

# Control of neuronal pathway selection by the *Drosophila* LIM homeodomain gene *apterous*

Scott E. Lundgren<sup>1</sup>, Christopher A. Callahan<sup>1,2</sup>, Stefan Thor<sup>1</sup> and John B. Thomas<sup>1,\*</sup>

<sup>1</sup>Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, PO Box 85800, San Diego, CA 92186, USA

<sup>2</sup>Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA

\*Author for correspondence

## SUMMARY

The *Drosophila apterous* gene encodes a LIM homeodomain protein expressed embryonically in a small subset of differentiating neurons. To establish the identity of these neurons and to study the role of *apterous* in their development, we made *apterous* promoter fusions to an axon-targeted reporter gene. We found that all *apterous*-expressing neurons are interneurons that choose a single pathway within the developing central nervous system. In *apterous* mutants, these neurons choose incorrect pathways and fail

to fasciculate with one another. Our results indicate that *apterous* functions to control neuronal pathway selection and suggest that other vertebrate and invertebrate members of the LIM homeodomain class of proteins may serve similar functions.

Key words: *Drosophila*, LIM domain, neuronal development, *apterous*, homeodomain

## INTRODUCTION

The LIM homeodomain genes encode a distinct family of proteins predicted to act as transcriptional regulators (Freyd et al., 1990; Karlsson et al., 1990). One distinguishing feature of this gene family is that all members appear to be expressed in discrete subsets of differentiating neurons (Barnes et al., 1994; Korzh et al., 1993; Li et al., 1994; Taira et al., 1993; Thor et al., 1991; Way and Chalfie, 1989; Xu et al., 1993), as well as in other tissues. The demonstrated role of the *Caenorhabditis elegans mec-3* gene in mechanosensory neuron function (Way and Chalfie, 1988), as well as the recent finding that combinations of vertebrate LIM homeodomain genes define distinct subclasses of motorneurons (Tsuchida et al., 1994), further suggest that this gene family might be involved in controlling neuronal differentiation and pathway selection. The *Drosophila* LIM homeodomain gene *apterous* (*ap*) is required for development of the wing and a subset of embryonic muscles (Blair et al., 1994; Bourgouin et al., 1992; Butterworth and King, 1965; Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993). In addition to being expressed in these tissues, *ap* is expressed in the embryonic brain and ventral nerve cord (VNC). In this paper, we show, using promoter fusions to a novel axon-targeting reporter (Callahan and Thomas, 1994), that *ap* is expressed by a small subset of developing interneurons that choose a single pathway and that, in *ap* mutants, these neurons choose incorrect paths. Our results provide evidence that a member of the LIM homeodomain gene family controls neuronal pathway selection.

## MATERIALS AND METHODS

Whole-mount in situ hybridizations were carried out essentially as described (Tautz and Pfeife, 1989) using a 3.7 kb *ap* cDNA (Bourgouin et al., 1992) as a template for an RNA probe. For the production of fusion protein, we constructed a modified pGEMEX (Promega) vector, pGEMEXΔSS, in which the 800 bp *SlyI-SfiI* fragment had been excised. A 1.6 kb *ApaI-HindIII* fragment from a 3.7 kb *ap* cDNA (Bourgouin et al., 1992) was cloned into pGEMEXΔSS restricted with *ApaI* and *HindIII*; fusion protein was gel purified and injected into rats. The specificity of the anti-Ap antibody was confirmed by lack of staining in embryos mutant for the transcriptional null *ap<sup>P44</sup>* allele. For antibody staining, embryos were dissected and processed as described (Callahan and Thomas, 1994; Thomas et al., 1984). Embryos were incubated overnight at 4°C with rat anti-Ap (diluted 1:200) and/or rabbit anti-β-gal (Cappel; diluted 1:10,000). Homozygous *ap* mutant embryos were independently identified by using a *CyO* balancer chromosome that contains a P element expressing β-gal from the *actin 5C* promoter (Bourgouin et al., 1992).

