are not frequent enough to account for the ISN crossovers, however. Furthermore, tracheal development proceeds normally and the pattern of muscles is also wild-type. It therefore seems likely that the defects in ISN projection are not secondary to changes in the usual substrates of the axons or to guidance errors in the CNS. Rather, the correlation of the area where guidance errors are observed with the areas of expression of *NetA* and *NetB* suggests that the ISN motoneurons are guided by Netrins in the periphery.

An alternative interpretation of our data is that the motoneuron pathfinding defects are due to the absence of another gene that is also deleted in *Df(1)NP5*. However, the phenotypes we observe are remarkably similar to those observed in embryos mutant for the gene *frazzled*, the Drosophila homolog of the C. elegans *unc-40* and vertebrate *deleted-in-colorectal carcinoma* (*DCC*) genes (P. A. Kolodziej, unpublished data). These genes encode putative receptors for Netrins, suggesting that the phenotypes we observe are indeed due to lack of Netrin, rather than another gene that is also deleted in *Df(1)NP5*. Furthermore, ectopic expression of Netrins by all muscles also causes misrouting of motor axons, showing that these axons are in fact responsive to the precise expression patterns of Netrin proteins by muscles.

The 24B–GAL4 line drives expression quite early in

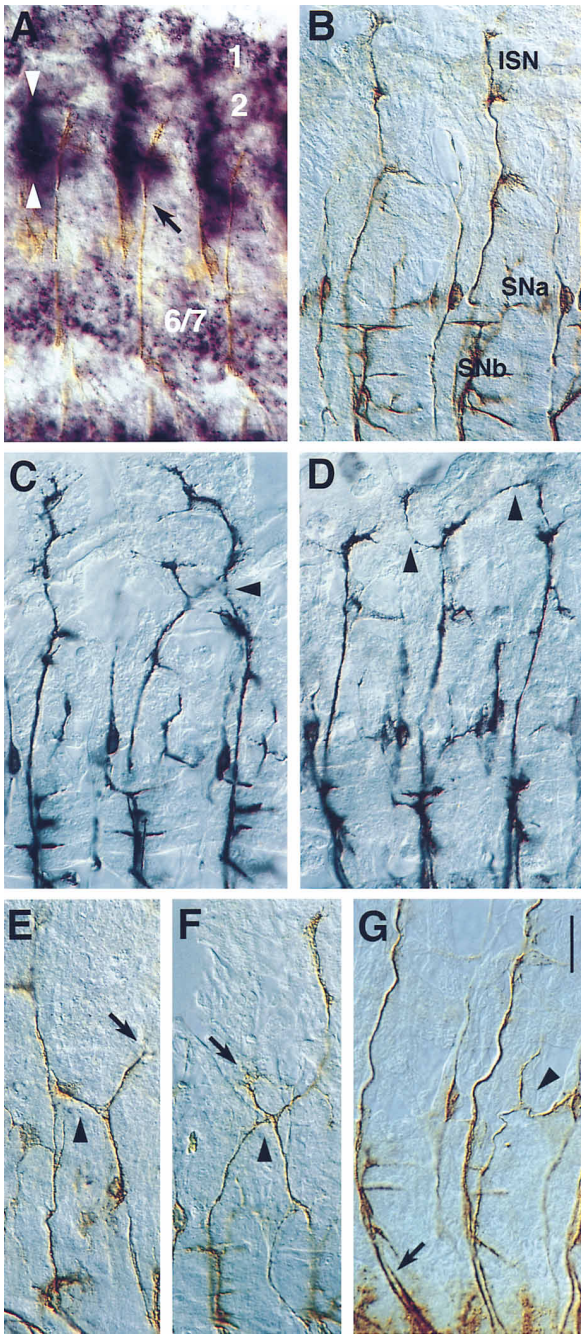

Figure 6. Motoneuron Loss-of-Function and Ectopic Expression *Netrin* Phenotypes

Each panel shows the motoneuron projections in two or three abdominal hemisegments of a filleted embryo. Anterior is to the left, dorsal is to the top, and the CNS is out of the frame of the picture at the bottom. Motoneurons are visualized with the monoclonal antibody 1D4, against Fasciclin II. All embryos are late stage 16, except (A), which is stage 15. (A and B): wild-type. (C and D): *Df(1)NP5/Y*. (E–G): 24B–GAL4/+; UAS–*NetB*/+ embryos ectopically expressing *NetB* by all muscles. (B)–(D) have been assembled from multiple focal planes.

