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Expression of the *disconnected* gene during development of *Drosophila melanogaster*

Kevin J.Lee, Matthew Freeman[1] and Hermann Steller

Howard Hughes Medical Institute, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 and [1]Department of Molecular and Cell Biology and Howard Hughes Medical Institute, 539 Life Sciences Addition, University of California, Berkeley, CA 94720, USA

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Proper development of the larval visual nerve, Bolwig's nerve, of *Drosophila melanogaster* requires the wild type function of the *disconnected (disco)* gene. In *disco* mutants, the nerve does not make stable connections with its targets in the larval brain. We have begun to explore the role of *disco* in the formation of the nervous system by examining the distribution of *disco* mRNA and protein in embryos and third instar larvae using *in situ* hybridization and antibody staining respectively. No differences between the distribution patterns of the two products are detected; *disco* is expressed in many tissues including both neural and non-neural cells. Many of the cells which express *disco* undergo extensive movement during development as they participate in major morphogenetic movements. Antibody staining shows that the protein is found in the cell nucleus. Products of the *disco* gene are detected in cells near the terminus of the growing Bolwig's nerve. In embryos homozygous for either of two mutant alleles of *disco*, the *disco* protein is absent near the nerve terminus, although protein distribution elsewhere is indistinguishable from wild type.

Key words: Drosophila melanogaster/neural development/neuronal connectivity/visual system

Introduction

Development of the nervous system poses a unique problem of pattern formation for the ontogeny of higher animals. Many cells in the nervous system extend axonal processes which traverse large distances following defined paths to form connections with other selected cells. Understanding the molecular mechanisms which play a role in the development of the nervous system is a goal which is being approached through the combined use of different methods. One such method is the application of classical genetic techniques to isolate genes which act to direct development of the nervous system. The development of the larval visual system of *Drosophila* is a simple model well suited to a study using this approach.

Mutations in the *disconnected* (*disco*) gene result in striking and relatively specific defects in neural cell patterning in the *Drosophila* visual system. Embryos homozygous for mutant alleles of this gene show defects in the projection of the larval visual nerve, Bolwig's nerve (Steller *et al.*, 1987). In these mutant embryos, Bolwig's nerve consistently fails to form stable connections with its synaptic partners and, in most cases, loses contact with the larval brain. Subsequent innervation of the optic lobes by the adult photoreceptors is also absent in the majority of *disco* mutants, due to an apparent pioneering function of Bolwig's nerve. In addition, the optic lobes of these mutants are extremely reduced in size. Since the development of the optic lobes is dependent on input from the developing eye (see, for example, Meinertzhagen, 1973; Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984), the phenotype of the optic lobes in *disco* mutants can be partly explained by the lack of retinal innervation. However, the degree of disruption in *disco* mutant optic lobes is more severe than that observed when eye innervation alone is absent (Steller *et al.*, 1987; Fischbach *et al.*, 1989). The difference could result from an organizing activity of Bolwig's nerve or from an autonomous requirement for *disco* in the optic lobes.

Mutations in the *disco* gene also give rise to defects in axonal patterning throughout the larval peripheral nervous system (PNS) (Steller *et al.*, 1987). These defects, although significant, are much more variable than the conspicuous abnormalities observed in the visual system of *disco* mutant larvae and adults.

We have begun a characterization of the *disco* gene product in order to explore its role in the development of the *Drosophila* nervous system. In the accompanying paper (Heilig *et al.*, 1991), the cloning of the *disco* gene and its sequence are presented. Here we describe the spatial and temporal pattern of expression of the *disco* transcript and protein as revealed by *in situ* hybridization and immunohistochemistry. The *disco* protein and mRNA are widely distributed; the gene products are detected in both neural and non-neural tissues. Immunohistochemistry shows that the protein is localized to the cell nucleus. Interestingly, many of the cells which express *disco* participate in morphogenetic movements such as dorsal closure, head involution and formation of the gut. The *disco* protein is expressed in a subset of cells in the central nervous system (CNS) and the peripheral nervous system (PNS) and in at least a portion of the primordium of the adult optic lobes, near the terminus of Bolwig's nerve. We observe that the expression of the protein in the region of the optic lobe primordium is largely absent in *disco* mutant embryos. These data are consistent with the proposal that the wild type *disco* gene product is required in cells near the terminus of Bolwig's nerve to direct the proper formation of the larval visual pathway.

Results

Expression of disco mRNA during embryonic development

Previous analysis (Steller *et al.*, 1987) showed that nervous system defects resulting from *disco* mutations can be detected after 10-12 h of embryonic development. We therefore

expected to find some level of *disco* gene expression in the embryo. This prediction was confirmed by Northern blotting experiments (Heilig *et al.*, 1991) which demonstrated that *disco* transcripts are present throughout much of embryogenesis. Transcripts of the same size are also found in larvae and, at somewhat lower levels, in adult heads and bodies. As phenotypic analysis suggested that the embryonic defects in Bolwig's nerve are responsible for later defects in the adult visual system, we initially focused on the embryonic expression. We sought to determine the spatial and temporal pattern of *disco* transcript accumulation by examining a collection of progressively older embryos hybridized with DNA probes from the *disco* gene.

This analysis was initiated using radioactively labeled DNA probes to detect mRNA in paraffin sections (data not shown). A complex and dynamic expression pattern was detected; spatially restricted expression was apparent in many tissues. Subsequently a whole-mount *in situ* hybridization technique using non-radioactive probes (Tautz and Pfeifle, 1989) was adopted. The results obtained with both techniques are virtually identical; some domains of expression are more clearly detected with the non-radioactive probes due to the greater resolution of this technique. Evidence that the signals detected result from products of the *disco* gene is provided by hybridization experiments using the deletion mutation Df(1)l9 (Steller *et al.*, 1987). This deficiency lacks a portion of the X chromosome containing the *disco* gene, and as expected, embryos hemizygous for the deletion show no staining (data not shown).

The results of whole-mount *in situ* hybridization to wild type embryos are shown in Figure 1. Expression of the *disco* transcript is first seen as a cap over the posterior pole of cellular blastoderm embryos (Figure 1A). Soon thereafter staining develops in the anterior region as well. After head segmentation becomes evident, staining is detected in the gnathal buds, the clypeolabrum, the antennal segment and a posterior region of the procephalic lobe. Additional staining is observed in the ectoderm of the thoracic segments and in the visceral mesoderm.

Many of the stained cells, especially those in the head region and the visceral mesoderm, participate in extensive morphogenetic movements. These movements are illustrated by Figure 1D–F. During head involution the gnathal segments move forward and into the anterior orifice, the atrium. At the same time, the staining cells in the posterior region of the procephalic lobe invaginate as a whole and move to join the brain lobe. It seems likely that this group includes the primordium of the optic lobe as described by Poulson (1950); see also Campos-Ortgega and Hartenstein (1985) and Hartenstein *et al.* (1985). Meanwhile, the cells of the visceral mesoderm elongate and contact the midgut primordia. They spread out, eventually flattening to form a sheet around the gut.

