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Guidance Cues at the Drosophila CNS Midline: Identification and Characterization of Two Drosophila Netrin/UNC-6 Homologs

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Summary

Netrins are chemotropic guidance signals that play important roles in circumferential axon guidance in C. elegans and in the developing vertebrate spinal cord. We have identified two Drosophila homologs of this protein family (*Netrin-A* and *Netrin-B*). Both Netrins are dynamically expressed throughout embryogenesis, including CNS midline expression at the time of commissure formation. Both *Netrin* genes map close to each other on the X chromosome, and embryos deficient for this region exhibit defects in commissure formation. This CNS phenotype can be rescued by expression of either Netrin at the CNS midline, confirming an important role for Drosophila Netrins in commissural growth cone guidance. A localized source of Netrin protein at the midline is apparently important for function, since ectopic expression of either Netrin throughout the CNS results in phenotypic defects similar to the loss-of-function phenotype.

Introduction

Neuronal growth cones utilize a variety of cues as they navigate along stereotypical pathways toward their specific synaptic targets during development. These cues are diverse and include both long-range diffusible factors and short-range contact-mediated signals. Furthermore, these signals can influence growth cones through either attractive or inhibitory mechanisms, effectively steering growth cones toward or away from specific sites or regions. Thus, the final projection of an axon is orchestrated by a variety of factors acting on the growth cone in different combinations, at different times and places, and through fundamentally different mechanisms (for recent reviews, see Goodman and Shatz, 1993; Keynes and Cook, 1995; Goodman, 1996).

In organisms with bilateral symmetry, the midline is a particularly interesting boundary for neuronal growth cones, since it exhibits both attractive and repulsive properties. Not surprisingly, the CNS midline from insects to vertebrates is comprised of a distinctive group of cells with special properties and functions. In vertebrates, the ventral midline of the developing spinal cord is a specialized group of cells called the floor plate (reviewed by Colamarino and Tessier-Lavigne, 1995a); in Drosophila, the analogous cells are the CNS midline glia (see Klämbt et al., 1991; Tear et al., 1993). These midline cells are attractive for commissural growth cones that extend toward and ultimately cross the midline. They also represent a repulsive boundary for many axons that do not cross, playing an active role in preventing these ipsilaterally projecting axons from crossing. For those axons that do cross the midline, crossing often corresponds with changes in the expression of cell surface proteins along with changes in affinity for specific axon pathways. Therefore, the midline likely provides specific signals that tell an axon when it has reached and crossed the midline and to proceed onward with the next step in its pathfinding program. Recently, a number of molecules that mediate some of these different responses of growth cones to the CNS midline have been identified or further characterized (or both) (Ishii et al., 1992; Leung-Hagesteijn et al., 1992; Serafini et al., 1994; Kennedy et al., 1994; Chan et al., 1995, Soc. Neurosci., abstract; Colamarino and Tessier-Lavigne, 1995b; Stoeckli and Landmesser, 1995; Tear et al., 1996).

In vertebrates, commissural neurons, which differentiate in dorsal regions of the developing spinal cord, extend their axons ventrally toward the floor plate located at the ventral midline. Both in vitro and in vivo studies indicate that this attraction of commissural axons to the floor plate is via a chemotropic mechanism (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b; Yaginuma and Oppenheim, 1991). That is, floor plate cells secrete a chemoattractant(s) for commissural growth cones that guides them to this first intermediate target. Two proteins, called netrins, were identified from chick that mimic the in vitro properties of the floor plate chemoattractant, and the genes encoding these two proteins were cloned (Serafini et al., 1994). Appropriately, both genes are expressed in ventral regions of the developing spinal cord at a time when commissural axons are first extending toward the floor plate; *netrin-1* is expressed specifically by the floor plate, while *netrin-2* is expressed in the ventral two-thirds of the spinal cord (Kennedy et al., 1994).

Sequence analysis of these two *netrins* indicates that they are homologous to each other and to a third gene, *unc-6* (Serafini et al., 1994). UNC-6, a laminin-related protein, is required for circumferential migrations of axons and other cells in Caenorhabditis elegans (Hedgecock et al., 1990; Ishii et al., 1992). The netrins and UNC-6 are secreted proteins with a modular organization. The N-terminal two-thirds is similar to domain VI and to the first three EGF-like repeats in domain V of laminin B subunits. The C-terminal third of UNC-6 and the netrins encodes a unique basic domain. UNC-6 functions in part by forming a gradient along the dorsal–ventral ectodermal circumference with highest concentrations of UNC-6 present at the ventral midline (Wadsworth et al., 1996). Axons and cells that migrate ventrally thus see UNC-6 as a chemoattractant, while dorsally migrating axons respond to UNC-6 as a chemorepellent. These three proteins, UNC-6 and the two vertebrate netrins, define a family of conserved chemotropic guidance molecules that play important roles in circumferential axon guidance in evolutionarily diverse organisms.

unc-6 is one of three genes in C. elegans that specifically affects circumferential growth cone guidance.

While null mutations in *unc-6* affect both dorsal and ventral migrations of axons and other cells, mutations in *unc-5* affect only dorsal migrations and mutations in *unc-40* primarily affect ventral migrations (Hedgecock et al., 1990; McIntire et al., 1992). The *unc-5* gene encodes an integral membrane protein of the immunoglobulin superfamily (Leung-Hagesteijn et al., 1992). Misexpression experiments have shown that UNC-5 expression is both necessary and sufficient for dorsally oriented cell movements that utilize the UNC-6 protein gradient (Hamelin et al., 1993). The *unc-40* gene also encodes an integral membrane protein of the immunoglobulin superfamily (Chan et al., 1995, Soc. Neurosci., abstract; Culotti and Kolodkin, 1996). Although direct demonstration of binding has not yet been reported, it seems likely that both UNC-5 and UNC-40 are receptors or components of a receptor mechanism that recognize and respond to the UNC-6 protein. Therefore, by utilizing distinct receptors, individual axons and cells may be able to recognize the bifunctional UNC-6 protein differentially, as either a chemoattractant or a chemorepellent.

Chick netrin-1, like UNC-6, has also been shown to operate as a bifunctional guidance cue. While netrin-1 is a chemoattractant for ventrally directed commissural axons, it is a chemorepellent for another group of neurons, the trochlear motor axons (Colamarino and Tessier-Lavigne, 1995b). Trochlear motoneurons differentiate from regions just lateral to the floor plate and subsequently extend axonal projections dorsally, away from the floor plate. This effect on trochlear axons can be replicated in vitro with either floor plate explants or COS cells secreting netrin-1 protein.

In this paper, we report the identification and characterization of two members of the netrin/UNC-6 protein family from Drosophila. Like other members of this gene family, Drosophila Netrins are expressed at the CNS midline and play an important role in commissural growth cone guidance. Mitchell and colleagues have also identified two Drosophila *Netrins* and report similar findings concerning the expression and function of these genes (Mitchell et al., 1994, Soc. Neurosci., abstract; Mitchell et al., 1996).