(A) This stage 15 embryo has been double-stained with MAb 1D4 (brown) and with antisense probes to both *NetA* and *NetB* together (purple). The ISN (black arrow) is growing out dorsally, just posterior and internal to the epidermal stripe of *NetA* expression (white arrowheads) toward the dorsal muscle group, which includes the *Netrin*-positive muscles 1 and 2. Ventral muscles 6 and 7, expressing *NetB*, are also marked.

(B) Wild-type embryo at late stage 16. The ISN has projected out to the dorsal muscle group. The SNa is slightly out of focus, projecting to the lateral muscle group, while the SNb can be seen projecting to the ventral muscle group.

(C) *Df(1)NP5/Y* loss-of-function mutant embryo. The ISN from the middle segment branches, projects inappropriately across the segment border, and fasciculates with the ISN in the posterior segment (arrowhead). The branches over the dorsal muscles are abnormal. (Some muscles have been damaged by dissection).

(D) *Df(1)NP5/Y* loss-of-function mutant embryo. Collaterals in the dorsal muscle region can be seen projecting across segment borders from the ISN in several segments (arrowheads). (Some muscles have been damaged by dissection).

(E) 24B–GAL4/+; UAS–*NetB*/+. The ISN in one segment has formed an anterior branch, which projects across the segment border (arrowhead), and a posterior branch, which has stalled short of its dorsal targets (arrow).

(F) 24B–GAL4/+; UAS–*NetB*/+. The ISNs from two segments have crossed each other (arrowhead) and one has stalled in the lateral part of the body wall (arrow).

(G) 24B–GAL4/+; UAS–*NetB*/+. Higher magnification view of the SNa projections. The SNa projects normally in the segment on the right (arrowhead), but is stalled near the CNS in the segment on the left (arrow).

Scale bar, 15 μm (A–F); 10 μm (G).

myoblasts and then in all muscles (Luo et al., 1994). We drove *Netrin* expression in this pattern by crossing UAS–*NetA* or UAS–*NetB* lines to this GAL4 line, and analyzed the motoneuron projections. Figures 6E–6G show several examples of embryos carrying 24B–GAL4 and UAS–*NetB*. As with ectopic expression in the CNS (see above), crosses to UAS–*NetA* lines yielded similar but weaker phenotypes, probably owing to lower expression levels. In crosses to UAS–*NetB*, the ISN can often (51% of segments, n = 96) be seen crossing segment borders, branching excessively, and sometimes even stalling short of the dorsal muscles (Figures 6E and 6F), suggesting that uniform *Netrin* expression renders these axons incapable of distinguishing their correct targets (see Discussion).

Another frequent phenotype that we observe when ectopically expressing *NetB* on all muscles is a failure of the SNa branch to innervate its normal targets, the lateral muscles, which in wild-type embryos do not express *Netrin*. Instead, the SNa axons can often (41% of segments, n = 98) be seen stalled at the edge of the CNS (Figure 6G) or fasciculated with the ISN and failing to enter the lateral muscle region. This suggests that the SNa axons may be repelled by Netrin, which in wild-type embryos could help to exclude them from inappropriate target regions.

Discussion

Netrins act as important signals to attract and repel different subsets of axons toward or away from the ventral midline in nematode and vertebrates. Here, we have shown that Netrins function in the same fashion in Drosophila. This genetic analysis in Drosophila also provides two novel insights into Netrin function. First, Netrins are expressed by subsets of muscles and function in motor axon targeting. Second, ectopic expression