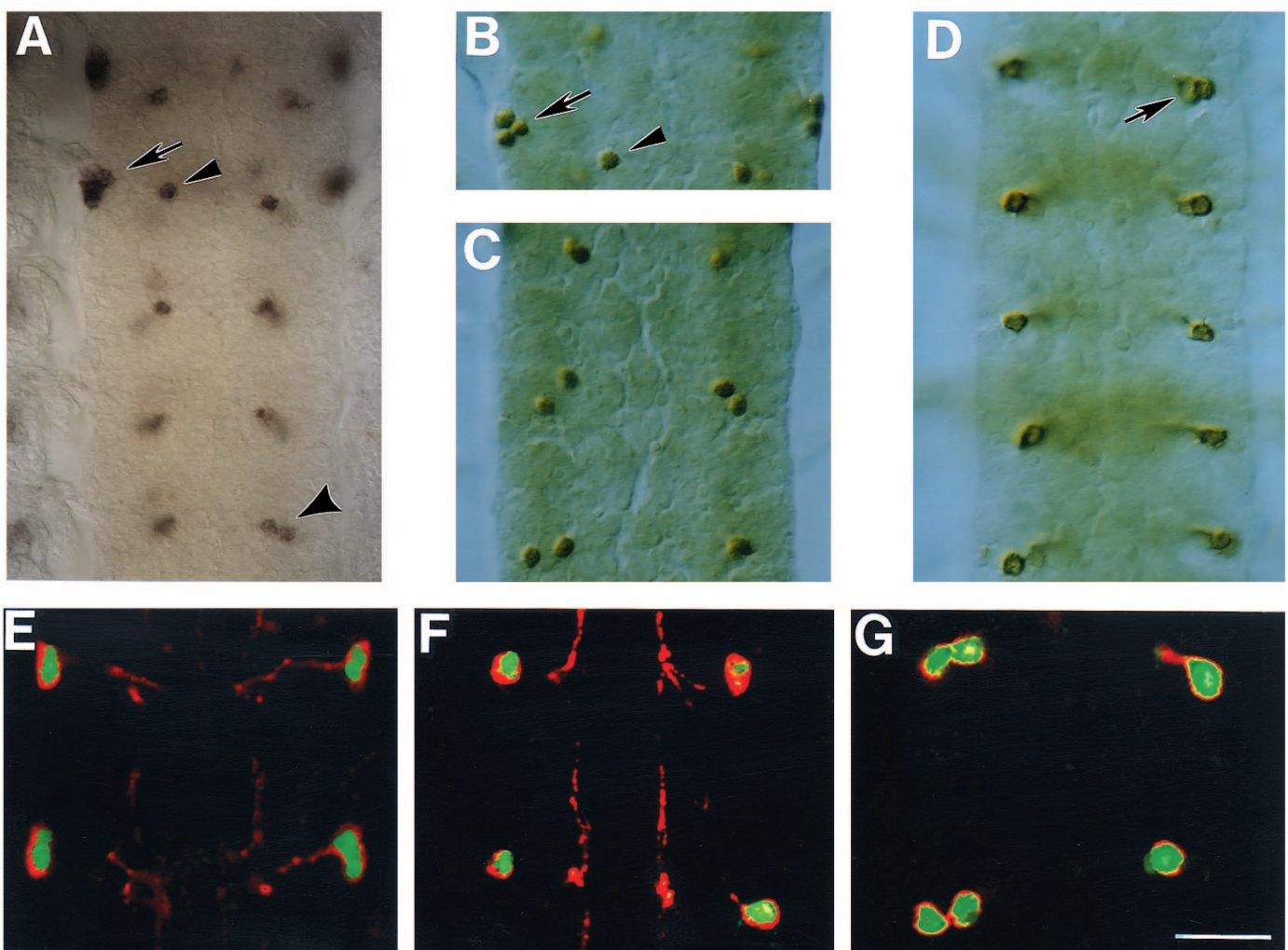
For testing promoter fragments, we constructed 2 *tau-lacZ* P element transformation vectors, PC4tLZ and PC4PtLZ, both based on PC4LZ (Wharton and Crews, 1993). PC4tLZ contains a polylinker directly upstream of the *tau-lacZ* fusion gene; PC4PtLZ is identical to PC4tLZ except that it contains P promoter sequences between the polylinker and the *tau-lacZ* fusion gene. Flies were transformed using standard techniques (Rubin and Spradling, 1982). Multiple independent insertions were isolated for each construct with the exception of apA, for which a single insertion was generated. Eleven independent transformants were isolated for the apC fragment. All showed the identical expression pattern, although some variation in the levels of expression was observed. For the experiments described in the text, we used a 3rd chromosome insert, *apC-tau-lacZ1.6*.

