Development 117, 431-440 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Specifying the path of the intersegmental nerve of the *Drosophila* embryo: a role for *Delta* and *Notch*

Edward Giniger[1], Lily Yeh Jan and Yuh Nung Jan

Howard Hughes Medical Institute and Departments of Biochemistry and Biophysics, and of Physiology, Room U-426, University of California, San Francisco, San Francisco, CA 94143, USA

*Present address: Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104, USA

SUMMARY

The intersegmental nerve (ISN) of the *Drosophila* embryo follows a reproducible course near the anterior border of each segment. Based on the experiments reported here, we suggest that growth of the axons constituting the nerve is guided, in part, by the transmembrane proteins Delta and Notch. In particular, we suggest that expression of Delta protein on a branch of the trachea provides a path for the nerve through the lateral part of the embryo, and that the growing axons use the Notch protein on their surfaces to recognize this path. Consistent with this idea, we show that disruption of the trachea abolishes the ability of the ISN to extend through this part of the embryonic periphery. Finally, we argue that the same regulatory network that directs these peripheral axons also specifies the trajectory of part of the axonal scaffold of the central nervous system.

Key words: *Delta*, *Notch*, axon guidance, peripheral nervous system

INTRODUCTION

How a neuron finds its synaptic targets is one of the fundamental issues in neural development (Ramon y Cajal, 1984). There is a wealth of data about the cell biology of axon guidance (reviewed in Dodd and Jessell, 1988; Bixby and Harris, 1991; Hynes and Lander, 1992), and the goal of much recent work in this area has been to identify molecules whose distribution, structure and activity all suggest that they are likely to be determinants of a choice in axon guidance (Bastiani et al., 1987; Cunningham et al., 1987; Tessier-Lavigne et al., 1988; Furley et al., 1990; Stahl et al., 1990; McIntire et al., 1992). One approach to uncovering a guiding molecule and its receptor is to look for mutations of *Drosophila* that give rise to specific aberrations in nerve trajectories. Such mutations may affect nuclear proteins required to specify the identity or transcriptional program of a neuron (Ghysen et al., 1983; Doe et al., 1988; Vaessin et al., 1991), or the cell surface and effector molecules that directly control axon extension (Gertler et al., 1989; Hedgecock et al., 1990; Grenningloh et al., 1991). However, there is no guidance decision for which the entire regulatory heirarchy is known: guiding molecule, receptor, and their regulators and effectors. We have therefore analyzed the development of a single nerve in the peripheral nervous system (PNS) of the fly embryo to identify the molecules that specify its trajectory.

In the *Drosophila* embryo, each side of each bilaterally symmetric thoracic and abdominal segment includes an axon bundle, termed the intersegmental nerve (ISN), that courses along a fixed path near the anterior boundary of that segment (Campos-Ortega and Hartenstein, 1985; Ghysen et al., 1986; Fig. 2A,B). The ISN contains both sensory axons that grow ventrally, towards the central nervous system, and motor axons that grow dorsally. While it is not known what genes or structures determine the trajectory of the ISN, it has been noted that this nerve grows, in turn, on three different substrata. In the most ventral part of the embryo, the ISN passes between layers of myoblasts (J. Johansen et al., 1989) whereas, in the most dorsal region, the motor axons of the nerve grow internal to the dorsal cluster of sensory neuron cell bodies. In between, in the lateral part of the embryo, the ISN grows in close apposition to the transverse branch of the embryonic trachea (Fig. 2A,B; Hartenstein, 1988), which develops prior to the initiation of peripheral axon extension. In fact, in this middle third of the pathway, the pioneer growth cone of the ISN migrates along the tracheal wall, leading to speculation that the trachea might define part of the ISN trajectory (Hartenstein, 1988).

The mechanisms that determine the shape of the trachea are not themselves understood, though it is known that a *Drosophila* homolog of the fibroblast growth factor receptor (D-FGFR) is expressed in the cells of the trachea and is specifically required for the morphogenesis of this tissue (Glazer and Shilo, 1991). If the trachea does in fact pro-

vide a guidance signal to the ISN, one might expect the trachea to produce a molecule for which there is a receptor on peripheral axons. One candidate for such a ligand-receptor pair is the proteins encoded by the genes *Delta* and *Notch*. In addition to their expression at other times and places in the developing fly, *Delta* is transcribed in the embryonic trachea (Vaessin et al., 1987), whereas Notch protein is expressed on the axons of the ISN (K. Johansen et al., 1989; Fehon et al., 1991).

Delta and Notch are large, transmembrane proteins that bear a number of EGF-like (epidermal growth factor-like) repeats in their extracellular domains (Wharton et al., 1985; Vaessin et al., 1987; Kidd et al., 1988; Kopczynski et al., 1988). These proteins are required for many of the cell-cell interactions that regulate the development of the embryonic and adult nervous system of the fly (Lehmann et al., 1983; Cagan and Ready, 1989; Hartenstein and Posakony, 1990), as well as being important for the development of several non-neuronal structures (Lindsley and Grell, 1968; Corbin et al., 1991; Ruohola et al., 1991). For example, in embryos mutant for either *Delta* or *Notch*, essentially all ectodermal cells in the neurogenic region become neurons (Lehmann et al., 1983; Campos-Ortega and Jan, 1991) and none become epidermal cells, due to a failure of cell-cell communication (Doe and Goodman, 1985; Technau and Campos-Ortega, 1987). It is generally believed that the Delta and Notch proteins interact directly with one another to effect those processes that require both genes, based on the following observations. First, lesions in these two genes give rise to very similar mutant phenotypes in a number of developmental contexts (Lindsley and Grell, 1968). Second, these loci interact genetically: the effect of a mutation in either gene is strongly influenced by the gene dosage of the other (Lindsley and Grell, 1968; Vaessin et al., 1985; de la Concha et al., 1988), and the phenotypes of some *Notch* mutations are suppressed by particular *Delta* mutations, in an allele-specific manner (Brand and Campos-Ortega, 1990). Third, in cultured cells, Notch protein can act as a receptor for Delta (Fehon et al., 1990; Rebay et al., 1991). This last result is consistent with data from genetic mosaics, which suggest that, in various situations, *Delta* generates the developmental signal for which *Notch* is part of the receiving mechanism (Hoppe and Greenspan, 1986, 1990; de Celis et al., 1991; Heitzler and Simpson, 1991). Most of the available data on the roles of *Delta* and *Notch* concern their activities in cell fate determination, but one report has described axonal defects in the adult peripheral nervous system in flies bearing particular mutant alleles of *Notch* (Palka et al., 1990). Furthermore, the *Notch* mutant phenotype has been interpreted as reflecting a primary role for the protein in cell adhesion (Hoppe and Greenspan, 1986), a cellular property that is believed to be a major determinant of axon guidance.

The experiments reported here suggest a mechanism for specifying the trajectory of the intersegmental nerve. In particular, we employ temperature shifts of temperature-sensitive mutant alleles of the genes *Delta* and *Notch* to show that, separate from their well-studied roles in the determination of cell fate, these genes are also required for the growth of ISN axons through the lateral region of the embryonic periphery. Since the ISN grows on the surface of the trachea through this part of the embryo and *Delta* is expressed in the trachea while its receptor, Notch protein, is expressed on the associated peripheral axons, we speculate that the trachea uses the interaction of Delta and Notch to direct the growth of ISN axons. We further test the idea that the trachea is important for peripheral axonogenesis by using a mutation of the D-FGFR to perturb tracheal development and show that the resulting tracheal defects are reflected in corresponding axonal defects.