After retraction of the germ band, staining is detected in many parts of the nervous system (Figure 1F). Groups of stained cells are seen in a regular array along the ventral nerve cord. There are a large number of these cells, ~ 30 in each segment. The pattern in each segment is invariant, with two pairs of cells located in a dorsal position on either side of the midline and two clusters of ~ 12 cells located more ventrally and laterally. In addition, there are many staining cells scattered throughout the brain lobes. A mass of stained cells is seen in the ventral portion of the brain lobes; this group includes the primordium of the optic lobes (see also above).

The pattern of staining remains largely constant after dorsal closure is complete. From that point until formation of the cuticle, which prevents further hybridization experiments, staining is detected in the atrium, surrounding the gut and in cells scattered throughout the CNS.

Production of antisera specific for disco protein

We produced *disco* polypeptide fusion proteins in *Escherichia coli* and used these bacterial products to generate *disco*-specific polyclonal antisera. A number of different expression systems were used to synthesize fusion proteins containing a 183 amino acid fragment from the N-terminal region of *disco*. This fragment includes a sequence with similarity to the 'zinc finger' motif consensus (Heilig *et al.*, 1991). By first immunizing rabbits with one fusion protein, boosting with a second fusion protein and affinity purifying the antibody preparations with a third, we produced antisera which are specific for the *disco* portion of the fusion proteins.

We tested the specificity of the antisera for the *disco* gene product by immunohistochemical staining of embryos hemizygous for Df(1)l9 which deletes the *disco* gene (see above). Most of the batches of antiserum we used showed a complete absence of staining in embryos which lack the *disco* gene. One preparation did show some slight staining in the deficiency mutant embryos (Figure 2J). In this case staining was seen in the lumen of the salivary gland and in all experiments using this antiserum, this signal was regarded as cross-reactivity with antigens not related to the *disco* gene.

Spatial and temporal expression of disco protein in wild type embryos

Expression of the *disco* protein was detected in wild type embryos of the Berlin strain using antisera drawn from three rabbits. Sera from all three rabbits gave qualitatively identical staining patterns with the exception of variable non-specific staining in salivary glands described above and shown in Figure 2J. Incubation of samples with the secondary antibody alone gave no staining (data not shown).

Results of whole-mount immunohistochemistry experiments with wild type embryos are shown in Figure 2. The pattern of *disco* protein distribution as detected by the antibodies is very similar if not identical to the mRNA distribution pattern revealed by *in situ* hybridization (compare Figures 1 and 2). The *disco* protein, like the mRNA, is first detected at the posterior pole of cellular blastoderm embryos. Later it is found in the gnathal segments and the antennal segment, in clusters in the thoracic segments, in the visceral mesoderm, in the central nervous system and in the region of the optic lobe primordium.

As can be seen in Figure 2, the *disco* protein is generally localized to the nucleus. In some tissues where nuclei occupy most of the cellular volume it is difficult to rule out cytoplasmic staining as well. In cells where a distinction can be made, such as in the nervous system and the visceral mesoderm, the staining is strictly nuclear.

Some structures not stained by *in situ* hybridization are recognized by *disco*-specific antisera. As these structures are not stained in *disco*-deficiency embryos and are recognized by the sera of all three rabbits we believe the staining results from antibody binding to the *disco* gene product in these tissues. The differences between the two staining results are

probably due to lack of adequate resolution with the *in situ* hybridization technique. At the dorsal margin of the lateral epidermis there are two longitudinal rows of conspicuously stained cells (Figure 2E and F). During the period of dorsal closure they move dorsally, eventually meeting at the midline (Figure 2G,H). These cells are the cardioblasts (Poulson, 1950; Campos-Ortega and Hartenstein, 1985), mesodermal derivatives which give rise to the dorsal vessel. In the lateral ectoderm there are also scattered stained cells arranged in a segmental array (Figure 2E,G). Double labeling experiments (see below) show that some of these cells are closely apposed to parts of the PNS. In a few cases PNS-specific antibodies stain some of these *disco*-containing cells (data not shown). Therefore, a subset of the stained cells may be neurons or support cells of the PNS, but due to technical limitations of the double labeling technique

