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Pigment-Dispersing Hormone-Immunoreactive Neurons in the Nervous System of Wild-Type *Drosophila melanogaster* and of Several Mutants With Altered Circadian Rhythmicity

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ABSTRACT

Antisera against the crustacean pigment-dispersing hormone (β-PDH) were used in immunocytochemical preparations to investigate the anatomy of PDH-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and in that of several brain mutants of this species, some of which express altered circadian rhythmicity. In the wild-type and in all rhythmic mutants (*small optic lobes, sine oculis, small optic lobes;sine oculis*), eight cell bodies at the anterior base of the medulla (PDFMe neurons) exhibit intense PDH-like immunoreactivity. Four of the eight somata are large and four are smaller. The four large PDFMe neurons have wide tangential arborizations in the medulla and send axons via the posterior optic tract to the contralateral medulla. Fibers from the four small PDFMe neurons ramify in the median protocerebrum dorsal to the calyces of the mushroom bodies. Their terminals are adjacent to other PDH-immunoreactive somata (PDFCa neurons) which send axons via the median bundle into the tritocerebrum. The results suggest a possible involvement of the PDFMe neurons in the circadian pacemaking system of *Drosophila*. The location and size of the PDFMe neurons are identical with those of neurons containing the *period* protein which is essential for circadian rhythmicity. Changes in the arborizations of the PDFMe neurons in *small optic lobes;sine oculis* mutants are suited to explain the splitting in the locomotor rhythm of these flies. In the arrhythmic mutant, *disconnected*, the PDFMe neurons are absent. The arrhythmic mutant *per*^{0}, however, shows normal PDH immunoreactivity and therefore, does not prevent the expression of PDH-like peptides in these neurons.

Key words: neuropeptides, circadian pacemakers, insect brain, immunocytochemistry

The pigment-dispersing hormones (PDHs) are a family of octadecapeptides which have been isolated from several crustacean species (Rao and Riehm, '89). PDHs released from the sinus gland of decapod Crustacea trigger chromatophoral pigment dispersion and evoke light adaptational migration of retinal screening pigment (Rao and Riehm, '88a). In insects, peptides with 78–83% sequence homology with β-PDH from *Uca pugilator* have been isolated from head extracts of the lubber grasshopper, *Romalea microptera* (Rao et al., '87), the cricket *Acheta domesticus* (Rao and Riehm, '88b, '89), and the cockroach, *Periplaneta americana* (Mohrherr et al., '92). These peptides were called pigment-dispersing factors (PDFs). Like the crustacean PDHs, the insect PDFs cause pigment dispersion in *Uca pugilator* when applied to the optic stalk of this crab (Rao and Riehm, '89).

With the aid of an antiserum against synthetic β-PDH (Dircksen et al., '87), PDH-immunoreactive neurons have been described in different insect species including locusts, crickets, cockroaches, a fly, a moth, and a beetle (Zahnow et al., '87; Homberg et al., '91a,b; Nässel et al., '91; Fleissner and Fleissner, '92). In all species studied, neurons with somata in the optic lobes show PDH-like immunoreactivity (PDHLI). In orthopteroid insects, three groups of neurons were immunostained in each optic lobe. Two of the three

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TABLE 1. Characterization of the *Drosophila* Mutants

Mutant	Optic lobe morphology	Locomotor activity
Small optic lobes (sol KS58)	Medulla and lobula complex reduced to about 50% (Fischbach and Heisenberg, '81)	Rhythmic (Helfrich and Engelmann, '83)
Sine oculis (so)[1]	Ocelli absent, compound eyes reduced to different degrees; in case of complete expression of the mutation the flies are eyeless, lamina and distal part of the medulla are absent, the lobula complex is reduced to about 40% (Fischbach, '83)	Rhythmic (Helfrich and Engelmann, '83)
Small optic lobes; sine oculis (sol;so)[1]	Eye phenotype same as "so"; optic lobes are reduced to less than 5%; the tiny rudiments seem to be formed exclusively by tangential neurons; the giant neurons of the lobula plate and medulla tangentials can be distinguished (Fischbach and Technau, '84)	Rhythmic, but most flies show splitting (Helfrich, '86)
Disconnected (disco 1)	Photoreceptor cells are disconnected from the optic lobe; "unconnected" phenotype: no innervation from the outer optic anlage, tiny rudiments of the optic lobes; "connected" phenotype: some innervation from the outer optic anlage, optic lobes of almost normal size but grossly disorganized (Steller et al., '87)	Arrhythmic (Dushay et al., '89)
Period^0 (per^0)	Normal	Arrhythmic (Konopka and Benzer, '71)