We observe that *Delta* or *Notch* mutations, which block normal development of the intersegmental nerve, also prevent formation of the longitudinal axon tracks between the segmental ganglia in the CNS. We propose, therefore, that the guidance system that we have defined for the lateral section of the ISN also specifies the trajectory of one element of the axon scaffold of the CNS.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster stocks were maintained on standard media at 25°C (or 18°C for temperature-sensitive mutants). In experiments involving temperature shifts, embryos were collected on grape-juice agar at 18°C and shifted to 32°C as specified for the particular experiment. For experiments at temperatures other than 25°C, times are given here as the equivalent developmental time at 25°C, not as the actual elapsed time (Ashburner, 1989). We assumed the rate of development to be decreased 50% at 18°C, and increased 10% at 32°C, relative to the rate at 25°C. The following mutant stocks were used: *fz* GF^{3b} (*Df3L 70B-70D6*)/*TM6B Tb e*; *fz* GS^{1a} (*Df3L 70C6-15 - 70E4-6*)/*TM3 Sb*; N^{ts1}/N^{ts1}; N^{ts1}/N^{ts1}, *SM1Cy Dp(1;2)*w^{+}*51b7*N^{+}/+; *st*$Dl^{6B37(ts)}$ *e*/*TM3 Sb Ser*; *st* Dl^{via1} *e*/*st* Dl^{via1} *e*. The wild-type stock was either Oregon R or *y w* iso 2,3 OreR (Bier et al., 1989). Temperature-shift experiments with *Delta* were performed using the *trans*-heterozygous combination *st* $Dl^{6B37(ts)}$ *e*/*st* Dl^{via1} *e*. The FGFR gene was deleted in the *trans*-heterozygous combination *fz GF3b*/*fz GS1a*; *fz* stocks were obtained from the Bloomington Stock Center. All stocks not otherwise specified are described in Lindsley and Grell (1968).

Histochemistry and microscopy

Immunocytochemistry and in situ hybridization were performed by standard methods (Bodmer and Jan, 1987; Bier et al., 1989; Tautz and Pfeifle, 1989) except samples for confocal microscopy were blocked with 5% normal donkey serum (in 0.1 M sodium phosphate, pH 7.2, 0.3% Triton X-100 with 2% bovine serum albumin) for 15 minutes at room temperature immediately prior to incubation with secondary antibody. Neurons were labelled either with anti-HRP (Jan and Jan, 1982), obtained from Cappel and affinity-purified, or with mAb 22C10 (Zipursky et al., 1984). Anti-tracheal antibody was mAb 68G5D3 (Sandra Barbel, Larry Ackerman, LYJ and YNJ, unpublished results). Peroxidase-coupled goat secondary antibodies were from BioRad, fluorescein- and Texas Red-coupled donkey secondary antibodies were from Jackson Immunologicals. Peroxidase-developed samples were cleared in toluene and mounted in Permount (Fisher) for Nomarski microscopy using a Nikon Optiphot; fluorescent samples were mounted in 90% glycerol with 2% n-propyl gallate and 20 mM Tris pH 7.6, and examined on a Zeiss Axioplan equipped with BioRad scanning confocal microscopy hardware and software. For in situ hybridization, Delta cDNA 3.2 (Vaessin et al., 1987) was labelled with digoxigenin (Boehringer Mannheim) and used to probe wild-type or mutant embryos. For different experiments,

either DNA probes were prepared by 'random-oligo' labelling, or antisense RNA probes were prepared by transcription in vitro. Staging of embryos for all experiments was as described by Campos-Ortega and Hartenstein (1985).

RESULTS

ISN guidance requires *Delta* and *Notch*

It has been observed that the axons of the intersegmental nerve (ISN) bear Notch protein (Fehon et al., 1991). We thus undertook a series of temperature-shift experiments with temperature-sensitive mutant alleles of *Notch*, and also of *Delta*, to uncover any specific function these proteins might have in nerve guidance. Embryos were grown for varying lengths of time at the permissive temperature (18°C) in order to provide the *Delta* and *Notch* activity required for neurogenesis, and were then raised to restrictive temperature (32°C) and maintained at that temperature until embryonic stage 15/16 (after the completion of peripheral axonogenesis). As background, we now briefly summarize the main events in neurogenesis and peripheral axonogenesis (Campos-Ortega and Hartenstein, 1985; Hartenstein, 1988; J. Johansen et al., 1989; Sink and Whitington, 1991). Neurogenesis begins at about stage 9 (4 hours of development) and the pioneers of the ISN are born by stage 13 (9 hours), though the full complement of motoneurons is not readily identifiable until stage 15. The motor pioneer of the ISN (aCC) exits the CNS during stage 13, at roughly the same time that the dorsal sensory pioneer of this nerve (dh1) initiates its ventrally directed axon outgrowth (about 9.5 hours). The two pioneer axons contact opposite ends of the transverse branch of the trachea late in stage 13. The axons meet along the trachea and fasciculate during stage 14. (Note that the transverse branch of the trachea has formed by the end of stage 12.)

Appropriate temperature-shifts of *Notch*^{ts} or *Delta*^{ts} mutants caused defects in the growth and guidance of the ISN (Fig. 1A-D). In representative segments, axons were found either to grow inappropriately, for example, extending into adjacent segments (Fig. 1B,D, arrows), or to cease extending altogether (Fig. 1A,C, open arrowheads). Temperature shifts done in parallel with wild-type embryos, or with *Notch*^{ts} embryos containing a wild-type copy of the *Notch* gene on another chromosome (Fig. 1E), did not produce such guidance errors. Unexpectedly, these defects were localized to roughly the lateral one-third of each segment: outside the lateral region of the embryo, the ISN did not appear to be adversely affected in these temperature-shift experiments (Fig. 1A-D, triangles). In our experiments, initiating the shift from permissive to restrictive temperature between 3 and 6 hours of development (equivalent time at 25°C; see Materials and Methods) reproducibly caused aberrations in axon elongation while minimizing neurogenic defects; shifting the temperature between 6 and 9 hours typically failed to produce a mutant axonal phenotype. The ISN grows through the lateral region of the embryo between about 10 and 11 hours of development. The delay between the initiation of the temperature shift and the onset of phenotypic aberrations presumably reflects perdurance of the mutant protein, and is consistent with the delay seen in analogous experiments examining *Notch*^{ts} effects in larvae and pupae (Cagan and Ready, 1989; Hartenstein and Posakony, 1990). In those experiments, it was necessary for animals to be at restrictive temperature for at least 4-8 hours (depending on the tissue) before any mutant phenotype was observed. Similarly, it has been observed in genetic mosaic experiments that Notch activity remains at significant levels for several hours following the generation of a mutant clone in embryos (Hoppe and Greenspan, 1990). The latency of *Delta*^{ts} effects in ovaries appears to be at least as long as is the case for *Notch*^{ts} (Ruohola et al., 1991).