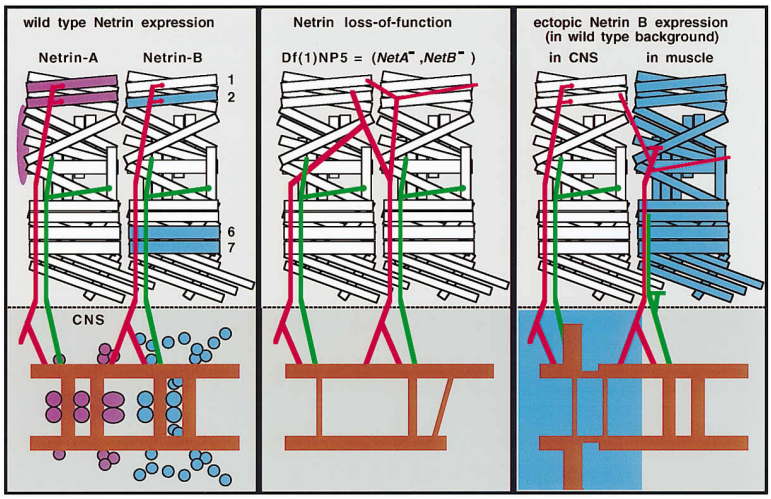

Figure 7. Summary of Netrin Expression, Loss-of-Function Mutant Phenotypes, and Ectopic Expression Phenotypes in CNS and Peripheral Motor Projections

Left: Expression of *NetA* (purple) and *NetB* (blue) in the CNS (bottom) and periphery (top) of a wild-type embryo (two segments). CNS axons are shown in brown, and motoneurons are shown projecting to the musculature in the ISN (red) and the SNa (green). *NetA* is expressed in more midline cells than *NetB*, while *NetB* is expressed in more cells in the lateral CNS. In the periphery, *NetA* is expressed in a patch of epidermal cells (oval) and in dorsal muscles 1 (stronger) and 2 (weaker). *NetB* is expressed in muscle 2 and in ventral muscles 6 and 7.

Middle: CNS and motoneuron defects in *Df(1)NP5* embryos, which lack both *Netrin* genes. In the CNS, commissures are thinner or absent, and there are occasional breaks in the longitudinal tracts. In the periphery, the ISN explores dorsal muscle regions excessively and can sometimes be seen crossing segment borders or sending collaterals into the adjacent segment. The SNa projections appear largely normal.

Right: Phenotypes due to ectopic expression of *NetB* in either the CNS (left) or in muscles (right). Ectopic expression throughout the CNS (blue) leads to thinning of the commissures and aberrant projection of axon bundles toward the nerve root exit points. Ectopic expression in all muscles (blue) causes the ISN to wander, branch, and sometimes stall short of its dorsal targets. The SNa often fails to enter its lateral target region, whose muscles normally do not express *Netrin*.

phenotypes in both the CNS and periphery show that the pattern of Netrin expression is crucial to correct patterning of axons, providing evidence that Netrins function as instructive, rather than simply permissive, guidance cues.

We have identified two tandem *Netrin* genes in Drosophila that appear to represent a gene duplication independent of that which occured in the vertebrate lineage. Both genes are expressed by midline glia. When both genes are deleted, the commissures are thin or missing. Expression of either gene alone at the midline can rescue this double mutant phenotype. Thus, at least for this midline function, the two *Netrin* genes in Drosophila appear to play largely redundant roles.

Although the two fly *Netrins* are highly conserved with netrins from nematode and chick, the fly genes contain several long stretches of amino acids not present in the other species, which could conceivably confer different properties on the two proteins (Figure 1). In particular, the insert in the first EGF repeat of NETB might affect receptor binding or specificity. At present, however, we have not identified any separable activities of NETA and NETB. This notion of functionally redundant genes with different patterns of expression has been observed for the two *engrailed* genes in mice (Hanks et al., 1995) and the two *gooseberry* genes in Drosophila (Li and Noll, 1994). Although this appears to be the case for Netrin function at the midline, we do not know whether the two genes are functionally equivalent for motor axons as well.

As in the developing spinal cord in vertebrates and the body wall of the nematode, the fly *Netrin* genes are expressed at the ventral midline of the CNS. Both *Netrin* genes are expressed strongly by the midline glial cells, while only one of them (*NetA*) is expressed strongly by cells in the VUM neuron cluster (Figure 7). The first axons, which pioneer the anterior and posterior commissures, project directly toward, and then make intimate contact with, these midline glia and VUM growth cones (Klämbt et al., 1991). By creating a small deficiency that deletes both *Netrin* genes, we have demonstrated that the *Netrin* genes are required for the normal formation of the axon commissures. But is their patterned expression required? Ectopic expression of either *Netrin* gene throughout the CNS provides an answer to this question.