## RESULTS

In addition to the wing disc and an embryonic muscle subset, *ap* is expressed in subsets of cells within the embryonic brain and VNC. Because of its relative simplicity, we consider here only the VNC, which by 12 hours of embryonic development, contains approximately 200 neurons per hemisegment. In situ hybridization using an *ap* RNA probe reveals that, within the VNC, transcription of *ap* is restricted to only 3 cells (1 dorsal and 2 ventral) per hemisegment and to an additional cluster of 4 lateral cells in each thoracic hemisegment (Fig. 1A). This same pattern of expression is revealed with antibodies to Ap (Fig. 1B,C).

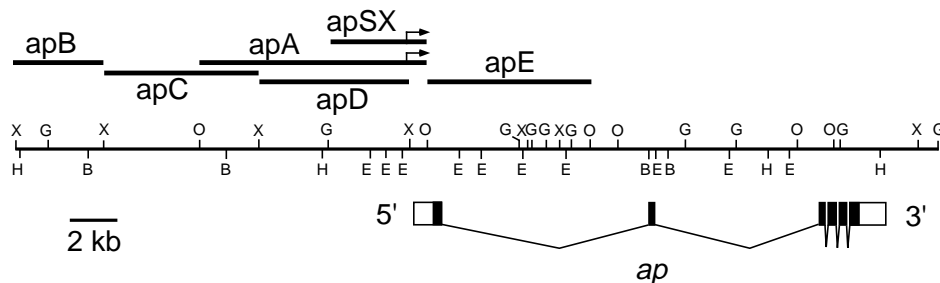
Since Ap is a nuclear protein, the anti-Ap antibody reveals only the cell body positions of the *ap*-expressing cells. Thus,

the identity of these cells (e.g., interneurons, motorneurons, glia) and any possible interactions among them cannot directly be determined. We therefore generated transformants carrying regulatory regions of the *ap* gene fused to *tau-lacZ*, a reporter gene whose product, tau- $\beta$ -galactosidase (tau- $\beta$ -gal), labels the entirety of neurons, including cell bodies and axonal projections (Callahan and Thomas, 1994). Genomic fragments covering 25 kb were tested for neuronal expression (Fig. 2). Fragment apC, which contains sequences from -6 to -12 kb upstream from the *ap* transcriptional start site, was found to direct tau- $\beta$ -gal expression in a pattern similar to *ap* itself (Fig. 1D). To confirm that the tau- $\beta$ -gal-expressing cells are indeed the *ap* cells, we carried out double-labeling with antibodies to both  $\beta$ -gal and Ap. As shown in Fig. 1E-G, the expression of Ap and tau- $\beta$ -gal is coincident, although we found that in some



**Fig. 1.** *ap* expression in the embryonic nervous system. (A) Whole-mount in situ hybridization of an *ap* RNA probe to a 12 hour embryo. Expression is detected in 1 dorsal cell per hemisegment (small arrowhead points to a dorsal cell in thoracic segment T3), a pair of ventral cells (large arrowhead points to ventral cells in abdominal segment A3), and a cluster of 4 lateral neurons in each thoracic hemisegment (arrow points to a cluster in T3). (B,C) HRP immunostaining with antibodies to Ap. The nuclei of one dorsal neuron (arrowhead), 4 clustered lateral thoracic neurons (arrow) and 2 ventral neurons (C) per hemisegment are stained. In three of the hemisegments shown in C, both of the ventral pair of neurons are in the same focal plane. (D) HRP immunostaining of an *apC-tau-lacZ* embryo with antibodies to  $\beta$ -gal shows the same pattern as revealed by anti-Ap antibodies. In some segments, tau- $\beta$ -gal expression is reduced in one of the two ventral neurons (arrow). (E-G) Confocal images of double fluorescence labeling of an *apC-tau-lacZ* embryo with antibodies to Ap (green nuclear signal) and to  $\beta$ -gal (red cytoplasmic signal). In the thoracic-specific neurons (E), the dorsal neurons (F) and in the ventral neurons (G), staining is coincident. For the ventral neurons on the right side in G, only a single cell body of the pair are in the focal plane. Scale bar is 25  $\mu$ m for (A-D), 15  $\mu$ m for (E-G).