We noted that, in the lateral part of the embryo, that is, the region in which we observed axonal defects arising from *Notch*^{ts} and *Delta*^{ts}, the axons of the ISN are known to extend along the surface of the trachea (Hartenstein, 1988; diagrammed in Fig. 2A). We therefore tested whether the defects that we observed might be indirect consequences of tracheal aberrations caused by the *Delta* or *Notch* mutations. *Notch*^{ts} and *Delta*^{ts} embryos were subjected to the same temperature-shift protocols described above and then labelled both with an antibody directed against the trachea and one directed against neurons. For comparison, the association of the ISN with the trachea in a wild-type embryo is shown in Fig. 2B. In this micrograph, the nervous system is in green and the trachea in red; their apposition in the lateral region is highlighted with a bracket. The anti-tracheal antibody labelled the lumen of the trachea, thus the apparent distance between the green- and red-stained structures is the thickness of the tracheal cells themselves. In the mutants (Fig. 2C-E), we readily found examples of segments in which tracheal development was normal (open arrowheads), but displaying defects in the growth and guidance of the ISN like those described in the previous section (arrows), suggesting that *Notch* and *Delta* are directly required for ISN guidance.

Guidance of the ISN by the trachea

The finding that *Notch* and *Delta* are required for growth of the ISN along the trachea recalled the report that Delta RNA is expressed in the trachea (Vaessin et al., 1987), which we have verified (E. G. and Y. N. J., unpublished observations). Taken together, these observations suggested the idea that Delta protein on the trachea might provide a path for the elongation of ISN axons. We tested this model further by disrupting tracheal development and examining the effect of such disruptions on axonogenesis. Specifically, we took advantage of the observation that mutation of a *Drosophila* homolog of the FGF receptor (D-FGFR) prevents tracheal morphogenesis but not the birth or differentiation of the tracheal cells (Glazer and Shilo, 1991; Klambt et al., 1992). In the embryonic periphery, the D-FGFR is expressed only in the trachea and is never detected in neurons. A subset of ventral midline cells also express the D-FGFR, but mutation of this gene has only very subtle effects on the CNS (Klambt et al., 1992). Moreover, the pioneer motor axon of the ISN (aCC) does not cross the midline. Thus, any peripheral neuronal defect in the mutants must arise as an indirect consequence of tracheal defects.

Mutant embryos lacking the D-FGFR suffered severe defects in tracheal morphology and had corresponding

defects in the associated nerves, as detailed below. The most apparent axonal aberrations were observed in segments that lacked trachea altogether, or in which the trachea was displaced dorsally beyond the filopodial reach of ISN motor axons. In these cases, the ISN either stopped growing or followed the trachea in an adjacent segment (Fig. 3A,A ,B,B), aberrent nerves indicated by arrows; trachea denoted by open arrowheads; segment lacking trachea highlighted with asterisk). We never observed a segment in which the ISN grew through the lateral part of the embryo in the complete