Results

Identification of Two *Netrin* Genes in Drosophila

The identification of vertebrate *netrins* and their similarity to the C. elegans gene *unc-6*, both in sequence and function, provides compelling evidence for a conserved chemotropic guidance mechanism operating at the midline of these diverse organisms. Since the attraction of commissural axons to the midline of the insect CNS has also been postulated to be via a chemotropic mechanism (see Tear et al., 1993), it seemed likely that a netrin/UNC-6 like protein(s) would exist in Drosophila as well. To test this possibility, we used a polymerase chain reaction (PCR)-based strategy with degenerate primers based on the amino acid sequence similarity of UNC-6 with the B chains of laminin (the Netrin sequences were not yet published when we initiated our studies).

Primers for PCR were designed from conserved regions at the end of domain VI and from the first EGF-like repeat, domain V-1 (see Experimental Procedures; Ishii et al., 1992). Using one pair of primers specific to the first EGF-like repeat, we were able to amplify a fragment of the expected size from Drosophila genomic DNA. Sequence analysis of this fragment indicated that it encoded an amino acid sequence typical of EGF-like repeats and with particularly high homology to UNC-6. More importantly, this fragment was not homologous to the previously characterized Drosophila laminin chains or any other characterized Drosophila gene.

This PCR fragment was used to recover corresponding cDNA clones from an embryonic cDNA library. Multiple cDNA clones were isolated and preliminarily characterized. From this initial analysis, it was clear that we had identified a Drosophila *Netrin* homolog that we have called *Netrin-A* (*NetA*). The largest *NetA* cDNA clone was selected and sequenced in its entirety. This clone is 3272 bp in length with a short poly(A) tail at its 3′ end. We believe this represents a full-length cDNA clone (or near full-length), since developmental Northern blots hybridized with *NetA*-specific probes identify a single predominant mRNA of ~3200 nt (data not shown). This cDNA encodes a single open reading frame (ORF) of 727 amino acids with 5′ and 3′ untranslated regions of ~590 and 490 bp, respectively (see Figure 1 for the NETA amino acid sequence). Finally, the initiating methionine is preceded by a favorable Drosophila translation initiation consensus sequence (AAACAUG versus the consensus sequence [C/A]AA[A/C]AUG) (Cavener, 1987).

During the course of isolating additional *NetA* cDNA clones from the embryonic cDNA library, we noted a number of weakly hybridizing clones. We further purified and characterized these clones. Although most of these weakly hybridizing clones were homologous to identified Drosophila laminin chains, one group was distinct and represented a second Drosophila *Netrin* gene, *Netrin-B* (*NetB*). We isolated additional *NetB* cDNA clones and sequenced the largest in its entirety. Our largest *NetB* cDNA is 3919 bp in length. It does not include a poly(A) tail at its 3′ end and does not represent a full-length cDNA, since developmental Northern blots hybridized with *NetB*-specific probes identify a single transcript of ~8300 nt (data not shown). However, this cDNA clone does encode a single long ORF of 793 amino acids with ~340 bp and ~1200 bp of 5′ and 3′ untranslated sequences, respectively (see Figure 1 for the NETB amino acid sequence). There are two potential initiating methionines for the NETB protein (see Figure 1), and neither AUG is preceded by a very favorable Drosophila translation initiation consensus sequence (the first methionine is preceded by the sequence GCCAAUG and the second methionine by AAGGAUG). Since it is difficult to predict which methionine is actually utilized, we have based our NETB amino acid numbering from the first methionine within this ORF.

Drosophila Netrins Are Highly Conserved

Both Drosophila Netrin proteins are clearly similar to each other and to other members of the netrin family (Figure 1). They share a common domain organization and extensive amino acid sequence similarity over the entire length of their ORFs (see Figure 1; Figure 2). Overall, Drosophila NETA is 41% identical to Drosophila

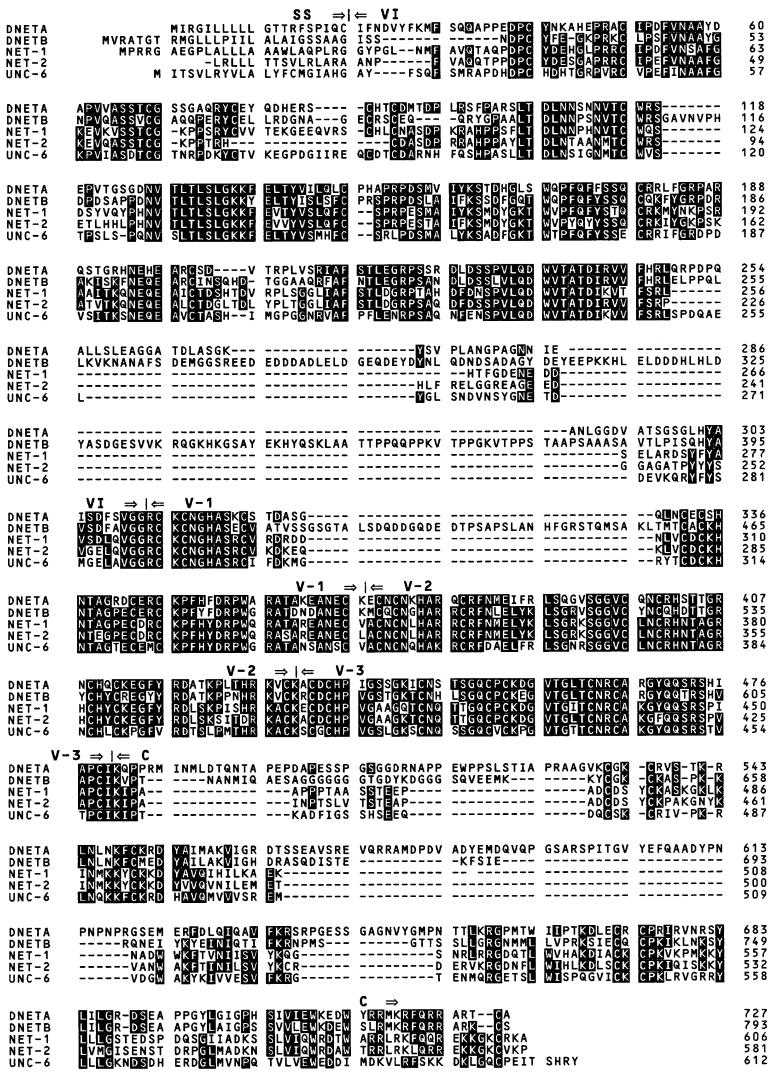

Figure 1. Sequence Alignment of Drosophila Netrins with Other Members of the Netrin Family

Predicted amino acid sequences of Drosophila NETA and NETB aligned with netrin-1 and netrin-2 from chick (Serafini et al., 1994) and UNC-6 from C. elegans (Ishii et al., 1992). Alignments were generated using sequence comparison programs of the GCG package. All amino acid residues shared by four or more family members are shown as white on black. The extent of various domains are indicated above the alignments (that is, signal sequence, domain VI, etc.) (see text and Figure 2). Amino acid numbering starts from the predicted initiating methionine. Signal sequence cleavage sites for NETA and NETB were predicted using rules proposed by von Heijne (1986).

NETB, 39% to UNC-6, and 40% to chick netrin-1; Drosophila NETB is 35% identical to UNC-6 and 38% to chick netrin-1. These comparisons underrepresent the degree of similarity of NETA and NETB with other members of the netrin family owing to the relative expansion in size of both NETA and NETB. If gapped positions are not included in these amino acid sequence comparisons, then NETA is 55% identical to NETB, 49% to UNC-6, and 51% to netrin-1; and NETB is 49% identical to UNC-6 and 54% to netrin-1. The relative conservation of the different Netrin domains is summarized in Figure 2. The three EGF-like repeats of domain V are the most conserved; the basic C-terminal domain C is the least conserved domain within this protein family.