**Fig. 2.** Structure of the *ap* gene. The *ap* gene contains 6 exons distributed over 25 kb. Non-coding regions are indicated by white boxes, coding regions by black boxes. The exon-intron structure was determined by Southern blot analysis and sequencing of genomic DNA. Above the restriction map are the genomic fragments tested for neuronal expression in *tau-lacZ*-based vectors. The 3' end of fragments apA and apSX is an *XhoI* site located within the 5' untranslated region of *ap* cDNAs (Bourgouin et al., 1992); arrows denote the putative transcriptional start site as determined by primer extension analysis. Fragments apA, apSX, apD and apE gave no reproducible expression. Fragment apB directed expression in a set of glial cells that do not express *ap*, and thus possibly contains an enhancer element for an adjacent gene. Fragment apC directs expression in the *ap* neurons and in the wing disc. B, *Bam*HI; E, *Eco*RI; G, *Bgl*III; H, *Hpa*I; O, *Xho*I; X, *Xba*I.



segments the levels of tau- $\beta$ -gal could vary between the 2 ventral cells, whereas the Ap protein levels usually appeared equivalent.

Analysis of *apC-tau-lacZ* embryos showed that the *ap* cells are interneurons that begin to elongate axons soon after they begin expressing Ap protein. Remarkably, the growth cones of all the *ap*-expressing interneurons choose a single pathway within the VNC. At hour 11 of embryogenesis, when many neurons are establishing a complex framework of discrete axon bundles (Thomas et al., 1984), the dorsal and ventral *ap* neurons have begun extending axons anteriorly and medially within the ipsilateral longitudinal connective (Fig. 3A). By hour 11.5, they have reached the adjacent anterior segments where they tightly fasciculate with their homologues, forming a discrete medial axon bundle running the length of the VNC in each connective (Fig. 3B). The 4 lateral thoracic *ap* neurons begin differentiating slightly later, but eventually they also fasciculate with the *ap* bundle (Fig. 3C).

At a gross level of analysis using antibodies that label all neurons (Jan and Jan, 1982), the overall structure of the nervous system in *ap* mutants is indistinguishable from wild type (data not shown). To visualize the effects of removing *ap* function on the differentiation of the *ap*-expressing neurons, we crossed the *apC-tau-lacZ* transgene into an *ap<sup>P44</sup>* null mutant background. In addition to *ap<sup>P44</sup>* homozygous individuals, we also examined embryos homozygous for *ap<sup>UGO35</sup>*, an independently isolated *ap* null allele (Cohen et al., 1992), as well as *ap<sup>P44</sup>/ap<sup>UGO35</sup>* embryos; all gave similar results. As assayed by the expression of tau- $\beta$ -gal, the *ap* neurons in *ap* mutant individuals are present, their cell bodies appear to be located in their normal positions, and axon elongation is initiated at the same time as in wild-type. However, the *ap* neurons project their axons along abnormal pathways and fail to fasciculate with one other (Fig. 3D,E). These pathfinding and defasciculation defects were detected in all of the *ap* mutant embryos examined (20/20), and in 90% (171/191) of the segments examined. In contrast, *ap<sup>+</sup>/apC-tau-lacZ* control embryos showed less than 1% (2/212) of segments with pathfinding or defasciculation defects of the *ap* neurons. Surprisingly, despite these striking pathfinding defects, some aspects of the behavior of the *ap* neurons appear to be relatively normal in *ap* mutants. For example, in virtually all segments examined (188/191), the *ap* neurons still project anteriorly within the connectives as they normally would in wild type, suggesting that a neuron's choice to project anteri-