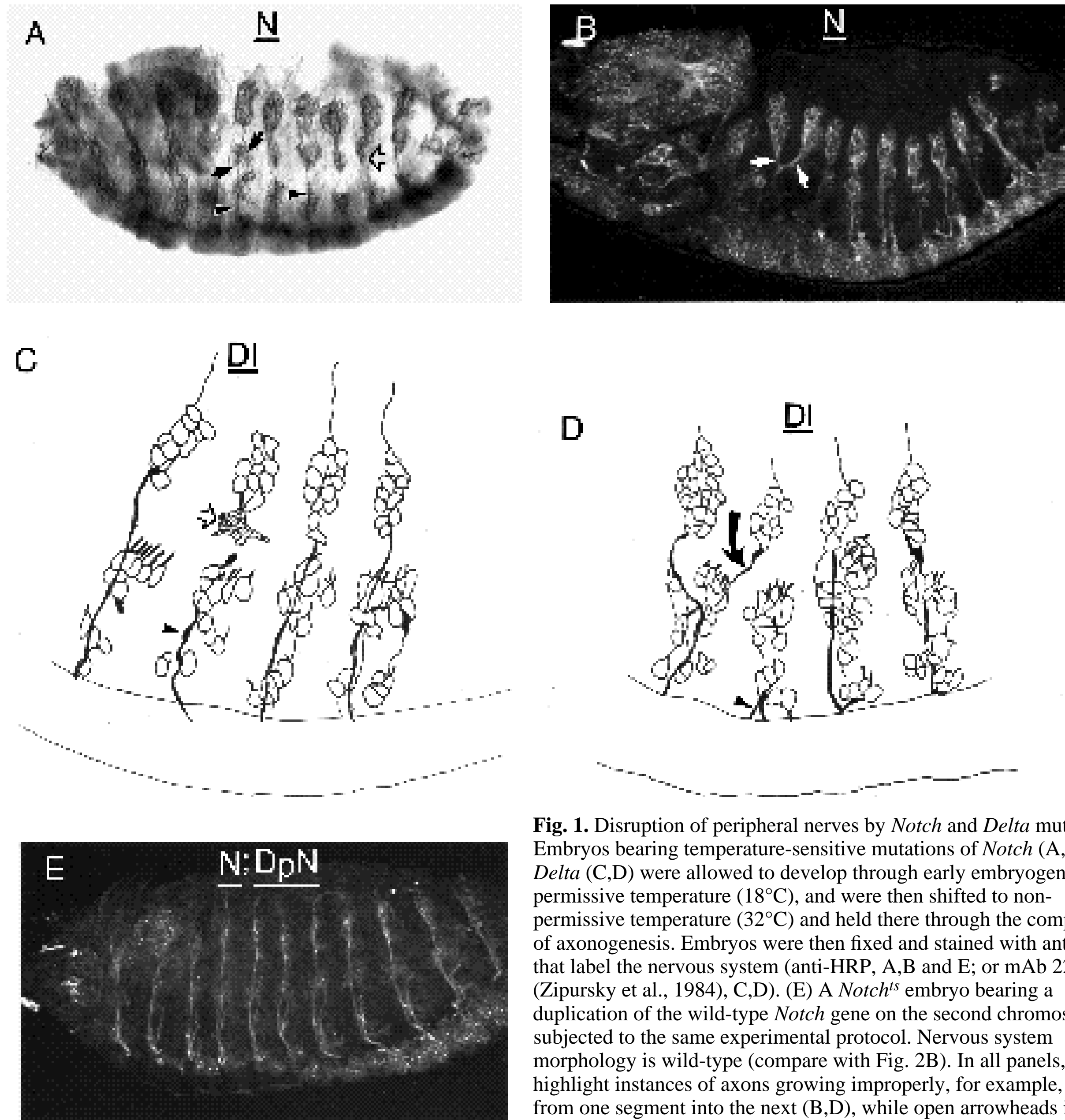

Fig. 1. Disruption of peripheral nerves by *Notch* and *Delta* mutations. Embryos bearing temperature-sensitive mutations of *Notch* (A,B) or *Delta* (C,D) were allowed to develop through early embryogenesis at permissive temperature (18°C), and were then shifted to non-permissive temperature (32°C) and held there through the completion of axonogenesis. Embryos were then fixed and stained with antibodies that label the nervous system (anti-HRP, A,B and E; or mAb 22C10 (Zipursky et al., 1984), C,D). (E) A *Notch*^{ts} embryo bearing a duplication of the wild-type *Notch* gene on the second chromosome, subjected to the same experimental protocol. Nervous system morphology is wild-type (compare with Fig. 2B). In all panels, arrows highlight instances of axons growing improperly, for example, crossing from one segment into the next (B,D), while open arrowheads indicate cases where axons have stopped growing altogether (A,C). In these mutants, ISN development typically appears normal outside the lateral region of the embryo (highlighted by triangles). Due to the rapid time course of embryonic development, *Notch*^{ts} and *Delta*^{ts} temperature shifts that were early enough to affect growth of the ISN in most segments of each embryo also caused a severe neurogenic phenotype (i.e. production of many extra neurons), while shifts late enough to minimize the neurogenic effects were also of more limited efficacy in disrupting axon guidance. For the experiments displayed in A-E, temperature shifts were initiated between 3 and 6 hours of embryonic development (equivalent developmental time at 25°C). See text for further discussion of timing of temperature shifts. (A,C,D) Samples were developed by peroxidase histochemistry; (C,D) camera-lucida traces of representative embryos; (B,E) Confocal fluorescence microscopy. In all cases, these are lateral views of stage 15/16 embryos. Anterior is to the left, dorsal at the top. In one representative experiment, of 99 appropriately staged and oriented *Notch*^{ts} embryos examined, 85 displayed, in at least one segment, a mutant axonal phenotype like those described here. The ISN appeared to form normally in all segments of the remaining 14 embryos.

absence of tracheal tissue. In segments where residual tracheal material developed, the ISN appeared to grow along this tissue, however irregular the morphology of that tracheal remnant might be. We never found an instance in which the ISN stalled after successfully contacting the trachea. Note that Delta RNA was expressed normally in the tracheal tissue that developed in mutant embryos (data not shown). In all cases, ISN development appeared normal in the ventral region of the embryo (triangles).

Our experiments employed overlapping chromosomal deficiencies that completely remove the receptor gene (see Materials and Methods). Thus, the receptor itself is not directly required for association of the ISN with the trachea. These deletions are also believed to remove several other genes (Glazer and Shilo, 1991), however, and thus we cannot rule out the possibility that one of these other genes might contribute to the mutant axonal phenotype. Nonetheless, the perfect correlation of tracheal and axonal defects

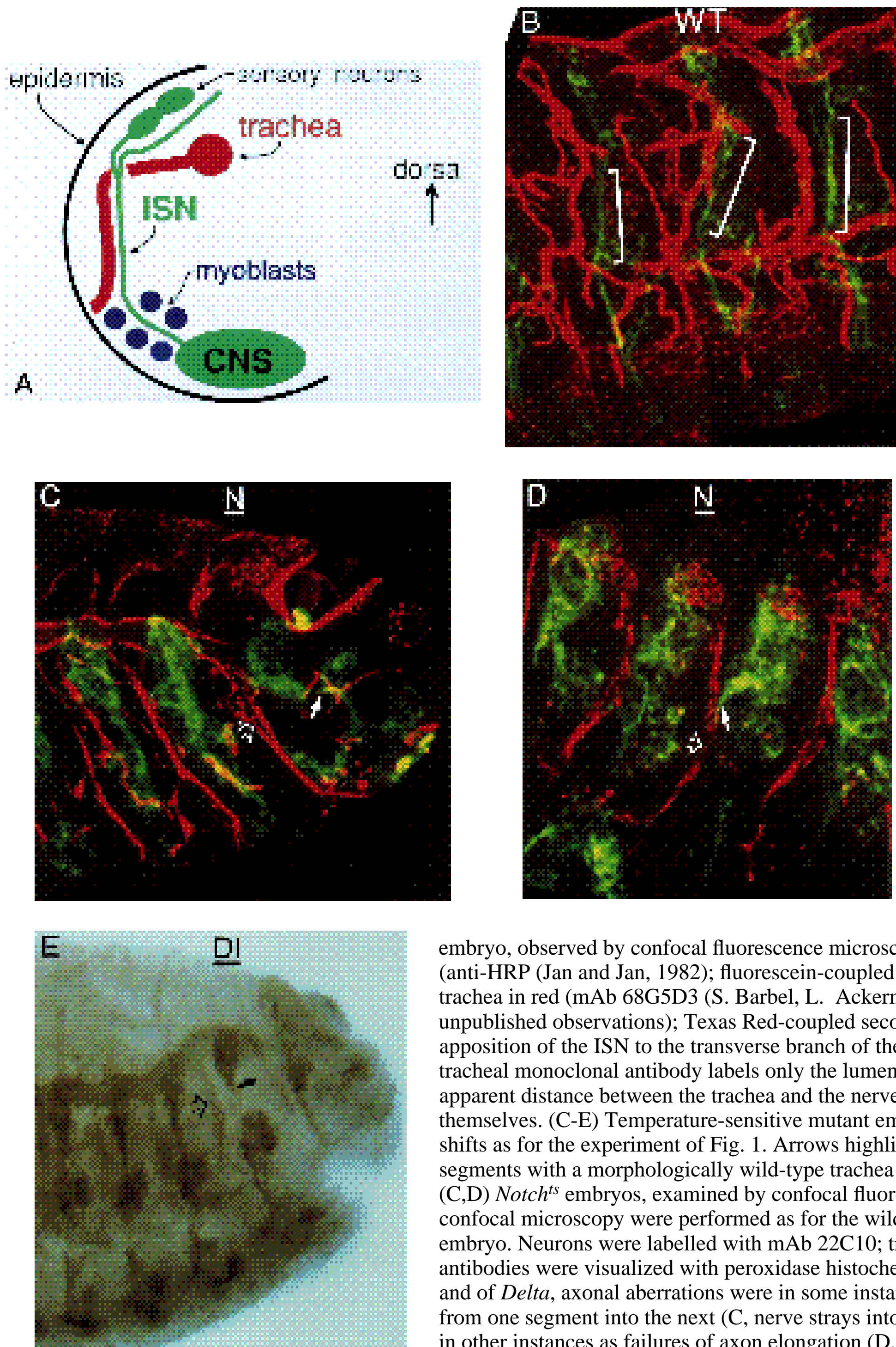

Fig. 2. Axonal defects in *Notch*^{ts} and *Delta*^{ts} are not secondary to tracheal aberrations. (A) The intersegmental nerve (ISN, in green) grows along three distinct substrata in different parts of the developing fly embryo. Ventrally, the nerve grows between layers of myoblasts (blue circles), laterally, the nerve grows along the transverse branch of the trachea (red line) and dorsally, the nerve grows internal to a cluster of sensory neurons (green ovals). Only one side of an embryo is depicted in this schematic cross-section through an abdominal segment. The outer black semicircle represents the embryonic bodywall. Dorsal is at the top. (B-E) Lateral views of embryos stained both with antibodies directed against the nervous system and against the trachea. (B) A wild-type stage 16 embryo, observed by confocal fluorescence microscopy. The nervous system is in green (anti-HRP (Jan and Jan, 1982); fluorescein-coupled secondary antibody) and the trachea in red (mAb 68G5D3 (S. Barbel, L. Ackerman, L. Y. J. and Y. N. J., unpublished observations); Texas Red-coupled secondary antibody). Note the close apposition of the ISN to the transverse branch of the trachea (brackets). The anti-tracheal monoclonal antibody labels only the lumenal surface of the trachea; the apparent distance between the trachea and the nerve is the thickness of the tracheal cells themselves. (C-E) Temperature-sensitive mutant embryos subjected to temperature shifts as for the experiment of Fig. 1. Arrows highlight instances of axonal defects in segments with a morphologically wild-type trachea (indicated by open arrowheads). (C,D) *Notch*^{ts} embryos, examined by confocal fluorescence microscopy. Labelling and confocal microscopy were performed as for the wild-type embryo in B. (E) *Delta*^{ts} embryo. Neurons were labelled with mAb 22C10; trachea with mAb 68G5D3. Both antibodies were visualized with peroxidase histochemistry. In the cases both of *Notch* and of *Delta*, axonal aberrations were in some instances recognized as nerves crossing from one segment into the next (C, nerve strays into next more posterior segment), and in other instances as failures of axon elongation (D,E).