[1]*So* and *sol;so* flies used in this paper were completely eyeless, unless specified otherwise.

groups have somata at the dorsal and ventral posterior edge of the lamina. Neurons of the third group have somata near the anterior proximal margin of the medulla (Homberg et al., '91a). In the fly, *Phormia terraenovae,* only the third group of neurons with somata anterior to the medulla was immunostained (Nässel et al., '91).

Increasing evidence from crustaceans and insects suggests that the PDH-immunoreactive neurons are involved in the circadian system of arthropods. In several crustaceans, PDH released from the sinus gland apparently synchronizes the adaptional migration of retinal screening pigment with respect to circadian rhythms (Arechiga and Mena, '75; Fingerman and Fingerman, '77; Larimer and Smith, '80). In cockroaches and crickets, the group of PDH-immunoreactive neurons with somata near the medulla fulfills several anatomical criteria proposed for circadian pacemakers in these insects (Homberg et al., '91a). After both optic stalks in cockroaches have been cut, the circadian locomotor rhythm disappears; at the same time, the PDH-immunoreactive terminals degenerate (Stengl and Homberg, '92). Furthermore, the regeneration of the PDH-immunoreactive terminals into the brain of cockroaches with severed optic tracts correlates well with the regaining of circadian locomotor activity (Stengl and Homberg, '92).

To investigate possible roles of PDH-immunoreactive neurons in the organization of circadian rhythms, we studied their morphology in the brain of *Drosophila melanogaster.* Molecular genetic studies, together with behavioral experiments, strongly suggest that circadian pacemakers in *D. melanogaster* reside in the lateral frontal brain close to the medulla. In this region, a small group of neurons, termed "lateral neurons" (LNs), contains the "*period* protein" which is essential for circadian rhythmicity (Siwicki et al., '88; Zerr et al., '90; review: Hall and Kyriacou, '90). Experiments with mosaic flies have shown that normal expression of the *period* protein in the LNs is required for normal rhythmicity of locomotor activity (Ewer et al., '92). In the structural brain mutant, *disconnected* (Steller et al., '87), which is behaviorally arrhythmic (Dushay et al., '89), the LNs seem to be absent (Zerr et al., '90; Hardin et al., '92). Studies on brain mutants of *D. melanogaster* that lack large parts of the optic lobes but are still rhythmic, suggest that the pacemaker neurons controlling locomotor activity are medulla tangential neurons (Helfrich and Engelmann, '83; Helfrich, '86), as these neurons are the only elements common to the optic lobes of these mutants (Fischbach, personal communication). Many medulla tangential neurons have somata at the frontal edge of the medulla and axons in the posterior optic tract (Fischbach and Technau, '84).

The present study investigates whether PDH-immunoreactive neurons in the brain of *D. melanogaster* fulfill the anatomical criteria for pacemaker neurons. PDH immunostaining was studied in wild-type *D. melanogaster* and compared with immunostaining in structural brain mutants, which have been previously well characterized with respect to their locomotor activity rhythms. Additionally, PDHLI was studied in the brain of *per^0*-mutants. The mutant *per^0* lacks the *period* protein and is arrhythmic at the behavioral and molecular level (Konopka and Benzer, '71; Hall and Kyriacou, '90). Parts of this study have been reported in an abstract form (Helfrich-Förster et al., '92).

MATERIALS AND METHODS

Animals

Experiments were performed on wild-type strains "Oregon" and "Canton S," and on several mutants of *Drosophila melanogaster* described in Table 1. The mutants *sine oculis (so), small optic lobes;sine oculis (sol;so)*; and *disconnected (disco 1)* were kindly supplied by K.-F. Fischbach, and the mutant *per^0* by C. Kyriacou. All strains were reared at 25°C in a cycle of 12 hours light and 12 hours dark (LD 12:12) on standard medium.