The two Drosophila Netrins do exhibit some interesting differences from other members of the netrin family. Both Drosophila Netrin proteins are considerably larger than UNC-6 and the two vertebrate netrins (see Figure 1; Figure 2). For NETA this increase in size is due to an expansion of domain C. This expansion has occurred at a number of different positions dispersed throughout this domain (see Figure 1). The NETB protein is larger owing to increases in the size of domains VI and V-1 (Figure 2). The insertion of 37 amino acids within the first EGF-like repeat of domain V is particularly striking, since the length of domain V has been absolutely conserved in the other four members of the netrin family.

Both *Netrins* Map to Polytene Band Position 12F1,2 on the X Chromosome

We have mapped both Drosophila *Netrins* to polytene band position 12F1,2 on the X chromosome (data not shown). We obtained P1 clones containing Drosophila genomic DNA that map to this region from the Drosophila Genome Project (also see Smoller et al., 1991). A set of overlapping P1 clones from this region hybridize with *NetA*- and *NetB*-specific probes, placing the *Netrins* quite close to one another on the molecular map (Figure 3A). *NetA* is located just distal to *NetB* on the chromosome, and both genes are transcribed in the same direction, from proximal to distal. Two P1 clones, DSO2078 and DSO5996, hybridize with the full-length *NetA* cDNA. Since the most distal P1 clone does not include the 3′ end of the *NetA* gene, we extended this cloned region to include all of the *NetA* gene by isolating λ phage clone A7 (Figure 3A). Our largest *NetB* cDNA hybridizes

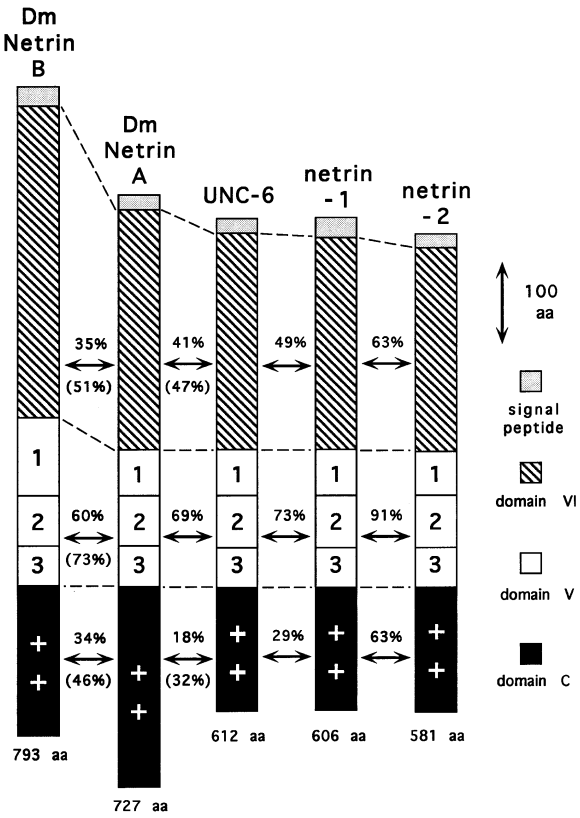

Figure 2. Schematic Representation of the Netrin Family

Netrin family members, divided into the various domains that comprise these proteins, are shown (adapted from Serafini et al., 1994). The relative size of different domains and among different family members is indicated. The percent identity in amino acid sequence between homologous domains is shown. The percent identity excluding gapped positions is also indicated in parentheses for a subset of these comparisons (see text).

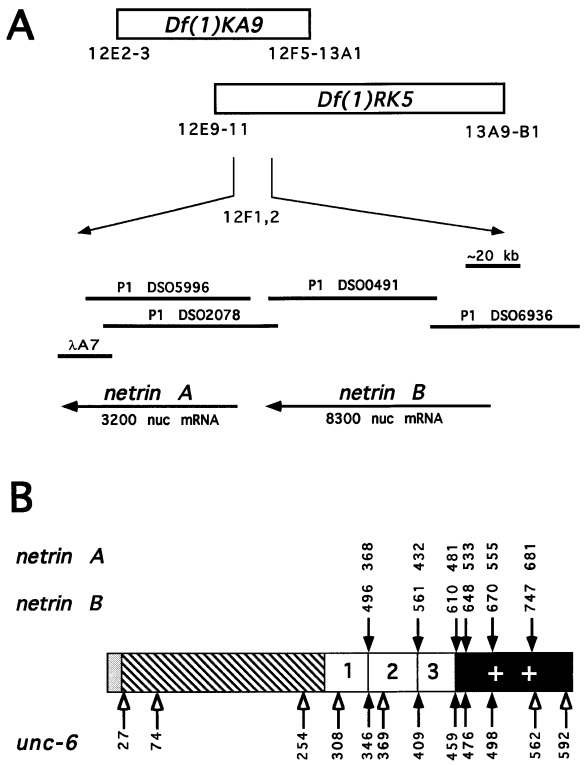

Figure 3. Summary of the Genomic Organization of Drosophila *Netrins*

(A) Genomic organization of the *Netrin* region at 12F1,2 on the X chromosome. The extent of several deficiencies for this region is indicated. A summary of overlapping genomic clones, including four P1 clones and one λ phage clone, is presented. The relative organization of the *NetA* and *NetB* transcription units is shown below the genomic clones that hybridize with *NetA* and *NetB* cDNA clones (see text).

(B) Locations of introns within the *NetA*, *NetB*, and *unc-6* ORFs projected onto a summary diagram of the Netrin protein structure. Solid arrows indicate intron positions that are found at homologous locations in *NetA* and *NetB* or between *unc-6* and the two Drosophila *Netrins*. Open arrows indicate the postions of introns that are not conserved between *unc-6* and the Drosophila *Netrins*. Numbers indicate the first uninterrupted codon following the intron/exons splice junction. Location of *unc-6* introns comes from Ishii et al. (1992).

to P1 clones DSO2078, DSO0491, and DSO6936. Since this cDNA encodes only 3900 nt of the ~8300 nt *NETB* mRNA, we cannot be certain how far the *NetB* gene extends either proximally or distally from what is indicated in Figure 3A.

We have determined the position of introns within the ORFs of both *NetA* and *NetB* by sequencing appropriate genomic DNA clones with specific oligonucleotide primers. Both Drosophila *Netrins* have six introns that disrupt their ORFs. All six of these introns occur at homologous sites within the NETA and NETB proteins (summarized in Figure 3B). There are 12 introns that disrupt the *unc-6* ORF (Ishii et al., 1992). Five of six Drosophila introns are in identical positions compared with the locations of introns within the *unc-6* ORF. Only the last intron within the Drosophila *Netrin* ORFs does not have an identical counterpart in *unc-6*.