in these tracheal mutants implies that ISN development depends, at least in part, upon the trachea. Also consistent with this idea was the observation that in a small subset of cases, temperature shifts of *Notch*^{ts} embryos blocked the development of the transverse branch of the trachea. In these rare cases, segments that lacked the transverse trachea due to the absence of *Notch* function did not support growth of the lateral section of the ISN (data not shown). This result provides independent confirmation of the idea that a critical role is played by the trachea in facilitating ISN development.

Requirement for *Notch* and *Delta* in the development of CNS axons

In the course of our analysis of the role of *Notch* and *Delta* in peripheral axonogenesis, we observed that these two mutations produced very similar mutant phenotypes in the axon scaffold of the CNS (Fig. 4B,C). Specifically, both

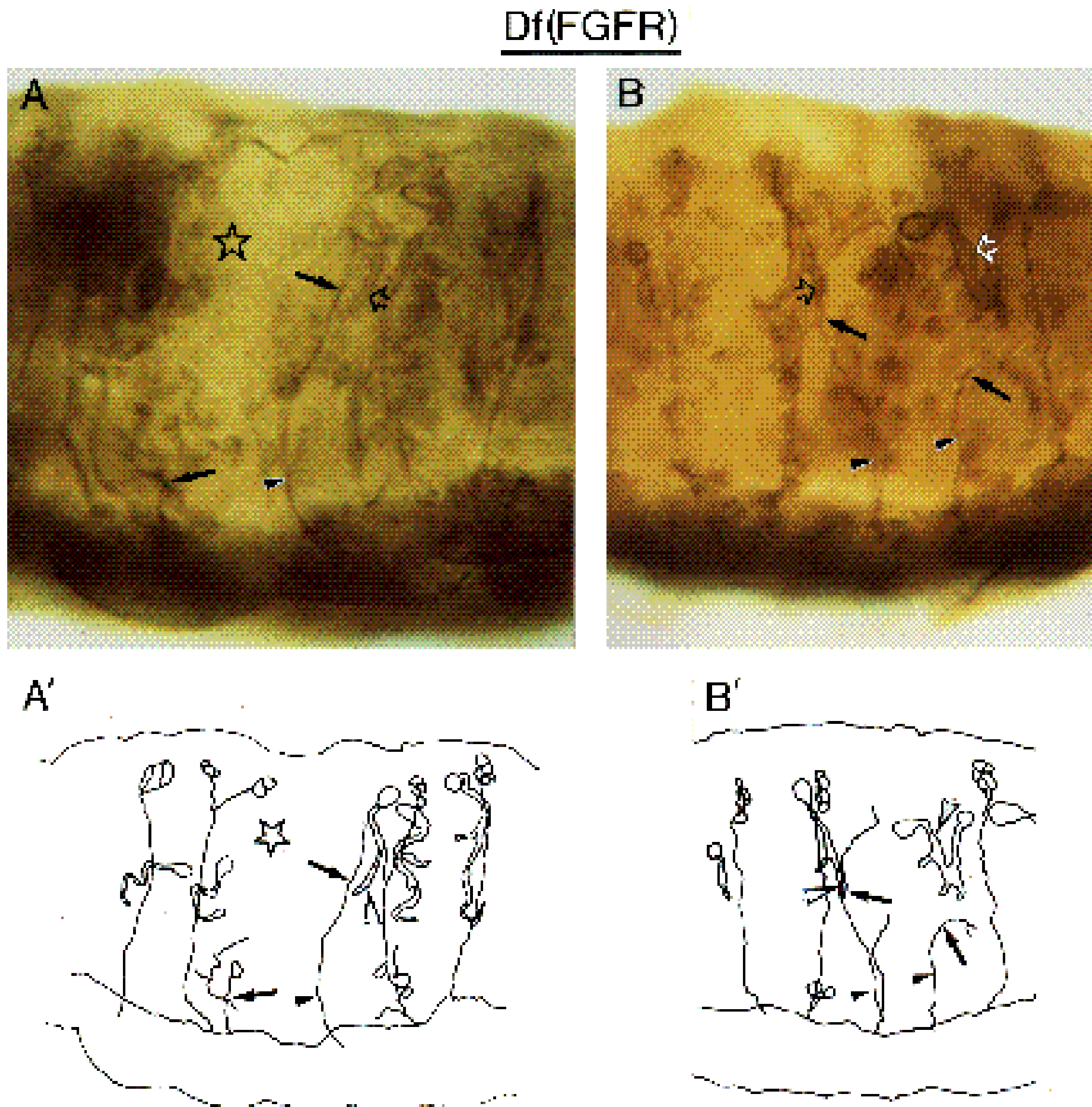

Fig. 3. Growth of the intersegmental nerve requires the trachea. Mutant embryos with tracheal defects as a consequence of deletion of the FGF receptor gene. (A ,B) Camera-lucida tracings of the same embryos shown in A and B. Embryos were double-stained with antibodies directed against both the nervous system and the trachea and developed by peroxidase histochemistry. Intersegmental nerve (thin brown line) is indicated with an arrow in affected segments; trachea is indicated with an open arrowhead. Asterisk highlights a segment that has no trachea; in this segment, the ISN does not grow through the lateral part of the embryo (A, segment A2). In segments where the ISN does extend through the lateral region, it does so along residual tracheal material (A, segment A3; B, segment A4 (second from left)). In most mutant individuals, a significant portion of the trachea develops in most segments and, in these cases, the ISN forms in a fairly normal way. In contrast to the lateral defects, this mutation does not disrupt ISN growth in the ventral region of the embryo (triangles). The cell bodies of most of the peripheral sensory neurons are not visible in these micrographs as they are in a more superficial plane of focus. For clarity, many of these have also been left out of the camera-lucida drawings. Anterior is to the left in all lateral views. A and A show 6 hemisegments; B and B show 5. (Total n>200 mutant hemisegments examined).