Immunocytochemistry

Immunocytochemistry was performed by the indirect peroxidase-antiperoxidase (PAP; Sternberger, '79) and the indirect immunofluorescence techniques. The PAP technique was carried out on vibratome sections, paraffin sections, or wholemounts of *Drosophila* brains. On vibratome sections, the PAP technique was performed as described previously (Homberg et al., '91a). The antiserum against β-PDH (Dircksen et al., '87) was diluted at 1:20,000. It was applied to the sections for 24 hours at room temperature. For paraffin embedding, the "collar" method of Heisenberg and Böhl ('79) was used. Up to 10 anesthesized flies were threaded by their necks in a metal collar. They were subsequently fixed for 4 hours at room temperature or overnight at 4°C in Boer's GPA fixative (Boer et al., '79), dehydrated in isopropanol and embedded in paraplast (melting point 58°C). Serial frontal sections (10 μm) were cut, deparaffinized, and immunostained following the PAP procedure. For the paraffin sections, the anti-PDH serum was diluted at 1:2,000.

For immunocytochemistry on wholemounts, brains or brains with thoracic and abdominal ganglia attached were dissected and fixed in 4% paraformaldehyde/7.5% picric acid in phosphate buffer (0.1 M, pH 7.4). Immunostaining was carried out as described for the vibratome sections but

with prolonged times for incubations and rinses. Brains were incubated with anti-PDH serum (1:5,000) for 72–96 hours, with goat anti-rabbit for 24–48 hours, and with the PAP complex for 24–48 hours. All steps of the staining procedure were performed at 18°C. Sodium azide (0.02%) was added to all solutions except to the PAP solution. The indirect immunofluorescence technique was carried out on vibratome sections with Texas Red- or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Dianova, Hamburg) as secondary antiserum.

The immunoreactive neurons were reconstructed from serial frontal or horizontal vibratome sections or from wholemounts immunostained by the PAP technique using a Zeiss microscope equipped with a camera lucida attachment.

Specificity controls

The specificity of the anti-PDH antiserum has been well documented (Dircksen et al., '87; Homberg et al., '91a; Nässel et al., '91). In *D. melanogaster*, immunostaining was abolished after omission of the primary antiserum or after preadsorption of the anti-PDH antiserum with 100 μM *Uca/Cancer* β-PDH.

RESULTS

Staining pattern in the wild-type

In wild-type *D. melanogaster*, three cell groups in the brain and one group in the abdominal ganglion exhibit PDH-like immunoreactivity (PDHLI). Usually eight perikarya at the anterior edge of the medulla show intense PDHLI. They are immunostained in wholemounts, vibratome sections, and paraffin sections, and we will refer to them as PDFMe neurons. Four of the eight cell bodies are large (diameter 9–12 μm) and are always intensely stained, and four have a smaller diameter (4–6 μm) and are less intensely stained. In some preparations, the smaller cells could not be detected. The four large somata lie either in a cluster or in a row between the anterior edge of the medulla and the lateral frontal protocerebrum (Figs. 1, 2). The four smaller somata lie more ventrally, close to the anterior base of the medulla (Figs. 1, 2). The PDFMe neurons send processes into the optic lobe and into the central brain. Owing to the superposition of immunoreactive profiles, the neurons could not be individually traced. Processes from the large PDFMe neurons run to the anterior base of the medulla, pass the somata of the small PDFMe neurons, and enter Cucatti's bundle (Figs. 1–3), which is composed of axons and dendrites of medulla tangential neurons (Fischbach and Dittrich, '89). Immunoreactive fibers are densely tangled within Cucatti's bundle and in a small area at the anterior base of the medulla. This area contains immunoreactive terminals and resembles the accessory medulla described in Trichoptera (Ehnbohm, '48; Hagberg, '86) and orthopteroid insects (Homberg et al., '91a). From the anterior base of the medulla, two sets of PDH-immunoreactive fibers, most probably derived from the large PDFMe somata, invade the medulla. The first set of fibers enters the serpentine layer (Fig. 3F), and the second set invades the distalmost layer of the medulla (Fig. 3B,E,F). Both sets of fibers form tangential arborizations extending throughout the retinotopic map of the medulla. The arborizations in the serpentine layer of the medulla are fine and only weakly immunostained. These processes were only seen in some vibratome preparations, but not in wholemounts or paraffin sections. The arborizations in the distalmost layer of the medulla, on the other hand, are strongly immunoreactive and were visible in all preparations. They form a tangential meshwork of ramifications with numerous varicosities (Fig. 3D). The immunoreactive arborizations in the medulla originate from fibers in Cuccati's bundle. Immunostained neurites in this bundle continue into the posterior optic tract (POT) and interconnect the two medullae without arborizations in the median protocerebrum (Fig. 1). Some preparations clearly show that the labelled fibers in the POT are derived from the large PDFMe cell bodies (Fig. 2).