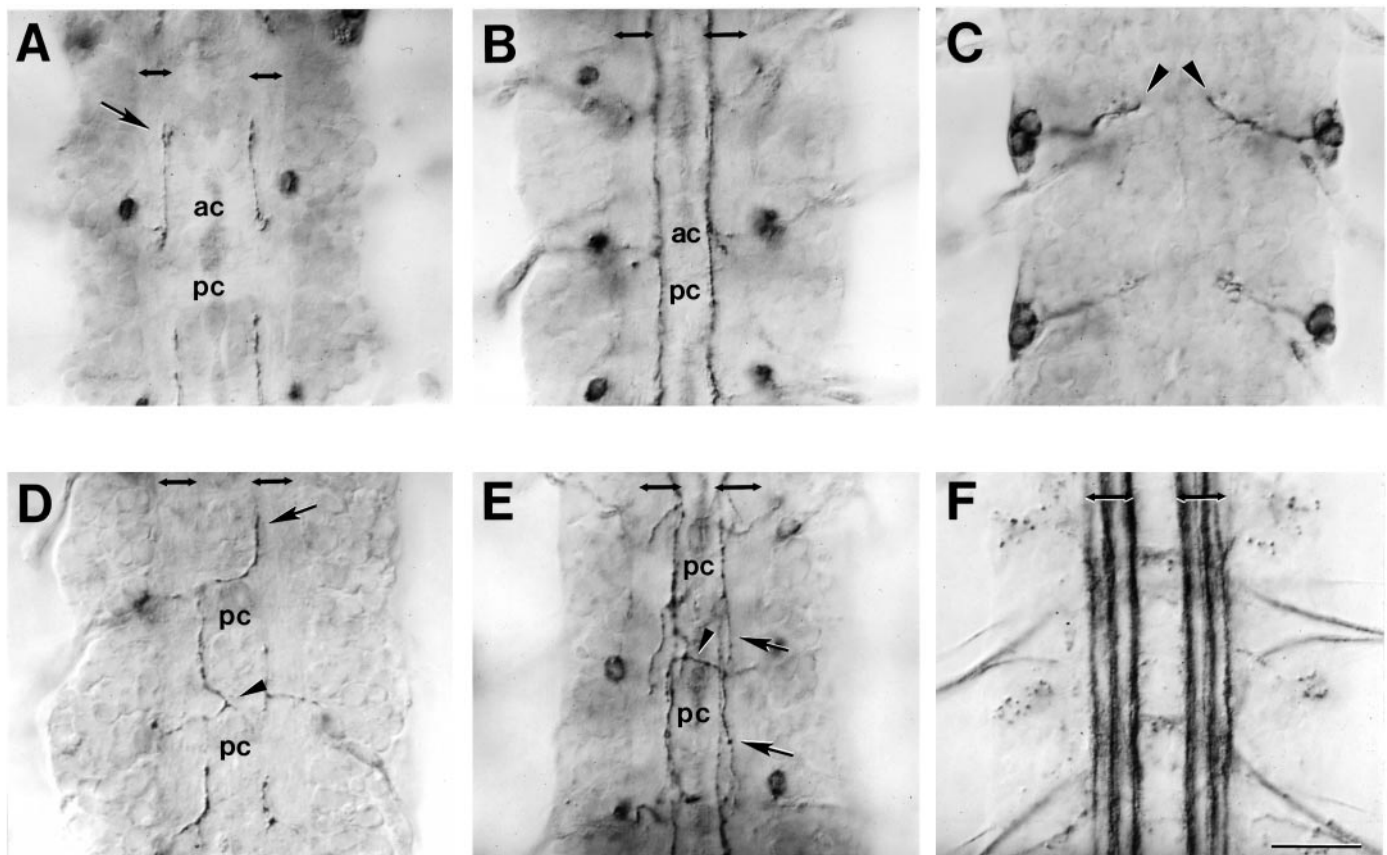
orly versus posteriorly is mediated by a mechanism separate from that underlying its choice of a specific pathway. Similarly, although in *ap* mutant individuals the *ap* neurons occasionally were found to project very laterally, in most hemisegments they projected within the medial half of the connective, suggesting that *ap*-independent mechanisms may determine the general mediolateral region of the connectives within which to project.

In contrast to the *ap* axon bundle, other axon fascicles are unaltered in *ap* mutants. Mutant embryos stained with monoclonal antibodies 7G10 (anti-Fasciclin III), 1D4 (anti-Fasciclin II) and 22C10, each of which labels subsets of axon bundles distinct from the *ap* fascicle (Patel et al., 1987; Van Vactor et al., 1993; Zipursky et al., 1984), are indistinguishable from wild type (Fasciclin II staining is shown in Fig. 3F). Thus, *ap* defects appear to be specific to the *ap* fascicle.

## DISCUSSION

Our results demonstrate that *ap* function is essential for the *ap* neurons to make their proper pathway choices and selectively fasciculate with one another. Although the molecular basis of pathway selection is poorly understood, it is generally thought to be mediated by some system of neuronal recognition molecules (Goodman and Shatz, 1993; Grenningloh et al., 1991; Lin et al., 1994). Since Ap likely acts as a transcriptional regulator and its expression commences only in postmitotic neurons, in contrast to the *Drosophila even-skipped* and *fushi tarazu* gene products which control neuronal identity but are also expressed within neural lineages (Doe et al., 1988a,b), we believe that Ap functions to regulate directly the expression of cell surface molecules mediating the specific recognition events leading to the formation of the *ap* fascicle. Given the conservation of protein structure and function between species, our results suggest a possible role in controlling neuronal pathway selection for the putative vertebrate homologue of *ap*, *LH-2* (Xu et al., 1993), and perhaps for other members of the LIM homeodomain class of proteins as well.