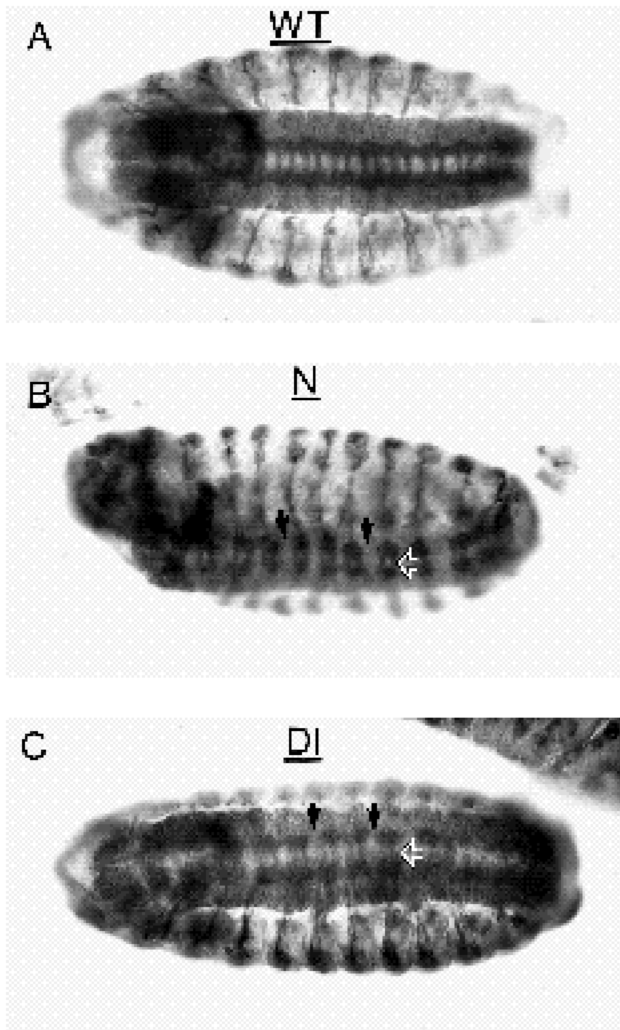

Fig. 4. CNS defects in *Notch* and *Delta* mutant embryos. Stage 16 embryos were stained with anti-HRP to label neurons, and developed with peroxidase histochemistry. (A) Wild type; (B) $Notch^{ts}$ and (C) $Delta^{ts}$, shifted to restrictive temperature between 3 and 6 hours of development, as for the experiments of Figs 1 and 2. In the mutants, longitudinal axon tracks failed to form (arrows) between the segmental ganglia, though other elements of the CNS axon scaffold formed normally (open arrowheads). Penetrance of the CNS axonal phenotype appears to be 100% for both mutations.

mutations prevented formation of the connectives (longitudinal tracks) between the segmental ganglia (arrows), while having far more modest effects upon the commissures (cross-tracks) and connectives within the ganglia (Fig. 4, open arrowheads). This phenotype is distinct from a variety of other classes of mutant phenotypes that have been described for the CNS (Mayer and Nusslein-Volhard, 1988; Rothberg et al., 1988; Thomas et al., 1988; Smouse et al., 1988). We therefore suggest that *Delta* and *Notch* are part of a regulatory system that determines a variety of individual choices in nerve guidance, including the trajectory of this element of the CNS and of the lateral third of the ISN.

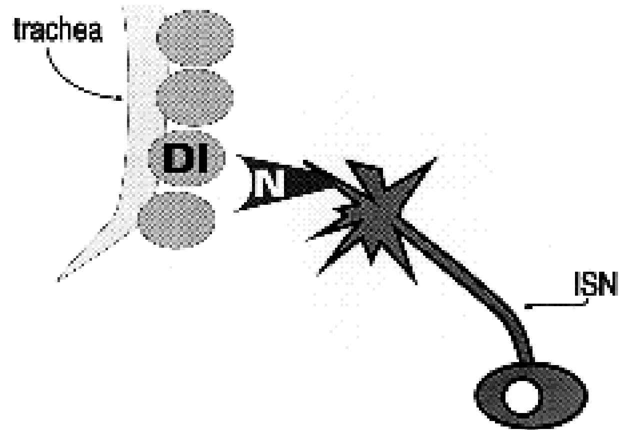

Fig. 5. Model for the specification of the trajectory of the ISN in the lateral region of the fly embryo. As detailed in the text, we suggest that expression of Delta protein on the transverse branch of the embryonic trachea provides a track to direct the growth of the ISN and that the axons of the ISN recognize this track by virtue of bearing the Delta receptor, Notch. In the figure, Delta is depicted by the light gray ovals on the surface of the trachea, while Notch is pictured as being on the axonal growth cone. Note, however, that we have examined only the distribution of Delta RNA, not of Delta protein, and, further that, while Notch has been detected on axons, its presence specifically on growth cones has not been examined. The trachea comprises the ectodermal cells of the tracheal epithelium as well as mesodermally derived peritracheal cells. For the purposes of this model, we do not distinguish between these cell types.

DISCUSSION

How is an axon guided to its synaptic targets? For one nerve, the intersegmental nerve (ISN) of the *Drosophila* embryo, the following model emerges from the experiments presented here (Fig. 5). Expression of Delta protein on the embryonic trachea defines a path for axons of the ISN and these axons use the Notch protein on their growth cones to recognize and follow that path. These conclusions follow, in part, from our observation that appropriate temperature shifts of $Notch^{ts}$ or $Delta^{ts}$ mutants prevent proper growth of the ISN through the lateral part of the embryo, and that this nerve growth is also blocked if tracheal morphogenesis is disrupted by mutation of the D-FGFR. The pattern of expression of these genes is also consistent with the proposed model, as *Delta* is expressed in the trachea (Vaessin et al., 1987), while its receptor, Notch, is on the axons of the intersegmental nerve (K. Johansen et al., 1989; Fehon et al., 1991). The regulatory system described here for guidance of the lateral part of the ISN evidently also specifies the trajectory of a section of each longitudinal nerve track in the CNS: mutations in *Delta* or *Notch* specifically disrupt the growth of CNS axons between the segmental ganglia.

One caveat to our interpretation of the $Notch^{ts}$ and $Delta^{ts}$ temperature-shift experiments is that we cannot formally rule out the possibility that some of the observed defects in axon guidance actually reflect transformations of neuronal identity, with consequent confusion of axonal rout-

ing. This is particularly a concern in the case of *Notch*, which is expressed in neurons throughout embryonic development. However, careful analysis of the embryonic PNS phenotypes of neurogenic mutations has provided no evidence for such changes in identity (Goriely et al., 1991). Furthermore, in the adult PNS, supernumerary neurons produced as a consequence of a *Notch* mutation are wild type in the pattern of their projections (Simpson and Carteret, 1990). Analogous detailed analyses of cell fate in the CNS of neurogenic mutants have not been reported, potentially complicating the interpretation of the mutant CNS phenotypes. For *Delta*, we consider it unlikely that we were observing axonal defects stemming from errors in neurogenesis, since late temperature shifts that did not produce the *Delta* neurogenic phenotype (neural hyperplasia) nonetheless resulted in defects in axon guidance (see, for example, Figs 1C, 2E) and those extra neurons that were produced appeared to be morphologically normal (Fig. 1C,D). Moreover, the level of Delta RNA in neurons is decaying rapidly at the stages of embryogenesis when the ISN is extending (Vaessin et al., 1987; Kopczynski and Muskavitch, 1989; E. G. and Y. N. J., data not shown).