A fascicle of PDH-immunoreactive fibers originating at the anterior base of the medulla joins Cuccati's bundle as it runs to the posterior lateral protocerebrum. These fibers do not enter the POT toward the contralateral hemisphere but project dorsally to the lateral horn of the protocerebrum. Some preparations show that these projections most likely originate from the four smaller PDFMe neurons (Fig. 2). In the lateral horns dorsofrontal to the calyx of the mushroom body, the immunostained fibers form varicose arborizations (Fig. 3C) which overlap with the processes of PDH-immunoreactive neurons in the median protocerebrum (PDFCa neurons, see below). No immunostained cell bodies and fibers were found in the lobula complex and in the lamina, but in some vibratome preparations, the lamina shows a slightly higher background staining than the other areas of the optic lobe.

In the central brain of *D. melanogaster*, faintly PDH-immunostained cell bodies were observed only in paraffin sections and in a few vibratome preparations. Up to four cells are immunostained dorsal to the calyx of each mushroom body (PDFCa neurons, Fig. 1) and up to six cells in the pars intercerebralis (PDFPi neurons, not shown). The PDFCa and the PDFPi neurons have variable sizes ranging from 4 to 8 μm. Immunoreactive processes, possibly originating from the PDFCa cells, run medially towards the pars intercerebralis (Fig. 1B). They turn frontally, project through the median bundle toward the esophageal foramen, and arborize in the tritocerebrum (Figs. 1B, 3A). In contrast to the PDFCa somata, the immunoreactive fibers in the median bundle and around the esophageal foramen are also immunostained in vibratome sections and in wholemounts.

The adult thoracic nervous system of *D. melanogaster* consists of the thoracic and the fused abdominal ganglia. The thoracic ganglia are free of PDHLI but, in the abdominal ganglia, four large (two on each side) and two small somata near the ventral midline exhibit PDHLI (Fig. 4). The large somata are strongly immunoreactive, whereas the small ones exhibit fainter staining and are not labelled in some preparations. Axons of these neurons project through the median abdominal nerve trunk into the posterior abdomen. Two PDH-immunoreactive fibers could be traced as far as 1 mm into the median nerve (Fig. 8), but their targets could not be identified.

Staining pattern in the mutants

The structural anomalies of the mutants of *D. melanogaster* which were studied immunocytochemically are listed in Table 1. The pattern of PDHLI in the thoracic nervous system of all mutant flies was indistinguishable from that of the wild-type but characteristic and reproducible differences from the immunostaining in the wild-type were found in the brain of the mutants (Figs. 5, 6).