Our results also suggest that *ap* may be serving similar functions in both the wing disc and the CNS by regulating the identities of cells in terms of their cell surface properties. During wing development, *ap* is required for cells of the dorsal compartment to assume a dorsal identity: *ap* mutant clones within the dorsal compartment take on a ventral identity and



**Fig. 3.** Pathfinding defects in *ap* mutants. Anti- $\beta$ -gal HRP immunostaining of wild-type (A-C) and *ap*<sup>P44</sup> homozygous (D-F) embryos carrying the *apC-tau-lacZ* transgene. The double-headed arrows delineate the mediolateral extent of the connectives; the levels of the anterior and posterior commissures are marked by ac and pc respectively. (A) An 11 hour *apC-tau-lacZ* embryo. The dorsal and ventral *ap*-expressing neurons have fasciculated together and have elongated axons anteriorly about half the distance through each segment. The axons project medially within the ipsilateral connective. The dorsal neuron cell bodies are in approximately the same focal plane as their axons in the connectives, but their initial axonal segments are ventral and out of the focal plane. Large arrow points to the growth cone of the dorsal neuron of the left hemisegment. (B) A 12.5 hour *apC-tau-lacZ* embryo. The *ap* neurons all tightly fasciculate with their homologues in the adjacent anterior segments, defining a single bundle of axons within the longitudinal connectives. (C) A 12.5 hour *apC-tau-lacZ* embryo. The lateral thoracic *ap* neurons develop slightly later than the 3 medial *ap* neurons, extending growth cones (arrowheads) dorsomedially toward the *ap* bundle (out of the focal plane) with which they will fasciculate. (D) An 11 hour *ap*<sup>P44</sup>; *apC-tau-lacZ* embryo. A dorsal *ap* neuron has extended an axon across the midline (arrow) in the anterior commissure; bifurcates (arrowhead), sending one process out of the focal plane and the other back across the midline. The projections of both neurons are also abnormally dorsal within the VNC and thus the cell bodies are not in the focal plane. (E) A 12.5 hour *ap*<sup>P44</sup>; *apC-tau-lacZ* embryo. The *ap* neurons fail to form the *ap* axon bundle. Axons from dorsal and ventral neurons project abnormally within the connectives and fail to fasciculate with one another (arrows). Arrowhead points to an axon projecting abnormally across the midline in the anterior commissure (F) A 12.5 hour *ap*<sup>P44</sup>; *apC-tau-lacZ* embryo stained with a monoclonal antibody against Fasciclin II (Grenningloh et al., 1991; Van Vactor et al., 1993). In contrast to the *ap* axon bundle, the FasII axon bundles appear indistinguishable from wild-type. Scale bar is 20  $\mu$ m.

are able to cross the dorsal-ventral boundary (Blair et al., 1994; Diaz-Benjumea and Cohen, 1993). Normally, cells in either compartment never cross the boundary, and this behavior is thought to be due at least in part to differences in the surface properties between cells of the two compartments.

Finally, an obvious question pertaining to the *ap* phenotype is whether the *ap* neurons manage to synapse with their normal targets. Although we do not know the normal synaptic targets of these neurons, many of the more extreme pathfinding defects that we observed, such as crossing the midline and extending in a very lateral position within the connective, would most likely compromise the ability of at least some of the *ap* neurons to reach their appropriate target areas. In this

regard, it is noteworthy that adult flies homozygous for *ap* null alleles, although viable and wingless, are also highly uncoordinated and have reduced levels of juvenile hormone (Altart et al., 1991), suggestive of a defect in nervous system structure and function.

We thank T. Jessell for sharing results prior to publication, G. Lemke for critical review of the manuscript, D. Peterson and F. Gage for use of and help with the confocal microscope. This work was supported by EMBO and HFSP Fellowships to S.T., an NIH/NIGMS Training Grant to C. A. C., who is a student in the Medical Scientist Training Program at UC San Diego, and grants from the NIH, a March of Dimes Basil O'Connor Scholar Research Award and a Pew Scholars Award from the Pew Memorial Trusts to J. B. T.