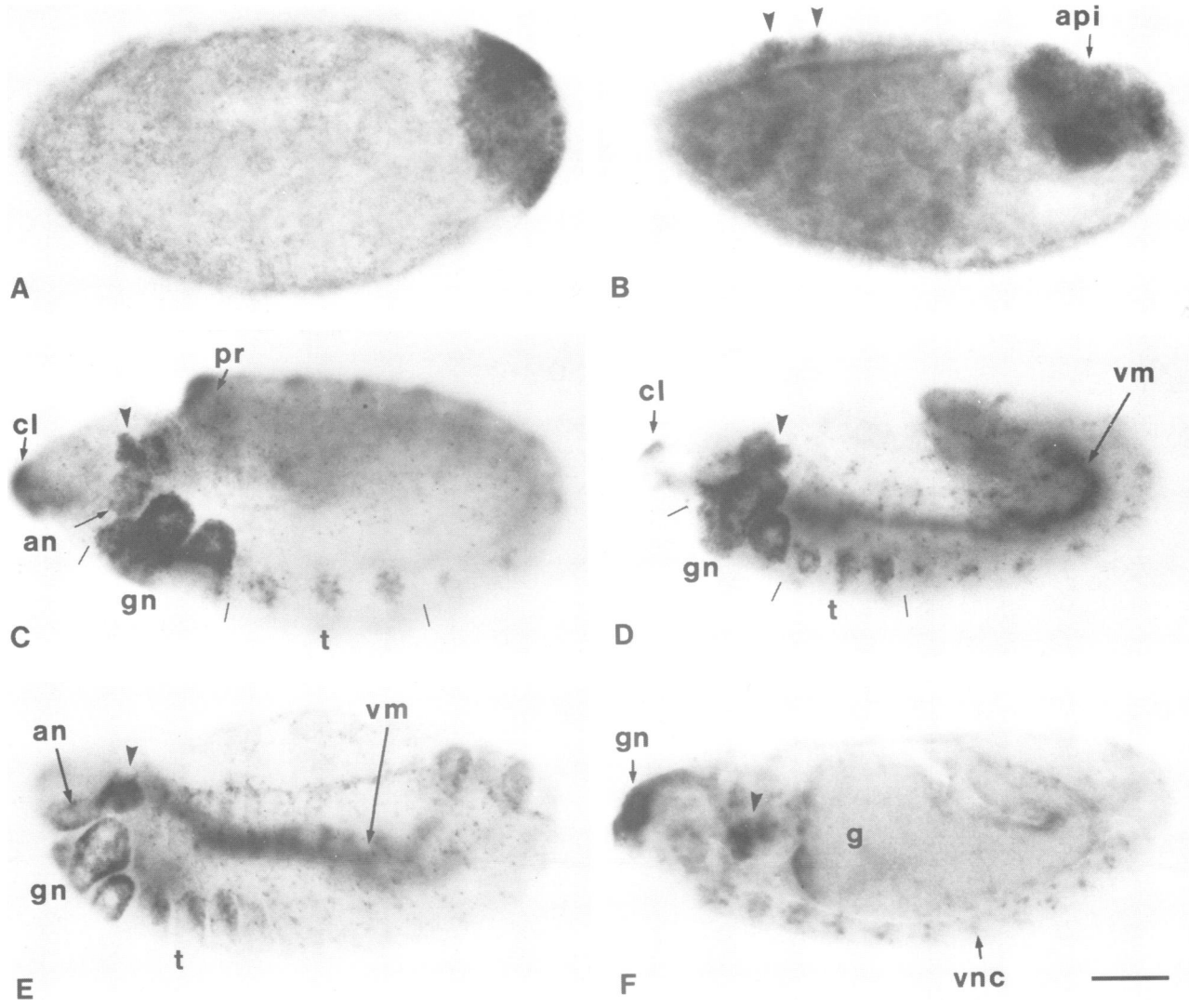
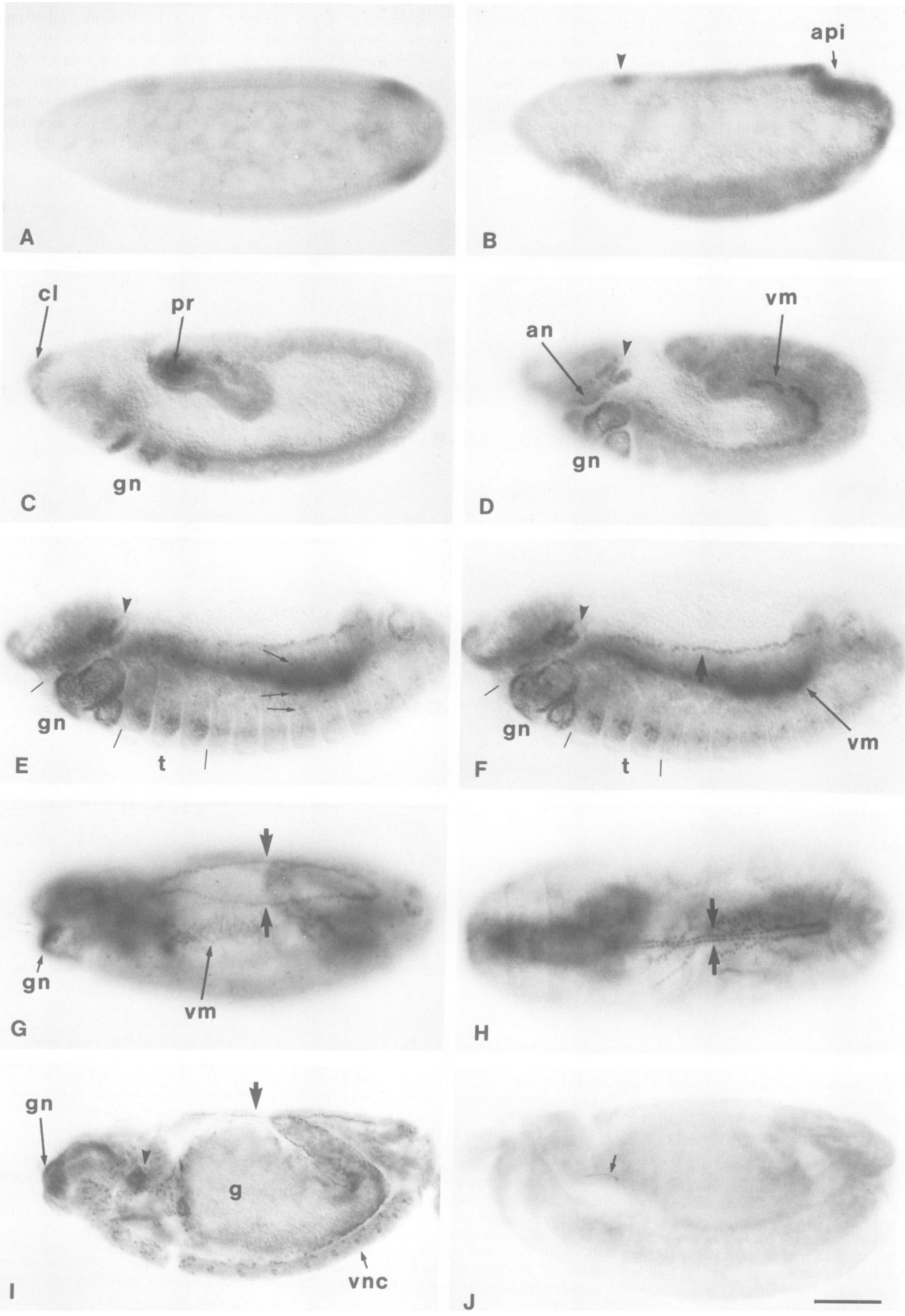


Fig. 1. Tissue distribution of *disco* mRNA during embryonic development. The *disco* transcript was detected in whole mount embryos by *in situ* hybridization using digoxigenin labeled probes. (A) and (C−F) are lateral views, (B) is a dorsolateral view. Anterior is to the left and dorsal is up. Staging follows the convention of Campos-Ortega and Hartenstein (1985). (**A**) cellular blastoderm embryo (stage 5). *disco* mRNA is found in a cap at the posterior pole surrounding the pole cells. (**B**) gastrulation (late stage 6). *disco* mRNA is found in the amnioproctodeal invagination (api) and in two dorsal transversal stripes (arrowheads) in and just anterior to the cephalic furrow. (**C**) extended germ band embryo (stage 11). *disco* mRNA is detected in the proctodeum (pr), in small clusters of cells in the ectoderm of abdominal segments, in larger clusters in the ectoderm of thoracic segments (t), in the three gnathal segments (gn), in the clypeolabrum (cl) and in parts of the procephalic lobe. Staining in the procephalic lobe comprises two domains: one group in the antennal segment (an) and one in and around the invaginating optic lobe primordium (arrowhead). (**D**) embryo during germ band retraction (stage 12). *disco* mRNA is distributed as in the previous panel with additional staining in the visceral mesoderm (vm). (**E**) stage 14 embryo. *disco* mRNA is found in the thoracic and gnathal segments as before. The gnathal segments are moving forward through the process of head involution. Staining persists in the antennal segment and in the optic lobe primordium (arrowhead). This latter domain has at this stage been internalized and is in contact with the brain lobe. The visceral mesoderm, which also continues to stain, is spreading out around the gut. (**F**) late stage 15 embryo. *disco* mRNA is found as before in the gnathal segments. By this time these segments are almost entirely contained within the anterior opening (atrium). Staining persists in the visceral mesoderm which has become a thin covering surrounding the gut (g). Staining is found in many parts of the nervous system including the ventral nerve cord (vnc) and the optic lobe primordium (arrowhead), now incorporated within the brain lobe. Bar = 50 μm.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(discussed below), more definitive identification has proved difficult.

disco expression during development of the larval visual system

Staining with *disco*-specific antisera shows that the gene is expressed in many parts of the embryonic nervous system. We wished to examine which of these expression domains could have a bearing on the Bolwig's nerve defects of *disco* mutants. To help resolve this question we used double-labeling techniques to study the relationship between domains of *disco* expression and elements of the developing visual system.