Pan-neural CNS ectopic expression leads to a thinning of the commissures similar to that seen in the loss-of-function embryos. This phenotype suggests that the precise pattern of *Netrin* expression (i.e., highest at the midline) is crucial and that when this pattern is obscured by uniform expression, commissural axons can no longer find their way. This provides strong in vivo evidence that Netrins do not simply function as permissive agents to promote axon outgrowth, but rather that their localization (presumably as a gradient emanating from the midline) is required for proper guidance, i.e., that Netrins function as instructive guidance cues.

We do not know for certain whether Netrins in Drosophila also function to repel certain growth cones, but one of the phenotypes of ectopic CNS expression provides suggestive evidence to support such a role. When either Netrin is ectopically expressed by many CNS neurons, we observe large bundles of axons projecting abnormally toward the edge of the CNS, suggesting that they may be repelled by Netrin and are trying to grow away from it. Interestingly, these abnormal axons stall at the edge of the CNS without leaving it, suggesting that there is some other guidance activity associated with the nerve root exit point.

In addition to their high level of expression at the midline, the *Netrin* genes are normally expressed by subsets of neurons throughout the CNS. This raises the possibility that Netrin proteins might be secreted and displayed on axon surfaces, and could label specific

axon pathways. This possibility has previously been proposed in the nematode (Wadsworth et al., 1996). In deficiency embryos that delete both *Netrin* genes, we observe occasional breaks in the longitudinal tracts. These are not significantly reduced in frequency in embryos in which midline *Netrin* expression has been restored and commissural pathways rescued, suggesting that they are not secondary to errors in midline guidance and may be due to lack of *Netrin* expression on longitudinal fascicles. Alternatively, it is possible that the longitudinal phenotype is due to the deletion of a different gene in this interval.

Netrins Are Expressed by Subsets of Muscles and Control Targeting of Specific Motor Axons

In addition to controlling midline guidance decisions within the CNS, Netrins also influence the peripheral projections of motor axons to their target muscles (Figure 7). *Netrins* are expressed by discrete subsets of muscles: *NetA* by dorsal muscles 1 and 2, and *NetB* by dorsal muscle 2 and ventral muscles 6 and 7. *NetA* is also expressed in a dorsolateral stripe in the epidermis. The clearest phenotypes that can be directly attributed to *Netrin* expression by muscles affect the projections of the ISN and SNa.

In *Netrin* double mutant embryos, the ISN axons project normally out of the CNS and no errors are apparent in the trajectory of this nerve until it reaches the dorsolateral region of the embryo, at which point the axons often appear to wander over more territory than usual. The entire nerve can sometimes be seen crossing the segment border in a dorsolateral or dorsal position and fasciculating with its homolog in the neighboring segment. These dramatic crossovers may result from stabilization of chance contacts with neighboring axons that are encountered during excessive exploration, in the absence of the guidance information normally supplied by Netrin. Even when the ISN axons correctly reach their dorsal muscle target region, they often appear to branch inappropriately over these muscles, stall, project past their dorsal muscle targets, or project collaterals into adjacent segments. The ISN phenotypes are remarkably similar to those observed in embryos mutant for the gene *frazzled*, the Drosophila homolog of mammalian *DCC* and a putative Netrin receptor (P. A. Kolodziej, unpublished data). This suggests that these phenotypes are due to lack of Netrins and not the deletion of another gene in *Df(1)NP5*.

Guidance errors are also observed in embryos that ectopically express Netrin on all muscles. In these embryos, the ISN branches excessively, often crosses segment borders, and sometimes stalls in the lateral muscle region. This suggests that these axons now do not sense their normal dorsal targets as an especially attractive region, explore more territory than normal, and will stop in inappropriate target regions.

The phenotypes of the double mutant and of ectopic *Netrin* expression are consistent with an attractive function for Netrin in the guidance of ISN axons to their target muscles. However, an alternative is that some ISN axons are repelled by Netrin in the periphery. Some of the crossover behavior in the double mutant embryos occurs in the dorsolateral region of the embryo, where *NetA* is normally expressed in a stripe of epidermal cells. The ISN axons normally grow past, but not over, these epidermal cells, which occupy a relatively internal position in the embryo at the deepest point of the segmental groove. It is conceivable that *NetA* expression by these cells acts as a barrier preventing crossing of the ISN axons into the adjacent segment. In the absence of *NetA*, the ISN axons are free to wander over this region and will tend to fasciculate with their counterparts in the next segment if they encounter them. It will be important to determine the role of this epidermal expression, and whether any of the ISN axons are indeed repelled by Netrin.