Both Drosophila Netrins Are Expressed at the Embryonic CNS Midline

We have analyzed the pattern of Drosophila Netrin expression using two approaches. First, nonradioactive probes were used to detect the spatial patterns of *NETA* and *NETB* mRNA accumulation in whole-mount embryos. Second, polyclonal antisera specific for each Netrin was used to examine the patterns of NETA and NETB protein accumulation throughout embryogenesis (see Experimental Procedures for details about generation of antibodies). All of the antibody staining is absent in embryos that are deleted for both Netrins (see below), confirming the specificity of the NETA and NETB antibodies. Also, since the patterns of protein accumulation for NETA and NETB do exhibit differences, we conclude that there is little or no cross-reactivity between the NETA- and NETB-specific antisera, an important point given the similarity of these two proteins.

The first detectable accumulation of *Netrin* mRNA within the developing nervous system begins at stage 12. During early stage 12 (stage 12/5) (for a description of CNS development, see Goodman and Doe, 1993), a

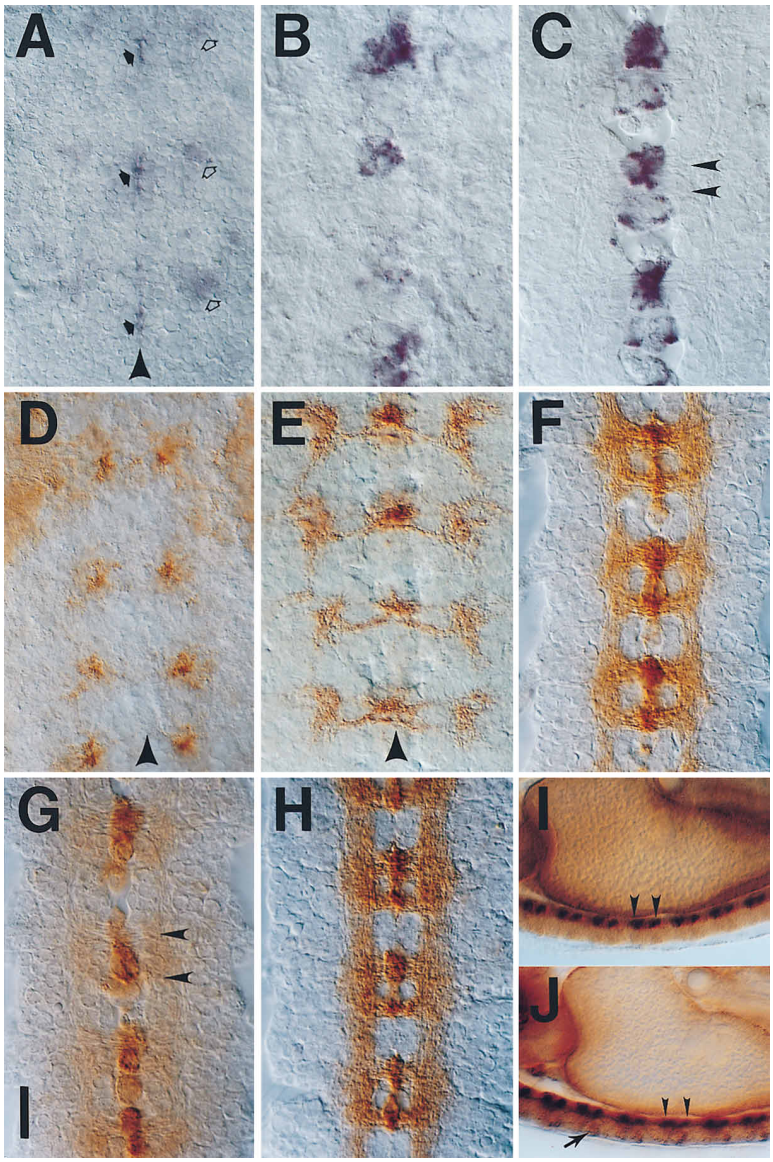

Figure 4. Drosophila Netrins Are Expressed within the Developing CNS

Expression of Drosophila Netrins within the developing CNS. (A)–(H) are dissected embryos where the CNS can be better visualized. (A)–(C) present the pattern of *Netrin* mRNA accumulation at three different stages as visualized by whole mount in situ hybridization (using alkaline phosphatase for detection). (D)–(J) present patterns of Netrin protein accumulation as visualized with anti-NETA or anti-NETB polyclonal antisera with horseradish peroxidase–immunocytochemistry. Scale bar: 10 μm in (B), (C), (E), (F), (G), and (H); 16 μm in (A) and (D); 25 μm in (I) and (J).

(A) A stage 12/5 embryo showing the first accumulation of *NETA* mRNA within the developing CNS. The arrowhead points to the midline, filled arrows to midline-expressing cells, and open arrows to the lateral expressing cells, which are slightly out of the focal plane.

(B) *NETA* mRNA accumulation in CNS midline cells of a stage 12/0 embryo. Expression by the lateral cell cluster is no longer detectable.

(C) *NETA* mRNA accumulation in the midline glia of a late stage 13 embryo. Arrowheads point to the anterior and posterior commissures within one segment.

(D) Expression of NETA protein in a stage 12/3 embryo. Accumulation of NETA is associated with the lateral cell cluster. The arrowhead points to the midline.

(E) Same as (D) except this is an older embryo (stage 12/0). Accumulation of NETA on midline cells is apparent, as is accumulation on axons of the first commissural pathways.

(F) NETA accumulation in a late stage 13 embryo. NETA protein is associated with midline glia and most axons.

(G) An embryo similar to (F) except NETA accumulation is predominantly associated with the midline glia and little axon staining is observed. Arrowheads point to the anterior and posterior commissures within one segment.

(H) A stage 14 embryo showing the pattern of NETB accumulation.

(I) A stage 15 whole-mount embryo showing the pattern of NETA accumulation within the ventral nerve cord. The arrowheads point to the two commissures within one segment. All NETA accumulation is present on axons or dorsally situated midline glia.

(J) A stage 15 whole-mount embryo showing the pattern of NETB accumulation. Expression of NETB by a cluster of ventrally located neuronal cell bodies (arrow) is apparent when compared with NETA expression in (I).

subset of cells along the midline begin to accumulate both *NETA* and *NETB* mRNA (Figure 4A). In addition, a small group of cells just lateral to the midline also express both *Netrin* mRNAs transiently (Figure 4A). At mid stage 12 (stage 12/3), expression by this lateral group of cells diminishes, and there is an increase in the level of midline Netrin expression (Figure 4B). By stage 13, it is clear that the midline Netrin expression is restricted to the midline glia; this midline expression continues throughout embryogenesis (Figure 4C).

This early pattern of mRNA accumulation within the CNS is mimicked by both the NETA and NETB proteins. The first protein accumulation is observed around the group of cells just lateral to the midline (Figure 4D). By late stage 12, accumulation by CNS midline cells is apparent and continues throughout embryogenesis (Figures 4E and 4F). We observe a variable degree of axon accumulation for both the NETA and NETB proteins. This is first apparent on axons associated with the lateral cluster of early expressing cells and continues as the ladder-like scaffold of axon pathways develops (Figures 4D–4F; Figure 4H). In some batches of antibody-stained embryos, we see little to no axon staining (compare Figure 4G with Figure 4F). It is not clear whether this axon accumulation represents specific interactions of the secreted Netrin proteins with these axons, nor is it clear what accounts for the variability that we observe in this axon staining. At stage 14, the first difference between NETA and NETB expression within the CNS becomes apparent. A lateral group of neurons begins to express NETB (Figure 4J), and no similar expression is observed for NETA (Figure 4I).