Wild type embryos were stained with antisera to the *disco* gene product and either a monoclonal antibody which labels the embryonic PNS, MAb 22C10 (Zipursky *et al.*, 1984),

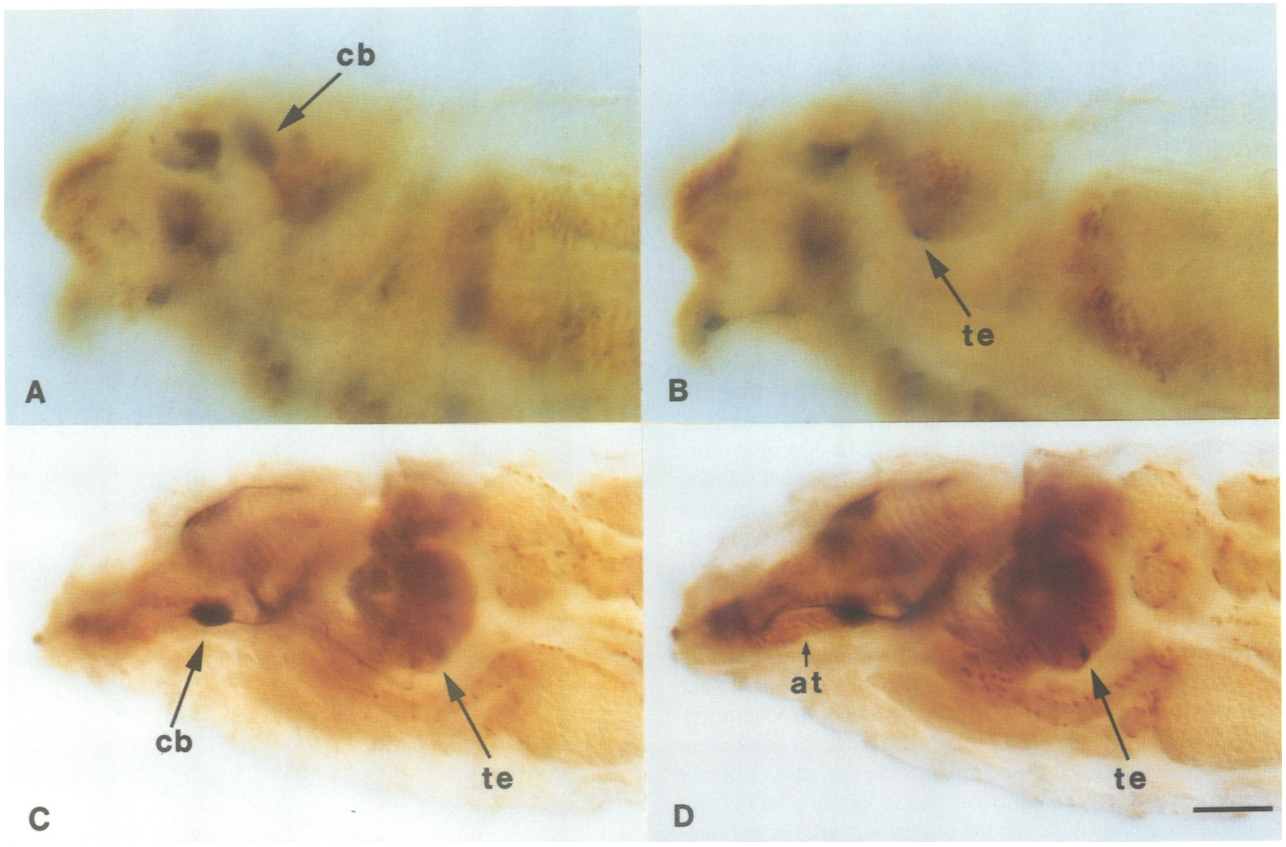

Fig. 3. Tissue distribution of the *disco* protein during development of the larval visual system. Embryos were stained with an affinity purified *disco*-specific polyclonal antiserum and either a PNS-specific monoclonal antibody, MAb22C10 (A, B), or a photoreceptor-specific monoclonal antibody, MAb24B10 (C, D). In both cases anti-*disco* staining is indicated by a brown reaction product and monoclonal antibody staining by a black reaction product. (**A**) and (**B**) two focal planes of the anterior region of a stage 14 embryo. At this stage of embryonic development, the neuronal cell bodies of Bolwig's nerve are only a short distance from the target region in the developing brain lobes. In (A) the position of these cell bodies (cb) is indicated and a short process is seen extending from them posteriorly and ventrally to the brain lobe surface. The terminal region of the nerve (te), shown in (B), is located at the edge of a large triangular domain of *disco* expression. This domain includes the primordium of the imaginal optic lobes. (**C**) and (**D**) two focal planes of the anterior region of a stage 17 embryo. In the period between the stage shown in the two previous panels and this stage, head involution has moved the neuronal cell bodies of Bolwig's nerve to their final position away from the brain lobes. These cell bodies are found in contact with the atrium (at) at a point near the larval mouthhooks. Bolwig's nerve extends back and penetrates the brain lobes. The domain of *disco* expression near the Bolwig's nerve terminal region is at this stage more dispersed and roughly ring shaped. Note also the extensive staining in the atrium near the Bolwig's nerve cell bodies. Bar = 19.5 μm in (A, B), 25 μm in (C, D).

Fig. 2. Tissue distribution of the *disco* protein during embryonic development. Embryos were stained with an affinity purified *disco*-specific polyclonal antiserum. Note the similarities between the staining shown here and that shown in Figure 1. (A–F, I and J) show lateral views, (G) a dorsolateral and (H) as dorsal view. Abbreviations are as in the preceding figure. (**A**) embryonic stage 5. anti-*disco* staining is present around the posterior pole. (**B**) stage 6. Staining can be seen in the amnioproctodeal invagination and in a transverse stripe (arrowhead) anterior to the cephalic furrow. (**C**) stage 10. *disco* protein is detected here in the proctodeum, in the clypeolabrum and ventrally on either side of the cephalic furrow in the primordia of the gnathal segments. (**D**) early stage 12. Staining can be seen in the gnathal segments, in the visceral mesoderm and in two parts of the procephalic lobe: the antennal segment and in and around the optic lobe primordium (arrowhead). This latter structure is clearly visible here as a groove in the posterior procephalon. (**E**) and (**F**) two focal planes of a late stage 13 embryo. *disco* protein is detected in the gnathal segments, in ventrolateral groups of cells in the thoracic segments and in and around the optic lobe primordium (arrowhead). (E) shows segmentally repeated staining in the lateral ectoderm (small arrows) which is near and likely includes a subset of the peripheral nervous system. (F) shows staining in the visceral mesoderm and in the cardioblasts, a longitudinal row of cells (large arrow) near the amnioserosa. (**G**) and (**H**) embryos during the process of dorsal closure. *disco* protein is detected in the rows of cardioblasts (large arrows) which meet at the dorsal midline and eventually give rise to the larval heart, the dorsal vessel. (**I**) late stage 15. Staining is seen in the gnathal segments, in the visceral mesoderm surrounding the gut, in the cardioblasts (large arrow) and in many parts of the nervous system including the optic lobe primordia (arrowhead). (**J**) embryo hemizygous for the deficiency Df(1)l9 which deletes the *disco* gene. The embryo was incubated with *disco*-specific antiserum as above and shows none of the previously described staining. in this case, non-specific staining is detected in the lumen of the salivary gland (arrow). Bar = 50 μm.

or a monoclonal antibody which labels all photoreceptors including the cells of Bolwig's nerve, MAb 24B10 (Zipursky *et al.*, 1984). Since we were unable to generate strong enough signals using fluorochrome-conjugated secondary antibodies, we used more sensitive horseradish peroxidase-conjugated secondary antibodies and employed cobalt–nickel enhancement to distinguish between the two antibody labels. The drawback of this method is that co-localization of both signals to a single cell may not be possible.