The SNa motor axons are also affected by Netrins. In wild-type embryos, these axons bypass the ventral muscles (which include muscles 6 and 7, which normally express *NetB*) and project to the lateral muscles, which normally do not express either *Netrin*. When Netrin is ectopically expressed on all muscles, the SNa axons often fail to enter their normal lateral muscle region and either stall near the CNS or fasciculate with the ISN. This suggests that the SNa axons may be repelled by Netrin proteins, which in wild-type embryos could help to exclude them from inappropriate ventral and dorsal target regions and restrict them to the lateral muscles, which do not express Netrins. However, the SNa projection appears relatively normal in *Netrin* deficiency embryos.

Similar results have been obtained in investigating the roles of Connectin and Semaphorin II in motor axon guidance (Nose et al., 1994; Matthes et al., 1995). Removal of either gene has little effect on the innervation of the normally Connectin-positive or Semaphorin-positive muscles, but ectopic expression on different muscles can repel the motoneurons that normally innervate them. These molecules may play a back-up role to prevent misinnervation in the event of another perturbation of the system, but may not be absolutely necessary in an otherwise wild-type environment.

Given the function of Netrins in Drosophila in controlling motor axon guidance, it will be of interest to determine whether netrin-1, which is expressed in a patterned fashion in the developing mouse limb (T. S., unpublished data), also controls motor axon guidance.

Relation of *Netrin* Genes to Other Drosophila Genes Controlling Midline Guidance

Numerous genes have been identified in Drosophila that appear to control important aspects of midline guidance. A large-scale genetic screen yielded many mutants that partially or completely disrupt commissural axon pathways (Seeger et al., 1993). Two genes identified in that mutant screen have particularly striking and penetrant mutant phenotypes that alter axon commissures: *commissureless* (*comm*) and *roundabout* (*robo*).

Mutations in *comm* lead to a dramatic loss of all commissural axon tracts, while mutants in *robo* display a complementary phenotype: normally ipsilaterally projecting axons now cross the midline inappropriately. Comm is a novel transmembrane protein expressed on midline glial cells (Tear et al., 1996). Robo is a transmembrane molecule expressed by neurons that may act as

a receptor for a repulsive signal from the midline (T. Kidd, K. J. M., C. S. G., and G. Tear, unpublished data). Other molecules have been implicated in the formation of axon commissures as well, including cell adhesion molecules (Elkins et al., 1990), cytoplasmic tyrosine kinases (Elkins et al., 1990), Ca^{2+}–calmodulin (Van Berkum and Goodman, 1995), and other cytoplasmic molecules (Gertler et al., 1995; Hill et al., 1995).

Thus, a number of molecules are known in Drosophila that may act as signals, receptors, or downstream signal transduction components that affect midline guidance decisions. The analysis of single and double mutant phenotypes, and the large number of uncharacterized mutants that affect the formation of axon commissures, make it clear that this process is likely to involve multiple signaling pathways.

It should now be possible to investigate the role of Netrins in commissural axon guidance using the powerful genetics in Drosophila and the wealth of knowledge about other genes and mutations that alter these events. For example, preliminary evidence already suggests that Netrins may function in the same pathway as Frazzled (P. A. Kolodziej, unpublished data) but in a separate pathway from Robo (T. Kidd, K. J. M., C. S. G., and G. Tear, unpublished data). It will be of interest to determine how growth cones simultaneously integrate several different guidance signals, transfer this information via different receptors, and how these signals converge on the cytoskeleton to control steering decisions.