## REFERENCES

- Altartz, M., Applebaum, S. W., Richard, D. S., Gilbert, L. I. and Segal, D. (1991). Regulation of juvenile hormone synthesis in wild-type and *ap* mutant *Drosophila*. *Mol. Cell. Endocrinol.* **81**, 205-216.
- Barnes, J. D., Crosby, J. L., Jones, C. M., Wright, C. V. and Hogan, B. L. (1994). Embryonic expression of *Lim-1*, the mouse homolog of *Xenopus Xlim-1*, suggests a role in lateral mesoderm differentiation and neurogenesis. *Dev. Biol.* **161**, 168-78.
- Blair, S. S., Brower, D. L., Thomas, J. B. and Zavortink, M. (1994). The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development* **120**, 1805-1815.
- Bourgouin, C., Lundgren, S. E. and Thomas, J. B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**, 549-561.
- Butterworth, F. M. and King, R. C. (1965). The developmental genetics of *apterous* mutants of *Drosophila melanogaster*. *Genetics* **52**, 1153-1174.
- Callahan, C. A. and Thomas, J. B. (1994). Tau-B-galactosidase, an axon-targeted fusion protein. *Proc. Natl. Acad. Sci. USA* **91**, 5972-5976.
- Cohen, B., McGuffin, M. E., Pfeifle, C., Segal, D. and Cohen, S. M. (1992). *apterous*, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**, 715-729.
- Diaz-Benjumea, F. J. and Cohen, S. M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell* **75**, 741-752.
- Doe, C. Q., Hiromi, Y., Gehring, W. J. and Goodman, C. S. (1988a). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science* **239**, 170-175.
- Doe, C. Q., Smouse, D. and Goodman, C. S. (1988b). Control of neural fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376-378.
- Freyd, G., Kim, S. K. and Horvitz, H. R. (1990). Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. *Nature* **344**, 876-879.
- Goodman, C. S. and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* **72**, 77-98.
- 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.
- 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.
- Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990). Insulin gene binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* **344**, 879-882.
- Korz, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118**, 417-425.
- Li, H. W., Witte, D. P., Branford, W. W., Aronow, B. J., Weinstein, M., Kaur, S., Wert, S., Singh, G., Schreiner, C. M., Whitsett, J. A., Scott, W. J. and Potter, S. S. (1994). Gsh-4 encodes a LIM-type homeodomain, is expressed in the developing central nervous system and is required for early postnatal survival. *EMBO J* **13**, 2876-2885.
- Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S. (1994). Genetic analysis of Fasciclin II in *Drosophila*: Defasciculation, refasciculation and altered fasciculation. *Neuron* **13**, 1055-1069.
- Patel, N. H., Snow, P. M. and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: A glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975-988.
- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- Taira, M., Haynes, W. P., Otani, H. and Dawid, I. B. (1993). Expression of LIM class homeobox gene *Xlim-3* in *Xenopus* development is limited to neural and neuroendocrine tissues. *Dev. Biol.* **159**, 245-256.
- 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.
- Thomas, J. B., Bastiani, M. J., Bate, C. M. and Goodman, C. S. (1984). From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* **310**, 203-207.
- Thor, S., Ericson, J., Brannstrom, T. and Edlund, T. (1991). The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. *Neuron* **7**, 881-889.
- Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
- Van Vactor, D., Sink, H., Fambrough, D., Tsou, R. and Goodman, C. S. (1993). Genes that control neuromuscular specificity in *Drosophila*. *Cell* **73**, 1137-1153.
- Way, J. C. and Chalfie, M. (1988). *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* **54**, 5-16.
- Way, J. C. and Chalfie, M. (1989). The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. *Genes Dev.* **3**, 1823-1833.
- Wharton, K. A., Jr. and Crews, S. T. (1993). CNS midline enhancers of the *Drosophila slit* and *Toll* genes. *Mech. Dev.* **40**, 141-154.
- Xu, Y., Baldassare, M., Fisher, P., Rathbun, G., Oltz, E. M., Yancopoulos, G. D., Jessel, T. M. and Alt, F. W. (1993). *LH-2*: A LIM/homeodomain gene expressed in developing lymphocytes and neural cells. *Proc. Natl. Acad. Sci. USA* **90**, 227-231.
- Zipursky, S., Venkatesh, T., Teplow, D. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

(Accepted 13 March 1995)