Figure 3A and B show double-labeling of an embryo with anti-*disco* antiserum and MAb22C10. This monoclonal antibody labels the embryonic PNS and a subset of CNS neurons and it stains Bolwig's nerve after ~ 10 h of embryonic development. The terminal region of Bolwig's nerve is adjacent to a large domain of *disco* expression in the brain lobe. This domain of expression originates as an invagination in the procephalic lobe and includes the optic lobe primordium (see above).

Results of double-labeling experiments with a *disco*-specific antiserum and MAb 24B10 are shown in Figure 3C and D. This monoclonal antibody clearly labels Bolwig's nerve from the cell bodies to the terminus. In older embryos, such as that shown in Figure 3C and D, the *disco* expression around the terminus of Bolwig's nerve has become more diffuse and is ring shaped. Several smaller clusters of staining cells are seen as well and many of these are only a short distance from the nerve terminus. However, at this level of resolution we cannot determine whether any of these cells are the primary targets of Bolwig's nerve.

Expression of disco gene products in third instar larvae

The tissue distribution of the gene product in third instar larvae was examined by whole mount immunohistochemistry with *disco*-specific antisera; the results are presented in Figure 4B and C. It was impossible to use Df(1)l9 hemizygotes to verify that the staining in larvae results from *disco* protein as was done for the embryonic analysis above. Individuals hemizygous for this large deletion die as embryos. However we believe that our staining results reflect the true distribution of the gene product since identical staining patterns are produced using sera drawn from three different rabbits. Moreover, *in situ* hybridization of *disco* gene probes to larval tissues produces staining patterns that are very similar, if not identical, to the immunohistochemical staining patterns (compare Figure 4A and B). A few scattered elements are stained by *disco*-specific antibodies but not by *in situ* hybridization (see, for example, Figure 4C). Presumably these differences result from the lower sensitivity of *in situ* hybridization for the detection of isolated positive cells.

Figure 4 shows that some regions stained by *disco*-specific antibodies in embryos are also stained in larvae. For example, mesodermal layers around the larval gut and some scattered groups of cells in the larval central nervous system are stained. Most conspicuous in the larval nervous system however, is a band of staining that wraps around each brain lobe (Figure 4A, B). This band extends from a point on the lateral surface posterior to the optic stalk (see Figure 4C) to the dorsal surface of the brain lobe. It is located in or near the superficial layers of the brain. This staining domain does not correspond exactly to any previously described brain structure. It is found near the boundary between central brain

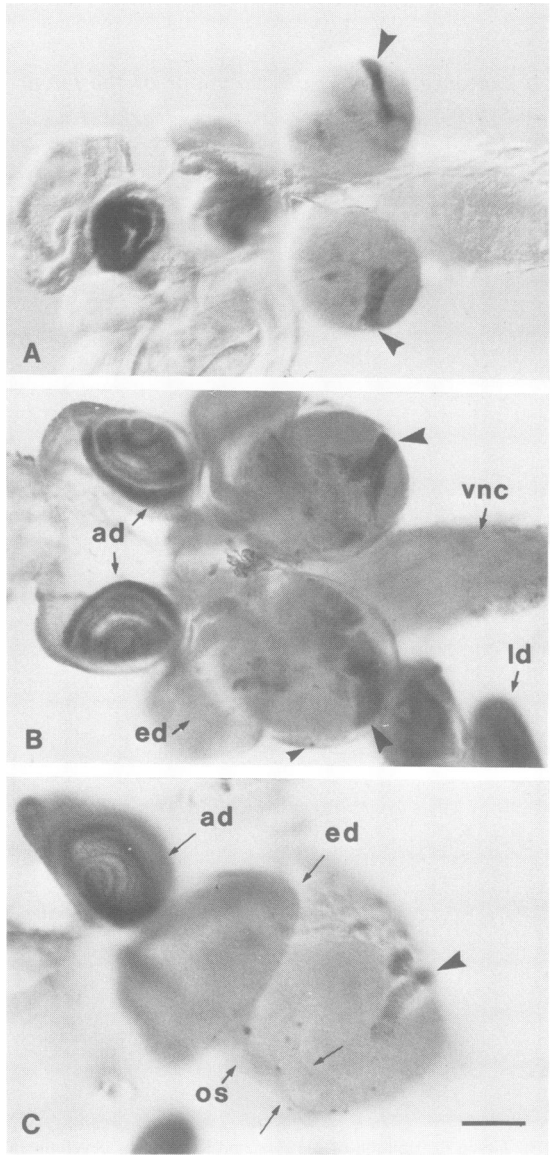

Fig. 4. Tissue distribution of *disco* mRNA and protein in third instar larvae. Third instar larvae were dissected and the head regions, imaginal discs and CNS were used for *in situ* hybridization (A) or stained with an affinity purified *disco*-specific polyclonal antiserum (B, C). (A, B) are dorsal views; (C) is a ventrolateral view. In all panels anterior is to the left. (**A**) the distribution of *disco* mRNA in third instar larvae. *disco* transcripts are seen in several imaginal discs and in parts of the larval brain lobe. A band of stained cells (arrowheads) is found on the surface of each brain lobe. This band extends from a ventrolateral position near the optic stalk to the dorsal surface. (**B**) the distribution of *disco* protein in third instar larvae. Staining is found in the antennal discs (ad), the leg discs (ld) and in parts of the CNS. As in (A), a band of staining is found wrapping around each brain lobe (large arrowheads). A regular array of stained cells is found in the ventral nerve cord (vnc). A small number of stained cells are found in the lamina primordium (small arrowhead shows one in this plane of focus). (**C**) distribution of the *disco* protein in the antennal-eye disc and brain lobe of a third instar larva. The antennal disc portion is extensively stained whereas the eye disc (ed) is unstained. Two large stained cells are found in the optic stalk (os), one of which is visible in this focal plane. At the base of the optic stalk is the primordium of the lamina, a crescent shape demarcated by furrows (between small arrows). In this individual seven stained cells are found extending across this crescent. The large band of stained cells described above (arrowhead) begins just posterior to this region and extends around the surface of the brain, out of the focal plane. Bar = 62.5 μm in (A), 50 μm in (B, C).