In summary, both the NETA and NETB proteins are expressed at the CNS midline. Expression by midline glia begins at a time when the first commissural growth cones are extending toward the midline and continues throughout embryogenesis. The greatest accumulation of the Netrin proteins is on the surface of these midline glia, although we do observe axon accumulation and some diffuse staining throughout the ventral nerve cord.

Both Netrins Are Expressed Outside of the Nervous System

Both Drosophila Netrins are expressed in a variety of tissues in a dynamic pattern throughout embryogenesis. Furthermore, both Netrins are expressed at other stages of development, as determined by developmental Northern blot analysis, including embryonic, larval, pupal, and adult stages (data not shown). Preliminary studies also suggest that at least some of this postembryonic expression is dynamic with differences in the patterns of expression for *NetA* and *NetB*.

The first expression of *NetA* during embryogenesis is at stage 6, the beginning of gastrulation. NETA protein accumulates on cells of the invaginating ventral furrow (Figure 5A). This early mesodermal expression continues through germband extension. By stage 12, separation of the visceral mesoderm, which continues to express NETA, is apparent (Figure 5B) (for a description of mesoderm development, see Bate, 1993). Expression of NETA by the visceral mesoderm continues through later stages of embryogenesis (Figure 5C). Patches of ectodermal cells that are likely primordia for the tracheal system express NETA from stage 12 onward (for a description of tracheal development, see Manning and Krasnow, 1993). This tracheal expression becomes clearer at later stages of development (Figures 5C and 5D). Finally, weak NETA protein accumulation is seen on motor axons as they transverse and synapse with muscles of the dorsal group (Figure 5E). This accumulation likely originates from earlier muscle expression.

The first expression of *NetB*, like *NetA*, is in the developing mesoderm. For *NetB*, this expression begins during germband extension, somewhat later than what is observed for *NetA* (Figure 5F). This expression continues in a variety of mesoderm derivatives, including the visceral mesoderm, somatic mesoderm, and cells of the dorsal vessel (Figures 5G; Figures 5J–5L). Expression of NETB by cells of the developing stomatogastric nervous system is apparent during stage 13 as these cells evaginate from the roof of the esophagus primordium (Figure 5H). During stage 14, accumulation of NETB is apparent in imaginal disc primordia, including cells that will give rise to the eye–antennal, labial, wing, haltere, and genital discs (Figure 5I) (for a description of imaginal disc primordia, see Cohen, 1993). There is NETB accumulation within the developing PNS, including the chordotonal organs (data not shown). Finally, muscles from both the dorsal and ventral muscle groups express *NetB* mRNA and protein. At earlier stages, expression of NETB by subsets of muscles is apparent; later, the NETB protein accumulates primarily on the motor axons as they explore and synapse with these specific muscle groups (Figures 5K and 5L). This accumulation of NETA and NETB on the motoneurons may indicate that they express specific receptors for the Netrins. A less likely alternative is that this Netrin protein accumulation originates from Netrin expression by the motoneurons themselves.

Embryos Deficient for Both Netrins Exhibit Defects in Commissure Formation

To address the role that Drosophila Netrins play in CNS development, we obtained a number of deficiency stocks that delete portions of the 12E–13A interval (Drysdale et al., 1991; Lindsley and Zimm, 1992). Two deficiencies are relevant for these studies, *Df(1)KA9* and *Df(1)RK5* (see Figure 3A). These two deficiencies delete both *NetA* and *NetB*, and when embryos from these deficiency stocks are stained with antisera specific for each Netrin, the expected 25% of embryos do not exhibit any immunoreactivity (data not shown).

Embryos hemizygous for either *Df(1)KA9* or *Df(1)RK5* undergo substantially normal development through the end of embryogenesis. The lack of any major defects (for example, in cell cycle, morphogenetic movements, or global patterning) allows us to examine the CNS of these deficiency embryos and to determine the phenotypic consequences of the total absence of both *NetA* and *NetB*. *Df(1)KA9* mutant embryos exhibit defects in the formation of commissural pathways, as visualized with MAb BP102, an antibody marker for all CNS axon pathways (Figure 6B). The phenotype of *Df(1)KA9* embryos is quite variable; it ranges from embryos that exhibit a complete absence of commissures in most segments to embryos that form many commissures, although these commissures are thin with a reduced number of axons included within the commissural bundle. *Df(1)RK5* mutant embryos also exhibit defects in commissure formation (Figure 6C); however, the phenotype of these deficiency embryos is generally less severe. In a typical D*f(1)RK5* mutant embryo, many commissures form, although they contain fewer axons.

Both of these deficiencies are quite large and remove an unknown number of genes in addition to *NetA* and *NetB*. To address whether these defects in commissure formation are due to the lack of either NETA or NETB protein, we took advantage of the upstream activating sequence (UAS)–GAL4 system developed by Brand and Perrimon (1993). *NetA* and *NetB* cDNA clones were inserted into the pUAST germline transformation vector, and a series of independent germline transformants were obtained for both UAS–NETA and UAS–NETB. To activate transcription of these UAS constructs along the CNS midline, we utilized a rho–GAL4 driver line that is expressed by CNS midline cells from stage 11 through at least stage 14 (Bier et al., 1990; Ip et al., 1992). By crossing flies containing the GAL4 driver line with the UAS–NETA or –NETB lines, we are able to express ectopically either Netrin along the CNS midline.

To access the ability of either *NetA* or *NetB* to rescue the commissure phenotypes associated with *Df(1)KA9*, we introduced both the rho–GAL4 driver and various UAS–NETA or UAS–NETB transformants into this deficiency background. Hemizygous deficiency embryos were identified by the absence of staining associated with a balancer chromosome carrying a P[*ftz–lacZ*] insertion. Significant rescue of the CNS phenotypes associated with *Df(1)KA9* was observed when either *NetA*