Might other molecules contribute to guidance of the ISN by the trachea? While we have shown that *Delta* is necessary to define this nerve path, we have not tested whether it is sufficient. One candidate for a co-regulator is the gene *Serrate*, which encodes a protein that is similar in structure to Delta and is expressed in parts of the trachea (Fleming et al., 1990; Thomas et al., 1991). As is the case for *Delta*, *Serrate* interacts genetically with *Notch* and cells expressing Serrate protein bind to Notch-expressing cells (Rebay et al. 1991). The embryonic *Serrate* mutant phenotype (Fleming et al., 1990) is rather different from the *Delta* and *Notch* phenotypes described here, but additional experiments will be required to determine whether *Serrate* plays a role in ISN guidance. Two lines of evidence argue that additional guidance information is indeed apt to reside in or near the trachea. First, expression of Delta RNA appears fairly homogeneous throughout the transverse part of the trachea (though we emphasize that we have not been able to examine the distribution of Delta protein, only of Delta RNA). Nonetheless, motor axons grow only in the dorsal direction and sensory axons grow only ventrally. We have not identified an explicit marker of directionality. Furthermore, axons of the ISN remain associated with the trachea over only a short distance, about 30 microns for the case of the motor axons, yet Delta RNA is present throughout this branch of the trachea. We have not identified the signal that separates axons away from the trachea at the appropriate point. Finally, *Notch* is expressed in the trachea, albeit at lower levels than it is in the nervous system. Our results do not exclude the possibility that this expression, or expression of *Delta* or *Notch* in tissues that we have not examined, may contribute to ISN guidance.

Implications for mechanisms of axon guidance

Notch protein is expressed throughout the ISN, yet *Notch* and *Delta* are required for the formation of only a short section of its trajectory. This observation suggests that the trachea-based *Delta*- and *Notch*-dependent guidance system may define an independent domain of guidance information, separable from the regulatory systems that guide the ISN through more dorsal and more ventral parts of the embryo. If this idea is correct, it should be possible to identify other, genetically separable, guidance systems for other sections of the ISN. The notion that different steps in the path of a single axon may be guided by separate regulatory mechanisms is consistent with the specificity of mutant phenotypes observed in the CNS in our experiments, as well as with results from other systems (Bastiani et al., 1987; Furley et al., 1990; Hedgecock et al., 1990; Grenningloh et al., 1991; McIntire et al., 1992).

The role that we propose for *Delta* and *Notch* in specifying nerve trajectory raises the question of how, mechanistically, these proteins control axon growth. In particular, it is striking that a pair of proteins that are central to the determination of the fates of many cells can produce such a different effect when active in axons. The Delta-Notch interaction could, in principle, guide the ISN by a purely adhesive mechanism (Hammerback et al., 1988), but we prefer the idea that Notch activates a specific intracellular effector system in axons (Gertler et al., 1989; Schuh et al., 1989; Silver et al., 1990). For example, it seems plausible that the signal transduction cascade that alters cell phenotype in response to *Notch* action elsewhere in development, that is, the 'neurogenic gene cassette' (Ruohola et al., 1991), includes some of the molecules responsible for effecting *Notch*-dependent growth of the ISN along the trachea. By this view, the next critical question is to identify those molecules that impart to the Delta-Notch regulatory system the particular ability to promote directed axon extension in the current developmental context.

We are grateful to many members of our laboratory, especially Harald Vaessin and Michael Brand, for helpful discussions at all stages of these experiments. We particularly acknowledge Kirsten Bremer, Michael Brand, Hannele Ruohola and Harald Vaessin for much advice about the *Delta* and *Notch* experiments, Larry Ackerman for assistance with confocal microscopy and in preparing the figures, and Sandy Barbel for performing the in situ hybridizations. We thank Ralph Greenspan, Sandy Johnson, Tom Jongens, Elaine Ostrander, Marc Tessier-Lavigne, Andrew Tomlinson and Robin Wharton for reading drafts of the manuscript. E. G. is an Associate, and L. Y. J. and Y. N. J. are Investigators, of the Howard Hughes Medical Institute.

REFERENCES

Ashburner, M. (1989). Drosophila. *A Laboratory Handbook.* Cold Spring Harbor, New York: Cold Spring Harbor Press.

Bastiani, M. J., Harrelson, A. L., Snow, P. M. and Goodman, C. S. (1987). Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell* **48**, 745-755.

Bate, C. M. (1976). Pioneer neurons in an insect embryo. *Nature* **260**, 54-55.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y. and Jan, Y. N. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-*lacZ* vector. *Genes Dev.* **3**, 1273-87.

Bixby, J. L. and Harris, W. H. (1991). Molecular mechanisms of axon growth and guidance. *Ann. Rev. Cell Biol.* **7**, 117-159.

Bodmer, R. and Jan, Y. N. (1987). Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. *Roux's Arch. Dev. Biol.* **196**, 69-77.

Brand, M. and Campos-Ortega, J. A. (1990). Second-site modifiers of the *split* mutation of *Notch* define genes involved in neurogenesis in *Drosophilamelanogaster*. *Roux's Arch. Dev. Biol.* **198**, 264-274.

Cagan, R. L. and Ready, D. F. (1989). *Notch* is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3**, 1099-1112.

Campos-Ortega, J. and Hartenstein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. New York: Springer-Verlag.

Campos-Ortega, J. A. and Jan, Y. N. (1991). Genetic and molecular basis of neurogenesis in *Drosophila melanogaster*. *Ann. Rev. Neurosci.* **14**, 399-420.

de Celis, J. F., Mari-Beffa, M. and Garcia-Bellido, A. (1991). Cell autonomous role of the *Notch* gene, an epidermal growth factor homolog, in sensory organ differentiation in *Drosophila*. *Proc. Natl. Acad. Sci., USA* **88**, 632-636.

de la Concha, A., Dietrich, U., Weigel, D. and Campos-Ortega, J. A. (1988). Functional interactions of neurogenic genes of *Drosophila melanogaster*. *Genetics* **118**, 499-508.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamp, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R. and Edelman, G. E. (1987). Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation and alternative RNA splicing. *Science* **236**, 799-806.

Dodd, J. and Jessell, T. M. (1988). Axon guidance and the patterning of axonal Projections in Vertebrates. *Science* **242**, 8692-699.

Doe, C. Q. and Goodman, C. S. (1985). Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev. Biol.* **111**, 206-219.

Doe, C. Q., Smouse, D. and Goodman, C. S. (1988). Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **336**, 376-378.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L, Xu, T., Muskavich, M. A. T. and Artavanis-Tsakonis, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. *Cell* **61**, 523-534.

Fehon, R. G., Johansen, K., Rebay, I. and Artavanis-Tsakonis, S. (1991). Complex cellular and subcellular regulation of *Notch* expression during embryonic and imaginal development of *Drosophila*: Implications for *Notch* function. *J. Cell Biol.* **113**, 657-69.

Fleming, R. J., Scottgale, T. N., Diederich, R. J. and Artavanis-Tsakonis, S. (1990). The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophilamelanogaster*. *Genes Dev.* **4**, 2188-2201.

Furley, A. J., Morton, S. B., Manalo, D., Karagogeos, D., Dodd, J. and Jessell, T. M. (1990). The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite-promoting activity. *Cell* **61**, 157-170.

Gertler, F. B., Bennett, R. L., Clark, M. J. and Hoffmann, F. M. (1989). *Drosophila abl* tyrosine kinase in embryonic CNS axons: A role in axonogenesis is revealed through dosage-sensitive interactions with *disabled*. *Cell* **58**, 103-113.