Experimental Procedures

Cloning of *NetA*

Primers were designed to conserved regions of chick netrins and *unc-6*, using Drosophila optimal codons where possible (Sharp and Lloyd, 1993). The successful primers were:
A: 5′ TGC AAG CCC TTC CAC TAC 3′
B: 5′ TGC GT(GC) GCC TGC AAC TGC AA 3′
D: 5′ GCT CTG CTG GTA (TGA)CC CTT GGC 3′
E: 5′ GGG GAT CTT GAT GCA GGG 3′

PCR reactions were performed at low stringency, using 1 ng of a cDNA library made from rough microsome poly(A)$^+$ RNA as a template (Kopczynski et al., 1996). These reactions yielded multiple bands that were probed with chick *netrin-1* cDNA. One band of the expected size (420 bp) for the primer pair A-E hybridized with this probe. In addition, internal primers B and D gave a product of the expected size using this band as a template. We cloned and sequenced the 420 bp band and found that it contained an open reading frame with high homology to the expected region of the chick and worm netrins. We used this 420 bp fragment to probe a 0–24 hr λgt11 cDNA library and isolated cDNA clones containing the complete ORF. The gene so identified was named *NetA*.

Cloning of *NetB*

NetB was cloned by an alternative PCR strategy, in which the conserved exon/intron structure of *unc-6* and *NetA* was used to design primers predicted to amplify a fragment from a single exon, using genomic DNA as a template. These primers, VIF and VIR (see Figure 1), with the sequences GCGCGAATTC(CT)TNGGNAA(AG)AA(AG)TT CGA and GCGCGGATCCAT(AG)TCNGTNGCNGTNACCCA, respectively, were used at a concentration of 1.0 μM each with ~1 μg genomic DNA in a PCR reaction. A single amplification product of ~300 bp resulted, approximately half of which could be cleaved with BglII, which was known to cut the expected PCR product from the *NetA* gene. The BglII-resistant fraction was subcloned into pBluescript and sequenced, and found to represent a second *Netrin* gene, which we named *NetB*. This fragment was then used to screen a 0–24 hr λgt11 cDNA library, yielding six overlapping cDNA clones, the longest of which contains the complete ORF shown in Figure 1.

Generation of *Df(1)NP5*

The deficiency NP5 was generated by recombination between two inversion chromosomes: In(1)N366.2 (12E9-11; 17C2), and In(1)P363 (12F2-5; 17C2) (provided by R. Davis). Both of these chromosomes are homozygous and hemizygous viable, both express *NetA* and *NetB*, and neither has a CNS phenotype in embryos. Recombination between the inverted portions of these chromosomes is predicted to yield a chromosome with a deficiency (Df) of the sequences from 12E9-11 to 12F2-5. We predicted the Df chromosome might be lethal and screened recombinants on that basis. N366.2 males were crossed to P363 homozygous females. F1 female progeny were then mated to FM7c(Act5C–β-gal)$_2$ males. We mated 150 F2 females singly to FM7c(Act5C–β-gal)$_2$ males, and these crosses were then scored for X-linked lethality. Two independent lethal lines (NP3 and NP5) were shown to carry a Df of the expected region, which deletes the two *Netrin* genes. We mapped the breakpoints of the Df to our genomic walks (see Figure 4).

Transformation Constructs

The P-element transformation constructs P[slit–*NetA*] and P[slit–*NetB*] are Carnegie20 derivatives in which a *slit* 1.0 kb HindIII–EcoRV enhancer (Wharton and Crews, 1993) is fused to a noninducible *hsp70* promoter to drive transcription of either a *NetA* or *NetB* cDNA, followed by the 3′untranslated region (3′UTR) of the α_1-*tubulin* gene. Both cDNAs were modified to add a C-terminal c-myc epitope to the mature protein. This epitope tag could not be detected using MAb9E10, perhaps because the C-termini of both *NetA* and *NetB* are either cleaved or inaccessible for the antibody. P[UAS–*NetA*] and P[UAS–*NetB*] were prepared by cloning the full-length *NetA* or *NetB* cDNA, including the 3′ myc epitope sequences, into the polylinker site of the vector pUASt (Brand and Perrimon, 1993). For transformation, these slit–*Netrin* and UAS–*Netrin* plasmids were injected at a concentration of 0.6 μg/μl, together with the helper plasmid pAc5CΔ2-3 at a concentration of 0.2 μg/μl into *ry* or *y w* embryos, respectively.

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GenBank Accession Numbers

The GenBank accession number for *NetA* is U60316 and for *NetB* is U60317.