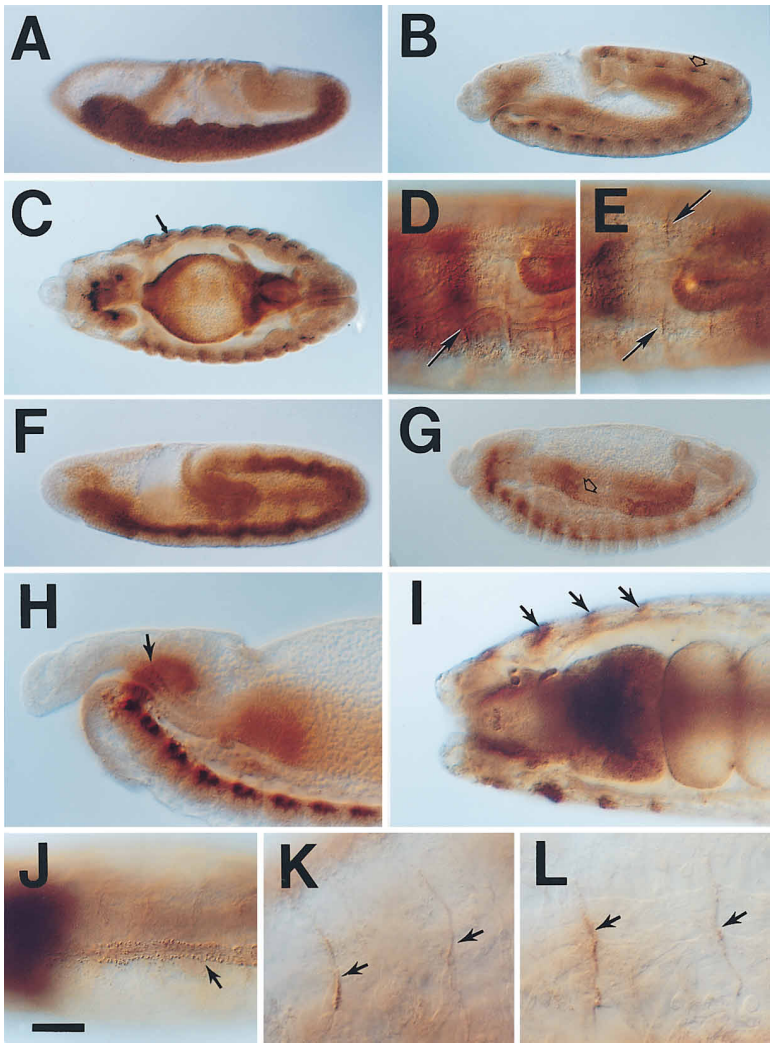

Figure 5. Drosophila Netrins Are Expressed Dynamically throughout Embryogenesis

Whole-mount embryos showing patterns of NETA (A–E) or NETB (F–L) protein accumulation throughout embryogenesis as detected with anti-NETA and anti-NETB specific antibodies and horseradish peroxidase–immunocytochemistry. Scale bars: 10 μm in (K) and (L); 25 μm in (D), (E), (H), (I), and (J); 50 μm in (A), (B), (C), (F), and (G).

(A) NETA accumulation in the invaginating ventral furrow of a stage 6 embryo.

(B) An early stage 12 embryo (stage 12/5) showing the first CNS NETA expression (open arrow) as well as visceral mesoderm accumulation.

(C) NETA accumulation in the developing tracheal system is apparent (arrow) in this dorsal view of a stage 14 embryo. Continued accumulation of NETA in the visceral mesoderm is also visible.

(D) NETA accumulation in the developing tracheal system of a stage 16 embryo. Staining of the longitudinal tracheal trunk and ventral branches is evident (arrow).

(E) NETA accumulation on motor axons located over the dorsal muscle group (muscle fibers 1 and 2). This muscle group on both sides of the embryo can be visualized in this dorsal view.

(F) A stage 10 embryo showing mesodermal accumulation of NETB.

(G) CNS and visceral mesoderm (open arrow) expression of NETB in a late stage 12 embryo.

(H) NETB expression in the developing stomatogastric nervous system (arrow) of a stage 13 embryo.

(I) NETB accumulation in imaginal disc primordia (arrows) of a stage 16 embryo.

(J) Dorsal vessel accumulation of NETB protein.

(K) Accumulation of NETB on motor axons located over the dorsal muscle group.

(L) Accumulation of NETB on motor axons located over the ventral muscle group (muscle fibers 6, 7, 12, and 13).

or *NetB* was expressed under control of the rho–GAL4 driver in these deficiency embryos (compare Figure 6B with Figures 6D–6F). The degree of phenotypic rescue is somewhat variable, although all embryos exhibit improvements in commissure formation relative to *Df(1)KA9* controls. Both *NetA* and *NetB* rescued the deficiency phenotypes to similar extents, suggesting that either Netrin can function equally well at the CNS midline. Similar results have been obtained with rescue of *Df(1)RK5* phenotypes (data not shown).

Ectopic Pan-Neural Netrin Expression Causes Defects in Commissure Formation

If Drosophila Netrins are chemotropic guidance cues, then a localized source of netrin protein should be critical for proper function of this guidance signal. To test this prediction, we utilized the UAS–GAL4 system to express ectopically either NETA or NETB throughout the developing nervous system. To drive expression in all neurons, we utilized a sca–GAL4 line that is expressed in all neuroblasts and their progeny, mimicking the expression pattern of the *scabrous* gene (Mlodzik et al., 1990).

Ectopic expression of either NETA or NETB by all neurons in an otherwise wild-type background leads to a variety of CNS defects (Figures 6G and 6H). Interestingly, these defects reflect the range of phenotypes seen in the deficiency embryos described previously. That is, embryos exhibit reductions in the number of axons in commissural bundles (Figure 6H) and more extreme phenotypes, such as missing commissures, in one or more segments (Figure 6G). These phenotypes are quite variable and appear to depend on the level of Netrin misexpression. Although we see phenotypes associated with either *NetA* or *NetB* misexpression, the mutant phenotypes are more prevalent and severe with *NetA* misexpression. Whether this represents a significant functional difference between *NetA* and *NetB* or simply reflects differences in the level of expression of the different UAS–Netrin lines is not clear.

Discussion

In this paper, we describe the identification and characterization of two Drosophila Netrin/UNC-6 homologs. Both Drosophila Netrins are expressed by CNS midline

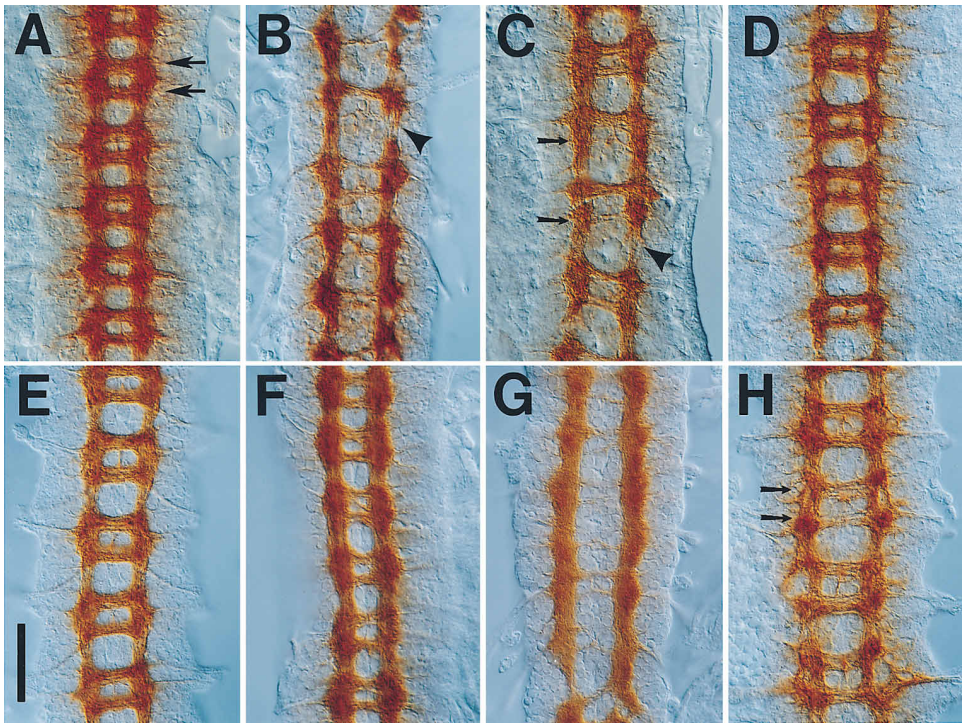

Figure 6. Drosophila Netrins Function in Commissural Growth Cone Guidance

Embryos of different genotypes stained with the MAb BP102 and horseradish peroxidase–immunocytochemistry to visualize CNS axon pathways. Scale bar, 25 μm.