Ghysen, A., Dambly-Chaudiere, C., Aceves, E., Jan, L. Y. and Jan, Y. N. (1986). Sensory neurons and peripheral pathways in *Drosophila* embryos. *Roux's Arch. Dev. Biol.* **195**, 281-289.

Ghysen, A., Janson, R. and Santamaria, P. (1983). Segmental determination of sensory neurons in *Drosophila. Dev. Biol.* **99**, 7-26.

Glazer, L. and Shilo, B.-Z. (1991). The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.* **5**, 697-705.

Goriely, A., Dumont, N., Dambly-Chaudiere, C. and Ghysen, A. (1991). The determination of sense organs in *Drosophila*: effect of the neurogenic mutations in the embryo. *Development* **113**, 1395-1404.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: Fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.

Hammerback, J. A., McCarthy, J. B., Palm, S. L., Furcht, L. T. and Letourneau, P. C. (1988). Growth cone guidance by substrate-bound laminin pathways is correlated with neuron-to-pathway adhesivity. *Dev. Biol.* **126**, 29-39.

Hartenstein, V. (1988). Development of *Drosophila* Larval Sensory Organs: Spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation. *Development* **102**, 869-86.

Hartenstein, V. and Posakony, J. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. *Dev. Biol.* **142**, 13-30.

Hedgecock, E. M., Culotti, J. G. and Hall, D. H. (1990). The *unc-5*, *unc-6* and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **4**, 61-85.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.

Hoppe, P. E. and Greenspan, R. J. (1986). Local function of the *Notch* gene for embryonic ectodermal pathway choice in *Drosophila*. *Cell* **46**, 773-783.

Hoppe, P. E. and Greenspan, R. J. (1990). The *Notch* locus of *Drosophila* is required in epidermal cells for epidermal development. *Development* **109**, 875-885.

Hynes, R. O. and Lander, A. D. (1992). Contact and adhesive specificities in the associations, migrations and targeting of cells and axons. *Cell* **68**, 303-322.

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

Johansen, J., Halpern, M. E. and Keshishian, H. (1989). Axonal guidance and the development of muscle fiber-specific innervation in *Drosophila* embryos. *J. Neurosci.* **9**, 4318-4332.

Johansen, K., Fehon, R. G. and Artavanis-Tsakonis, S. (1989). The *Notch* gene product is a glycoprotein expressed on the cell surface of both epidermal and neuronal precursor cells during *Drosophila* development. *J. Cell Biol.* **109**, 2427-2440.

Kidd, S., Kelley, M. R. and Young, M. W. (1988). Sequence of the *Notch* locus of *Drosophilamelanogaster*: Relationship of the encoded protein to mammalian clotting and growth factors. *Mol. Cell. Biol.* **6**, 3094-3108.

Klambt, C., Glazer, L. and Shilo, B.-Z. (1992). *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.

Kopczynski, C. C., Alton, A. K., Fechtel, K., Kooh, P. J. and Muskavitch, M. A. T. (1988). *Delta*, a *Drosophila* neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. *Genes Dev.* **2**, 1723-1735.

Kopczynski, C. C. and Muskavitch, M. A. T. (1989). Complex spatio-temporal accumulation of alternative transcripts from the neurogenic gene *Delta* during *Drosophila* embryogenesis. *Development* **107**, 623-636.

Lehmann, R., Jimenez, F., Dietrich, U. and Campos-Ortega, J. A. (1983). On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **192**, 62-74.

Lindsley, D. L. and Grell, E. H. (1968). *Genetic Variations in* Drosophila melanogaster. Washington, DC: Carnegie Institution of Washington, Publ. 627.

Mayer, U. and Nusslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* **2**, 1496-1511.

McIntire, S. L., Garriga, G., White, J., Jacobson, D. and Horvitz, H. R. (1992). Genes necessary for directed axonal elongation or fasciculation in *C. elegans. Neuron* **8**, 307-322.

Palka, J., Schubiger, M. and Schwaninger, H. (1990). Neurogenic and antineurogenic effects from modifications at the *Notch* locus. *Development* **109**, 167-175.

Ramon y Cajal, S. (1984). *The Neuron and the Glial Cell.* (de la Torre, J. and Gibson, W.C., transl. and ed.). Springfield, Illinois: Charles C. Thomas.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonis, S. (1991). Specific EGF-repeats of Notch mediate interactions with Delta and Serrate: Implications for Notch as a multifunctional receptor. *Cell* **67**, 687-699.

Rothberg, J. M., Hartley, D. A., Walther, Z. and Artavanis-Tsakonas, S. (1988). *slit*: An EGF-homologous locus of *Drosophila melanogaster* involved in the development of the embryonic central nervous system. *Cell* **55**, 1047-1059.

Ruohola, H., Bremer, K. A., Baker, D., Swedlow, J. R., Jan, L. Y. and Jan, Y. N. (1991). Role of neurogenic genes in sstablishment of follicle cell fate and oocyte polarity during oogenesis in *Drosophila. Cell* **66**, 433-50.

Schuh, U., Lohse, M. J. and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. *Neuron* **3**, 13-20.

Silver, R. A., Lamb, A. G. and Bolsover, S. R. (1990). Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones. *Nature* **343**, 751-754.

Simpson, P. and Carteret, C. (1990). Proneural clusters: equivalence groups in the epithelium of *Drosophila*. *Development* **110**, 927-932.

Sink, H. and Whitington, P. (1991). Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons. *Development* **112**, 307-316.

Smouse, D., Goodman, C. S., Mahowald, A. and Perrimon, N. (1988). *polyhomeotic*: A Gene required for the embryonic development of axon pathways in the central nervous system of *Drosophila*. *Genes Dev.* **2**, 830-842.

Stahl, B., Muller, B., von Boxberg, Y., Cox, E. C. and Bonhoeffer, F. (1990). Biochemical characterization of a putative axonal guidance molecule of the chick visual system. *Neuron* **5**, 735-743.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Technau, G. M. and Campos-Ortega, J. A. (1987). Cell autonomy of expression of neurogenic genes of *Drosophilamelanogaster*. *Proc. Natl. Acad. Sci., USA* **84**, 4500-4504.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J. and Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775-778.

Thomas, J., Crews, S. and Goodman, C. S. (1988). Molecular genetics of the *single-minded* locus: A gene involved in the development of the *Drosophila* nervous system. *Cell* **58**, 133-141.

Thomas, U., Speicher, S. A. and Knust, E. (1991). The *Drosophila* gene *Serrate* encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. *Development* **111**, 749-761.

Vaessin, H., Vielmetter, J. and Campos-Ortega, J. A. (1985). Genetic interactions in early neurogenesis of *Drosophila melanogaster*. *J. Neurogen.* **2**, 291-308.

Vaessin, H., Bremer, K. A., Knust, E. and Campos-Ortega, J. A. (1987). The Neurogenic locus *Delta* of *Drosophilamelanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. *EMBO J.* **6**, 3431-3440.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. and Jan, Y. N. (1991). *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell* **67**, 941-953.

Wharton, K. A., Johansen, K. M., Xu, T. and Artavanis-Tsakonis, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-581.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: Monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

(Accepted 16 October 1992)