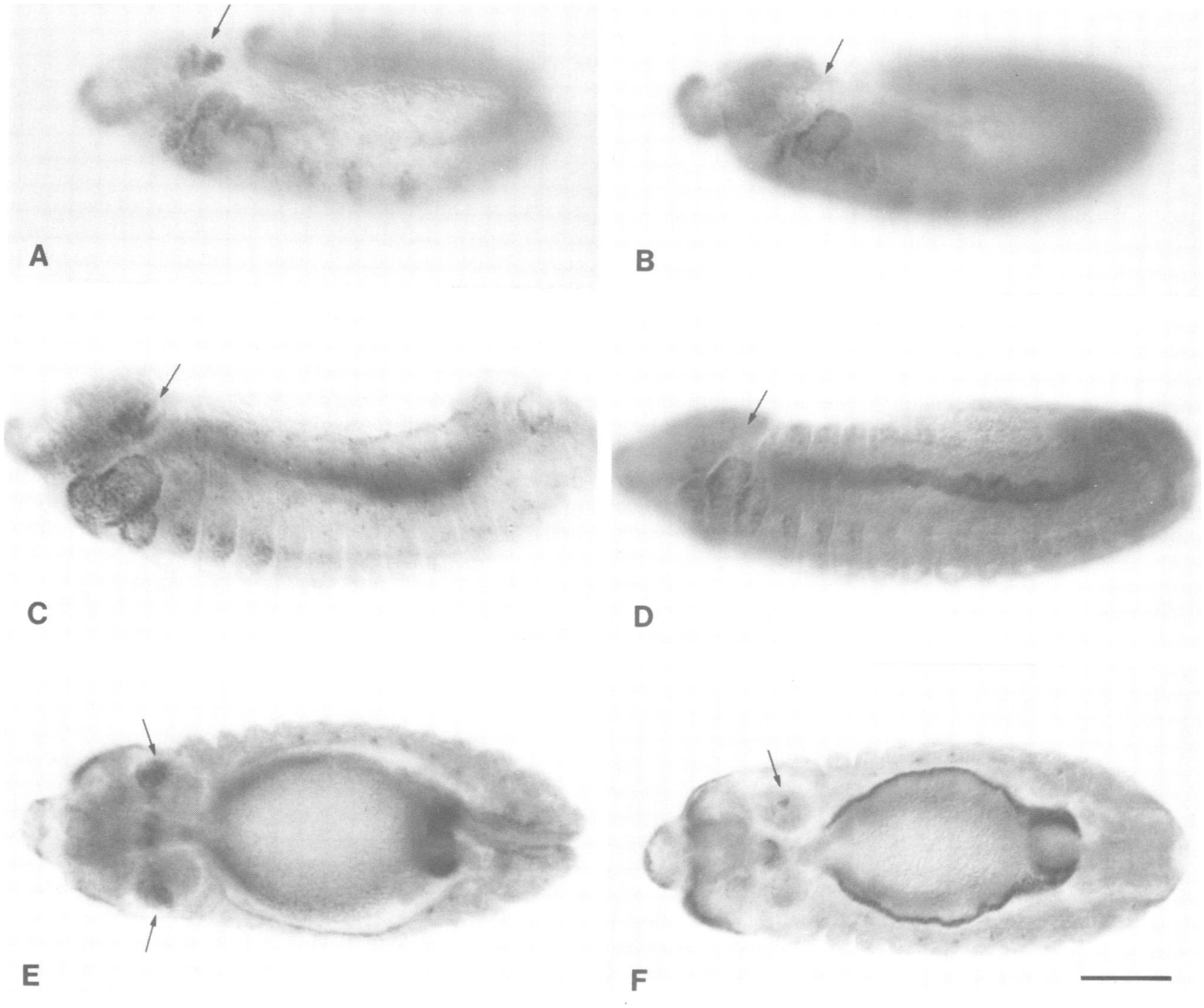

Fig. 5. Tissue distribution of the *disco* protein in wild type and *disco* mutant embryos. Wild type (A, C, E), $disco^{1}$ homozygous (B, F) and $disco^{1656}$ homozygous (D) embryos were stained with an affinity purified *disco*-specific polyclonal antiserum. (A–D) show lateral views; (E, F) show dorsal views. (**A**) and (**B**) stage 11 embryos. In the wild type embryo *disco* expression is found in the region of the optic lobe primordium (arrow). In the mutant staining is absent in this region. In all other tissues staining in the mutant is identical to that observed in the wild type. (**C**) and (**D**) stage 13 embryos. Again the only difference in staining between the wild type and mutant is a lack of staining in the region of the optic lobe primordium (arrow) in the mutant. (**E**) and (**F**) stage 14 embryos. Staining in the two embryos again differs only in the region of the optic lobe primordia (arrows). In this case the mutant embryo shows a tiny rudiment of staining in the right brain lobe (arrow). Bar = 50 μm.

and optic lobes and is at least partially contained within the primordia of the inner medulla and lobula complex of the optic lobes. This staining is not seen in second instar larvae (not shown) but develops during the third larval instar. In addition, *disco* gene product is detected in sparsely distributed nuclei in two other areas of the larval visual system (Figure 4C). First, a very small number of stained nuclei are found scattered across the developing lamina. This structure is easily distinguished as a crescent shape demarcated by furrows in the brain surface. The number of these stained nuclei varies from one to seven in different brains, perhaps in an age-related manner. Secondly, two large stained nuclei are found at the base of the optic stalk near its connection with the eye disc.

We also examined the imaginal discs of third instar larvae for *disco* gene products. Figure 4B and C show the extensive staining detected in the antennal disc. Staining was also detected in the leg discs. The eye disc, however, is unstained. The latter result is consistent with mosaic studies which showed that wild type *disco* function is not required in the eye for its wild type development (Steller *et al.*, 1987).

Expression of disco protein in disco mutant embryos

Two point mutant alleles of the *disco* gene have been isolated and sequenced. Both of these are mis-sense mutations altering codons that specify cysteine residues within the putative 'zinc finger' domain. Northern analysis reveals that wild type amounts of *disco* mRNA are transcribed in embryos carrying

these mutant alleles (Heilig *et al.*, 1991). These mutations could however affect stability or processing of the protein product. We wished to determine whether the abundance, subcellular localization and tissue distribution of *disco* protein in these mutants differ from that in wild type embryos.

Collections of homozygous *disco*^{1} and *disco*^{1656} embryos were stained with *disco*-specific antiserum. The results, shown in Figure 5, are identical for the two mutant alleles. Neither mutant differs significantly from wild type in terms of overall level or in terms of subcellular localization of *disco* protein. We do find one small but nonetheless important difference in the tissue distribution of the protein in *disco* mutant embryos. The mutant embryos consistently lack the domain of staining which originates from the posterior portion of the procephalic lobe (Figure 5B and D). As mentioned above, we believe that this structure corresponds to the optic lobe primordium. Some mutant individuals do exhibit residual traces of staining in this area (Figure 5F). The absence of staining in this region represents the only difference in *disco* protein distribution between the wild type and *disco* mutant embryos; in all other tissues, including other parts of the nervous system, the staining patterns are perfectly identical.

Discussion

In the present study we have examined the spatial and temporal distribution of the *disco* mRNA and protein. We have found no significant differences between the distribution of the two gene products. Both are present in many different tissues throughout the embryo and in these tissues the distribution pattern of the gene products is complex and dynamic. Much of the expression is in groups of cells that are involved in major morphogenetic events such as gastrulation, head involution, dorsal closure and formation of the gut. The gene products are found in a subset of cells throughout the nervous system, in both the CNS and PNS.