(A) Wild-type CNS. Note the well formed anterior and posterior commissures within each segment (indicated by arrows).

(B) A moderate *Df(1)KA9* mutant phenotype (*Df(1)KA9*/Y embryo). Normal commissures never form, although partial commissures are observed. In addition to the commissure phenotypes, there are often breaks in the longitudinal connectives (arrowhead). The phenotype of the embryo in (G) is typical of a strong *Df(1)KA9* mutant phenotype.

(C) A typical *Df(1)RK5* mutant phenotype (*Df(1)RK5*/Y embryo). Partial commissures often form with some looking relatively normal. Arrows point to several missing commissures. Disruptions of the longitudinal connectives are often apparent (arrowhead).

(D) Rescue of the *Df(1)KA9* phenotype by expression of *NetA* at the CNS midline with the UAS–GAL4 system (*Df(1)KA9*/Y; P[UAS–NETA]/P[rho–GAL4]).

(E) Rescue of the *Df(1)KA9* phenotype by expression of *NetB* at the CNS midline (*Df(1)KA9*/Y; P[UAS–NETB]/P[rho–GAL4]).

(F) An example of partial rescue by *NetB* (embryo is the same genotype as in [E]).

(G) A strong phenotype caused by ectopic pan-neural expression of *NetA* in an otherwise wild-type background (P[UAS–NETA]/P[sca–GAL4] embryo).

(H) A more subtle misexpression phenotype (same genotype as in [G]). Arrows point to several commissures that have fewer axons than normal.

cells at a time when commissural growth cones are extending toward the midline. Embryos deleted for both *NetA* and *NetB* exhibit defects in commissure formation; this deficiency phenotype can be rescued by directed expression of either Netrin at the CNS midline. Therefore, the role of netrins as important guidance cues that function at the midline is conserved from insects to nematodes to chordates.

Netrin-A, *Netrin-B*, and Other Members of the Netrin Family

The presence of two netrins in Drosophila raises questions about the generation of diversity in the netrin family and the potential for additional netrins in flies and other organisms. The two chick netrins are very similar to each other (compared with other family members) and most likely represent a fairly recent duplication event. A number of features suggest that the two Drosophila Netrins also arose from a gene duplication event, although this event is likely more ancient. First, *NetA* and *NetB* share greater amino acid sequence similarity with each other than with other members of the netrin family. Second, there is a striking conservation in the location of introns within the Drosophila *Netrin* ORFs. Finally, the tandem organization of the Netrins is very suggestive of a duplication event. The eventual cloning of netrins from a diversity of species should help shed additional light on the evolution of this gene family.

For all their similarities with other members of the netrin family, the two Drosophila Netrins also exhibit some interesting differences. Both the NETA and NETB proteins are considerably larger than other family members. In the case of NETA, this is due to expansions within the C-terminal domain. NETB is larger owing to expansions of the first domain, domain VI, and from an insertion within the first EGF-like repeat. Given that the EGF-like repeats are the most highly conserved portions of the Netrin proteins, this insertion of 37 amino acids

is particularly intriguing. No specific function for the first EGF-like repeat has been described for any of the netrins; however, insights about the role of the second EGF-like repeat in UNC-6 have come from the molecular characterization of different *unc-6* mutations (Wadsworth et al., 1996). A collection of independent *unc-6* alleles that specifically affect dorsal migrations all result in the same mutant protein product: an UNC-6 protein that is missing the second EGF-like repeat owing to errors in splicing of the primary *unc-6* transcript. These observations have led to the hypothesis that the chemorepulsive role of UNC-6 is mediated at least in part by this second EGF-like repeat and that the putative chemorepulsive receptor, UNC-5, will require this repeat for its interaction with UNC-6 (Wadsworth et al., 1996). Perhaps other specific functions are mediated by the other EGF-like repeats, and if so, the rather divergent first EGF-like repeat of NETB might confer different functional properties.

Will other components of the UNC-6-mediated circumferential guidance mechanism be conserved in Drosophila as well? That is, are there UNC-5- and UNC-40-like molecules in flies? The answer is apparently yes, at least for UNC-40. UNC-40 is a transmembrane protein of the immunoglobulin superfamily with greatest similarity to vertebrate Deleted in Colorectal Cancer (DCC) (Chan et al., 1995, Soc. Neurosci., abstract; Culotti and Kolodkin, 1996). The Drosophila gene *frazzled* has been cloned and shares significant similarity with vertebrate DCC as well (Kolodziej et al., 1995, Soc. Neurosci., abstract). Furthermore, embryos mutant for the *frazzled* gene exhibit defects in commissural growth cone guidance (P. Kolodziej, personal communication). Thus, it seems likely that *frazzled* will be an important part of the mechanism by which commissural growth cones are attracted toward the CNS midline.

Expression and Function of Drosophila Netrins

Drosophila Netrins are expressed at the CNS midline and play important roles in commissural growth cone guidance. The failure to form commissures, commissures with less than normal numbers of axons, and disruptions of longitudinal connectives are phenotypes that are associated with the deletion of both Netrins. *NetA* and *NetB* apparently play redundant roles at the embryonic CNS midline, since both proteins are similarly expressed within the embryonic CNS and both proteins can rescue the phenotypes associated with deficiencies that delete *NetA* and *NetB*. Critical testing of this potential redundancy will require individual null mutations in *NetA*, *NetB*, and the corresponding double mutant that is not complicated by the loss of additional genes as in the case of *Df(1)KA9* and *Df(1)RK5*.

The rescue of Netrin loss-of-function defects in longitudinal pathways by Netrin expression at the midline is quite interesting. This may indicate that failure to reach the midline results in subsequent disruptions of the pathfinding program. The absence of such longitudinal pathway defects in *commissureless* mutants is consistent with this hypothesis, since the defect in a *commissureless* mutant is the inability to cross the midline boundary, not in reaching and contacting the midline (Tear et al., 1996).

More robust genetic tools will also allow us to look at the function of Netrins in other tissues and at other times of development. For instance, the expression of Netrins in subsets of muscle fibers raises the possibility of a role for Netrins in motoneuron pathfinding and target recognition. Since the Netrins differ at least partially in the subset of muscles that they are expressed in, *NetA* and *NetB* may provide unique signals that guide different motoneurons to their appropriate synaptic target or target region.

The phenotypes associated with *Df(1)KA9* and *Df(1)RK5* raise a number of puzzling questions. Somewhat surprisingly, the complete elimination of both Drosophila Netrins does not completely disrupt commissure formation. In *Df(1)RK5* mutant embryos, many commissures form and look relatively normal. This would suggest that other signals are present that help attract commissural growth cones to the midline. These other signals may be additional members of the netrin family (we have looked for additional netrins without success) or could represent a new class of proteins. This evidence for additional signals at the midline is not unique to Drosophila. In C. elegans, null mutations in *unc-6* do not completely disrupt circumferential guidance (Hedgecock et al., 1990; McIntire et al., 1992). If this additional signal(s) plays a minor role or if there are multiple proteins that are functionally redundant, then this signal may have been missed in genetic screens for mutations that disrupt proper formation of commissural pathways, as were the Netrins (for example, see Seeger et al., 1993). In this case, other approaches will be required to identify these additional guidance cues.