The pattern of *disco* expression in embryos is more widespread than might be predicted from the relatively specific phenotype of the existing *disco* mutant alleles. Mutant embryos exhibit defects in placement and projection of Bolwig's nerve and variable abnormalities in the patterning of the peripheral nervous system, but appear otherwise anatomically normal. Although the mutant alleles we have examined here may not be nulls, the result of complete loss of *disco* gene function can be determined by examining embryos hemizygous for Df(1)l9, which removes a portion of the X chromosome containing the *disco* locus. These embryos lack between 5 and 10% of the X chromosome, but they develop until late in embryogenesis and show nervous system defects no more severe than those of the *disco* point mutants (Steller *et al.*, 1987). Moreover, the Df(1)l9 mutant embryos do not show consistent gross defects in head involution or dorsal closure although significant amounts of *disco* gene product are found in cells participating in these processes. Finally, P-element excision has been used to generate small deletions removing the first exon of the *disco* gene. Flies homozygous for these small deletions are viable and have a phenotype like the existing point mutants (Heilig *et al.*, 1991).

Results from a number of studies in different organisms indicate that many of the molecules involved in development of axonal pathways and cellular connections have more general roles in development. The majority of molecules shown by *in vitro* studies to affect axonal growth and connectivity can be found in non-neural tissues. Examples include laminin, N-CAM, NG-CAM, L1, and tenascin (see reviews by Jessel, 1988; Lander, 1989). Molecules that are involved in these processes in *Drosophila,* for example, amalgam (Seeger *et al.*, 1988), fasciclin I (Zinn *et al.*, 1988; Elkins *et al.*, 1990), fasciclin II (Snow *et al.*, 1988; Harrelson and Goodman, 1988), and neuroglian (Bieber *et al.*, 1989) are similarly expressed in many parts of the embryo outside the nervous system. Only a small subset, if any, of the tissues which express these genes show discernable defects in the corresponding mutants. Likewise, many of the tissues which normally express *disco* are not phenotypically abnormal in *disco* mutants. One possible explanation for the lack of obvious abnormalities in these regions is that their morphogenesis is mediated by functionally redundant systems.

We have begun to address the role of the *disco* gene product in the development of the larval system by relating the gene expression to the time and location of the outgrowth of Bolwig's nerve. Previous mosaic studies located the focus of the *disco* mutant phenotype in an anterior dorsal position (Steller *et al.*, 1987). This location is far removed from the site of origin of the optic lobes as determined by a previous fate mapping study (Kankel and Hall, 1976). Steller *et al.* (1987) also observed that the cell bodies of the neurons of Bolwig's nerve are frequently dispersed and abnormally positioned in *disco* mutants. This fact, together with the results of mosaic analysis, led to the proposal that the requirement for wild type *disco* function is in the neurons of Bolwig's nerve. Consistent with this hypothesis, we find expression of the *disco* gene in and around the antennal segment which is the likely site of origin of the cells of Bolwig's nerve (Hartenstein, 1988). However, we have not identified significant *disco* expression in the cells of Bolwig's nerve after axonal outgrowth.

We have also found significant *disco* expression in the primordium of the adult optic lobes near the terminal region of Bolwig's nerve. Therefore, there could be non-autonomous requirements for *disco* gene function to direct normal Bolwig's nerve connectivity. Whereas expression of *disco* in the cells of Bolwig's nerve may be transient, expression in the optic lobe primordia persists well beyond the period of Bolwig's nerve outgrowth. This domain of expression is altered in *disco* mutant embryos (see discussion below). In addition, significant expression is detected in the cells of the atrium. These cells are adjacent to the Bolwig's nerve cell bodies in their final position (see Figure 3D). Requirements for wild type *disco* function in the atrium and in cells near the terminus of Bolwig's nerve could explain both the cell body and axon positioning defects seen in *disco* mutants. At present we cannot determine in which cells *disco* function is required for proper connectivity and positioning of the larval photoreceptor cells. Mapping the *disco* focus with a marker for the genotype of internal tissues is needed to resolve this question.

We have observed that the *disco* protein is found in the cell nucleus in embryonic and larval tissue. Analysis of the sequence of the *disco* gene (Heilig *et al.*, 1990) revealed a short region with similarity to the zinc finger class of nucleic acid binding proteins, many of which are transcription factors. These findings raise the possibility that the *disco*

gene product may be a regulatory protein. If so, *disco* could, for example, be directing the expression of molecules necessary for recognition of synaptic partners. More generally it could affect the development or differentiation of cells that are required for some step in the patterning process.

We have found that overall levels of *disco* gene expression in embryos homozygous for either mutant *disco* allele are normal. Subcellular localization of the gene product in these embryos is also indistinguishable from wild type. However, a single domain of gene expression, namely, expression in the optic lobe primordium near the Bolwig's nerve terminus, is missing in the mutant embryos. The absence of staining in this single region in mutant embryos demonstrates that this tissue has a different requirement for wild type gene function than the other tissues which show no obvious alteration in staining. This indicates that, at some level, the gene influences either directly or indirectly its own expression in this region. Given the nuclear localization and the previously described sequence similarity to a motif found in transcriptional regulators, it is possible that the alteration in staining represents a direct autoregulatory function. An alternate explanation is that the cells which normally express *disco* in this region are absent or are ectopically located. However, there is no evidence of morphological abnormalities in the optic lobe primordium in *disco* mutant embryos.

Tix *et al.* (1989) have identified a cluster of cells (named OLPs) in the optic lobes which differentiate early, probably during embryonic development. Their location and projection pattern, together with their time of origin, suggest they might interact with Bolwig's nerve during embryonic development or larval life. Preliminary evidence indicates that these cells are missing in *disco* mutant larvae (S.Tix and K.-F.Fischbach, personal communication). One question raised by these data is whether *disco* is expressed in the OLPs during formation of the larval visual system. The experiments described here do not show expression of *disco* in these cells in third instar larvae. As the OLPs can currently be identified only during late larval stages, it is unknown whether *disco* gene products are found in these cells earlier.

Finally, we have found that *disco* expression continues beyond embryogenesis in many tissues. The gene is expressed in late third instar larvae in or near the developing imaginal optic lobes. These expression data indicate a need for further experiments to ascertain whether this later *disco* expression plays an autonomous role in development of the optic lobes.

Materials and methods

In situ hybridization

Whole mount *in situ* hybridization to embryos and larvae was performed essentially as previously described (Tautz and Pfeifle, 1989). In all experiments the probe was an embryonic cDNA isolate (Heilig *et al.*, 1991) which contains most of the *disco* open reading frame. This DNA was labeled using methods and reagents supplied by Boehringer Mannhein Biochemicals.

For embryonic staining we used a slightly modified protocol provided by L.Dickinson and R.Lehmann. This protocol differed from the method of Tautz and Pfeifle (1989) only in the hybridization solution [which was 50% formamide, 1× salts (from a 10× solution: 3 M NaCl, 100 mM Tris, 100 mM sodium phosphate, 2% polyvinylpyrrolidone, 2% Ficoll 400, 50 mM EDTA, pH 6.8), 10% dextran sulfate, 1 mg/ml yeast tRNA, 0.1% Tween 20] and the inclusion of an additional 1 h, 45°C washing step in hybridization solution after the overnight hybridization.

For larval staining, we used a slightly modified protocol provided by S.Campuzano and J.Modolell. This protocol differed from the method of Tautz and Pfeifle (1989) chiefly in the initial fixation step. Larvae were dissected and fixed in 4% paraformaldehyde in PBS for 20 min, washed in PBS, then fixed in 0.5% glutaraldehyde in PBS for 2 min on ice.

Production of bacterial fusion proteins

Briefly, a 541 bp *Bam*HI fragment, corresponding to nucleotides 615–1156 of the mature *disco* message (Heilig *et al.*, 1991), was ligated into the *Bam*HI site of the expression vectors pRIT2 (Nilsson *et al.*, 1985), pUR291 (Rüther and Müller-Hill, 1983) and pET-3a (Studier *et al.*, 1990). After transformation into the appropriate bacterial hosts, cultures were grown and fusion proteins were induced as previously described. In the case of the pET derivative, it was necessary to use the BL21 (DE3) host carrying the pLysS plasmid (Studier *et al.*, 1990) in order to reduce loss of the expression vector during culture. The pRIT fusion protein was purified by affinity chromatography over a rabbit IgG agarose (Sigma) column. The pUR and pET fusion proteins were partially purified by preparative SDS–PAGE.

Generation of antisera

Typically, standard procedures were used for preparation and purification of antisera [see, for example, Harlow and Lane (1988)]. The purified pRIT fusion protein (100 μg) was mixed 1:1 with MPL + TDM + CWS adjuvant (RIBI Immunochem) and injected subcutaneously into three female New Zealand White rabbits. These rabbits were boosted several times at four week intervals with the same immunogen. The same rabbits were then boosted at four week intervals with the partially purified pET product (125 μg) mixed 1:1 with MPL + TDM + CWS adjuvant. Serum was collected two weeks after each boost and stored at −20°C. The sera were purified by chromatography over a column containing the partially purified pUR fusion protein coupled to Affigel 10 (Bio-Rad). Antibodies were eluted from the column with 50 mM glycine pH 2.5, 150 mM NaCl, then neutralized, dialyzed against PBS and stored at 4°C.

Immunohistochemistry

Staining of embryos and larvae was performed essentially as previously described (Steller *et al.*, 1987) with the following modifications: tissues were fixed in PLP fixative (McLean and Nakane, 1974) for 20–30 min at room temperature. Subsequent washing steps were done in BSS (Ashburner, 1989) and antibody incubations and blocking steps were in BSN (0.9× BSS, 0.2% Triton X-100, 10% filtered goat serum). These antibody incubation steps were for 4–6 h at room temperature or overnight at 4°C. Monoclonal antibodies were used at a dilution of 1:1, affinity purified anti-*disco* antibodies at a dilution of 1:5 and HRP-conjugated secondary antibodies (Bio-Rad) at a dilution of 1:100 for embryonic tissues, 1:500 for larval tissue. Double-labeling experiments were performed using the method of Lawrence *et al.* (1987). Briefly, embryos were incubated simultaneously with polyclonal anti-*disco* sera and monoclonal antibodies, followed by washing, blocking, incubation with HRP-conjugated goat anti-rabbit IgG and reaction with DAB as before. This was followed by washing, blocking and another incubation with HRP-conjugated goat anti-mouse IgG. After this incubation, the samples were reacted with DAB in the presence of 0.03% nickel sulfate and 0.03% cobalt chloride, then dehydrated and mounted as before.

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References

Ashburner,M. (1989) *Drosophila: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bieber,A.J., Snow,P.M., Hortsch,M., Patel,N.H., Jacobs,J.R., Traquina,Z.R., Schilling,J. and Goodman,C.S. (1989) *Cell*, **59**, 447–460.

Campos-Ortega,J.A. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster*. Springer Verlag, New York.
Elkins,T., Zinn,K., McAllister,L., Hoffman,F.M. and Goodman,C.S. (1990) *Cell*, **60**, 565–575.
Fischbach,K.-F. and Technau,G. (1984) *Dev. Biol.*, **104**, 219–239.
Fischbach,K.-F., Barleben,F., Boschert,U., Dittrich,A.P.M., Gschwander,B., Houbé,B., Jäger,R., Kaltenbach,E., Ramos,R.G.P. Schlosser,G. (1989) In Singh,R.N. and Strausfeld,N.J. (eds), *Neurobiology of Sensory Systems*. Plenum Press, New York, pp. 171–194.
Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Harrelson,A.L. and Goodman,C.S. (1988) *Science*, **242**, 700–708.
Hartenstein,V. (1988) *Development*, **102**, 869–886.
Hartenstein,V., Technau,G.M., and Campos-Ortega,J.A. (1985) *Roux's Arch. Dev. Biol.*, **194**, 213–216.
Heilig,J.S., Freeman,M., Laverty,T., Lee,K.J., Campos,A.R., Rubin,G.M. and Steller,H. (1991) *EMBO J.*, **10**, 809–815.
Jessel,T.M. (1988) *Neuron*, **1**, 3–13.
Kankel,D.R. and Hall,J. (1976) *Dev. Biol.*, **48**, 1–24.
Lander,A. (1989) *Trends Neurosci.*, **12**, 189–195.
Lawrence,P.A., Johnston,P., Macdonald,P. and Struhl,G. (1987) *Nature*, **328**, 440–442.
McLean,I.W. and Nakane,P.K. (1974) *J. Histochem. Cytochem.*, **12**, 1077–1083.
Meinertzhagen,I.A. (1973) In Young,D. (ed.), *Developmental Neurobiology of Arthropods*. Cambridge University Press, New York, pp. 51–104.
Meyerowitz,E.M. and Kankel,D.R. (1978) *Dev. Biol.*, **62**, 112–142.
Nilsson,B., Abramsén,L. and Uhlén,M. (1985) *EMBO J.*, **4**, 1075–1080.
Poulson,D.F. (1950) In Demerec,M. (ed.), *Biology of Drosophila*. Wiley, New York, pp. 168–274.
Rüther,U. and Müller-Hill,B. (1983) *EMBO J.*, **2**, 1791–1794.
Seeger,M.A., Haffley,L. and Kaufman,T.C. (1988) *Cell*, **55**, 589–600.
Snow,P.M., Zinn,K., Harrelson,A.L., McAllister,L., Schilling,J., Bastiani,M.J., Makk,G. and Goodman,C.S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5291–5295.
Steller,H., Fischbach,K.-F. and Rubin,G.M. (1987) *Cell*, **50**, 1139–1153.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Tautz,D. and Pfeifle,C. (1989) *Chromosoma*, **98**, 81–85.
Tix,S., Minden,J.S. and Technau,G.M. (1989) *Development*, **105**, 739–746.
Zinn,K., McAllister,L. and Goodman,C.S. (1988) *Cell*, **53**, 577–587.
Zipursky,S.L., Venkatesh,T.R., Teplow,D.B. and Benzer,S. (1984) *Cell*, **36**, 15–26.

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