The greater severity of the *Df(1)KA9* embryonic CNS phenotype relative to *Df(1)RK5* presents an interesting paradox. Both deficiencies delete both *NetA* and *NetB*, and the CNS phenotype of either deficiency can be substantially rescued by midline expression of either *NetA* or *NetB*. This suggests that some gene or genes that are removed by *Df(1)KA9* but not by *Df(1)RK5* lead to an enhancement of the CNS phenotype. There must not be an absolute requirement for this gene or genes since the Netrins can rescue the *Df(1)KA9* phenotype. Additional studies will be required to determine whether this enhancement is specific (that is, some gene that functions in commissural growth cone guidance) or simply some nonspecific effect.

A Localized Netrin Signal Is Important for Commissural Growth Cone Guidance

A critical requirement for any chemotropic signal should be a localized source of the signal with a concentration gradient of the signal emanating from the source. Both Drosophila Netrin proteins are primarily associated with the surface of the cells that are expressing them, a situation similar to what has been observed for UNC-6 in C. elegans (Wadsworth et al., 1996). Although we have not observed any clear global Netrin gradient originating from the CNS midline, it is possible and likely that neuronal growth cones are responding to differences in Netrin protein concentrations.

The observation that ectopic pan-neural expression of either NETA or NETB induces CNS phenotypes similar

to the loss-of-function phenotype is consistent with the prediction that a localized Netrin source is critical for function. These experiments suggest that there are two ways to inactivate a chemotropic signal: one is to eliminate the signal itself, and the second is to eliminate the polarity of the signal by flooding the system with ectopic signal. This hypothesis should be testable. For instance, increased expression of Netrin at the midline should be able to rescue subtle commissure defects associated with pan-neural ectopic expression. That is, ectopic expression phenotypes that are just above a critical threshold should be suppressed by increasing the Netrin signal originating from the midline and conversely enhanced by decreasing the midline Netrin signal.

It will be quite important to test these hypotheses concerning chemotropic functions of Drosophila Netrins, since there are alternative, although less likely, explanations of the Netrin loss-of-function and misexpression phenotypes. For instance, the Netrins may be providing specific local guidance cues in contrast to more global signals. There is evidence from C. elegans that some activities of *unc-6* are mediated through functions as a local guidance cue (Wadsworth et al., 1996).

Will Drosophila Netrins Have Chemorepulsive Functions?

Chemorepulsive roles have been described for both UNC-6 and chick netrin-1 in addition to their functions as chemoattractants. It would not be surprising if Drosophila Netrins functioned as chemorepellents as well. Perhaps other functions will become apparent as we expand our understanding about the role of *NetA* and *NetB* in other tissues, define important functional domains of these proteins, identify receptors, and isolate individual null mutations in these two interesting genes. The ability to eliminate the function of these two proteins individually and in combination is an essential next step in our analysis of Drosophila Netrins.

Experimental Procedures

PCR of Drosophila *Netrins*

Drosophila *NetA* was identified by PCR from Drosophila genomic DNA using the following primers: primer 1, TGG GT(C/G/T) AC(A/C/G) GC(A/C/G/T) AC(A/C/G) (A/G)A(C/T) (A/C)T; primer 2, TG(C/T) (A/C)G(A/C/G/T) TG(C/T) AA(C/T) GG(A/C/T) CA(C/T) GC; primer 3, GC (A/G)TG (A/G/T)CC (A/G)TT (A/G)CA (A/C/G/T)C(G/T) (A/G)CA; and primer 4, GT (A/G)TT (A/G)TG (A/C/G/T)(C/T)(G/T) GCA (A/C/G/T)T(C/T) GCA. These primers correspond to the following amino acid sequences conserved between UNC-6 and laminin B2 polypeptides: primer 1, WVTAT(D/N)(I/L), end of domain VI; primers 2 and 3, CKCNGHA, beginning of the first EGF-like repeat; and primer 4, C(D/N/E)C(R/K)HNT, middle of the first EGF-like repeat (see Ishii et al., 1992). PCR was performed using Vent polymerase (New England Biolabs, Beverly, MA) and a "touchdown" PCR strategy where the annealing temperature was decreased over successive cycles. Primer pair 2 and 4 generated the initial *NetA* fragment.

cDNA Isolation, Sequence Analysis, and Molecular Characterization

Standard molecular techniques were carried out following Sambrook et al. (1989). cDNAs were isolated from a λgt11 phage library made from 9–12 hr embryonic poly(A)$^+$ RNA (Zinn et al., 1988). *Netrin* cDNA clones were sequenced initially from a set of Exonuclease III nested deletions using the fmol DNA cycle sequencing system (Promega, Madison, WI). Genomic sequence and confirmation of the cDNA sequence was determined using a set of specific oligonucleotide primers. Sequences were analyzed using programs of the Wisconsin GCG sequence analysis package, and database searches were performed using the BLAST program (Altschul et al., 1990).

Generation of Fusion Proteins and Antibodies

Fusion proteins were constructed by cloning the C-terminal portions of the *NetA* and *NetB* ORFs into two different expression vectors. The C-terminal portions of the Netrins were chosen, since this region exhibits the least degree of homology between the Netrins and is not conserved in the laminin polypeptides. For *NetA*, a ScaI to EcoRI fragment (encoding amino acids 524–727) was cloned into the fusion protein vectors pRSET (a His_6-tag vector) (Invitrogen, San Diego, CA) and pMAL (a maltose binding protein [MBP] vector) (New England Biolabs). For *NetB*, a ScaI to EcoRI fragment (encoding amino acids 649–793) was cloned into the same fusion protein vectors. Goats were injected with 1–1.5 mg of the His-tag fusion proteins in Freund's adjuvant and boosted at monthly intervals. Affinity purified antibodies were isolated from the goat sera as generally described by Harlow and Lane (1988). Serum was purified over an AffiGel (Bio-Rad, Richmond, CA) NETA–MBP or NETB–MBP fusion protein column.

In Situ Hybridization and Immunocytochemistry

Polytene in situ hybridizations were done according to Ashburner (1989) with minor modifications. Whole-mount embryo RNA in situs were done as in Tear et al. (1996). Immunohistochemistry was performed as described by Patel (1994).

Germline Transformation and Genetic Analysis

NetA and *NetB* cDNA clones were inserted into the EcoRI site of the P-element transformation vector, pUAST (Brand and Perrimon, 1993). Purified DNA (Qiagen kit, Quiagen Inc., Chatsworth, CA) was injected into embryos carrying the constitutively active source of P-element transposase, *Δ2–3* (Robertson et al., 1988). Surviving adults were outcrossed to *white* flies and germline transformants were identified in the F1 generations by rescue of the *white* eye phenotype. UAS–NETA and UAS–NETB inserts on either the second or third chromosomes were crossed into a *Df(1)KA9* or *Df(1)RK5* mutant background. Hemizygous deficiency embryos were identified by the absence of β-galactosidase staining associated with the FM7c balancer chromosome that carries a P[*ftz–lacZ*] insertion.

All markers and balancer chromosomes used are described by Lindsley and Zimm (1992). Deficiencies were obtained from the Drosophila stock center at Bloomington. *Df(1)RK5* (and several others) were provided by B. Ganetzky. The rho–GAL4 and sca–GAL4 driver lines were provided by C. Klämbt.

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