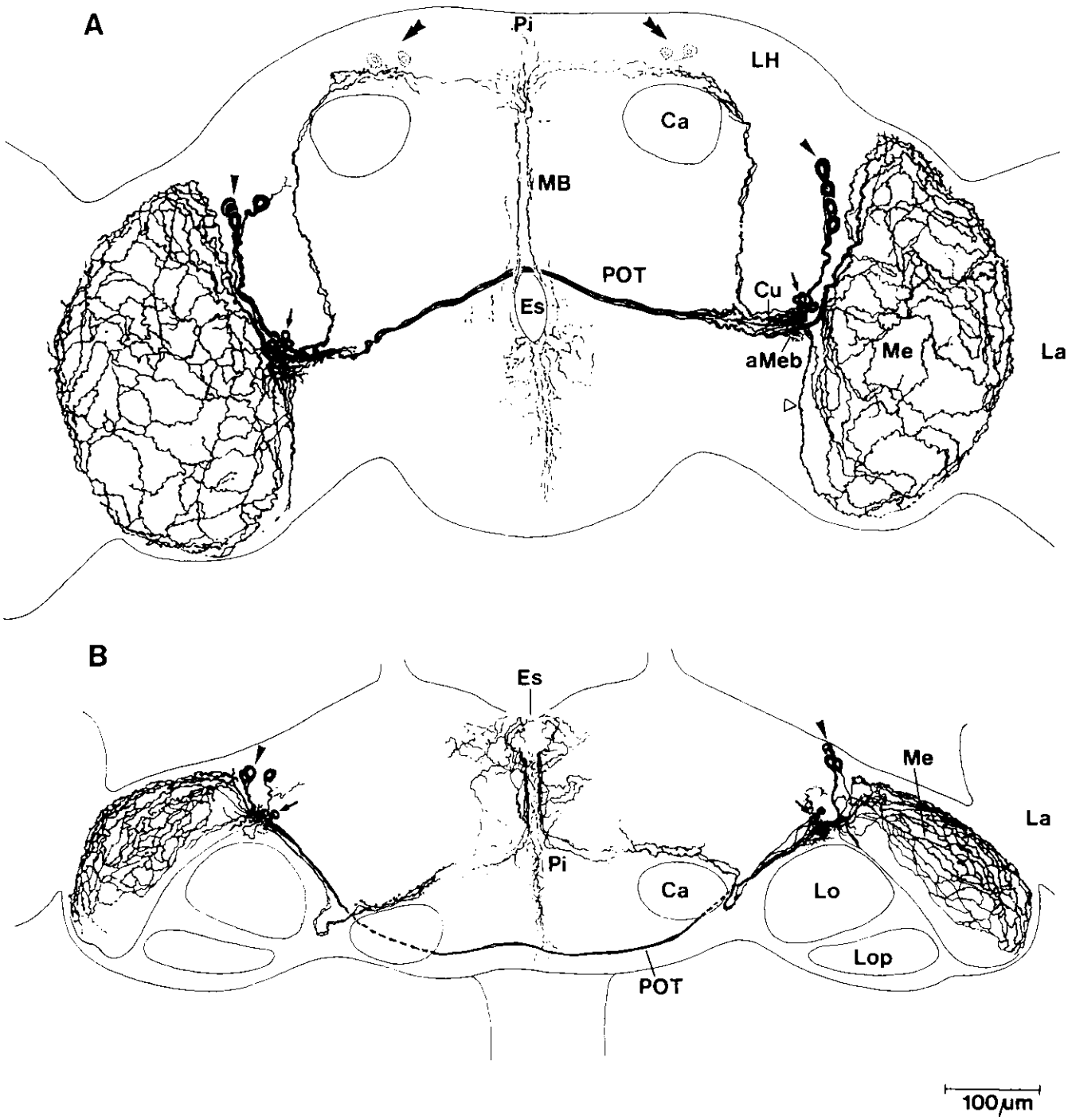

Fig. 1. Frontal (**A**) and horizontal (**B**) reconstruction of pigment-dispersing hormone-like immunoreactivity (PDHLI) in the brain of wild-type *D. melanogaster*. The ocelli and the ocellar tract have been omitted in this and in all other reconstructions. Arrowheads point to the large PDFMe neurons and arrows to the small PDFMe neurons. The small PDFMe neurons lie at the anterior base of the medulla (aMeb), a region with dense immunoreactivity. From here, PDH-immunoreactive fibers invade the medulla (Me). Other fibers project dorsally and innervate a small area dorsofrontal to the calyces of the mushroom bodies (Ca), and some axons enter the posterior optic tract (POT). The right hemisphere in A shows that immunostained fibers enter the distalmost layer of the medulla via the POT apparently from the contralateral PDFMe neurons. Only one fiber bundle running ventrally (open arrowhead) might be derived from the ipsilateral PDFMe neurons. Immunoreactive arborizations, probably from PDFCa cells (double arrowheads), are near the pars intercerebralis (Pi) and via the median bundle (MB) in the tritocerebrum around the esophageal foramen (Es). Cu, Cuccati's bundle; La, lamina; LH, lateral horn; Lo, lobula; Lop, lobula plate.

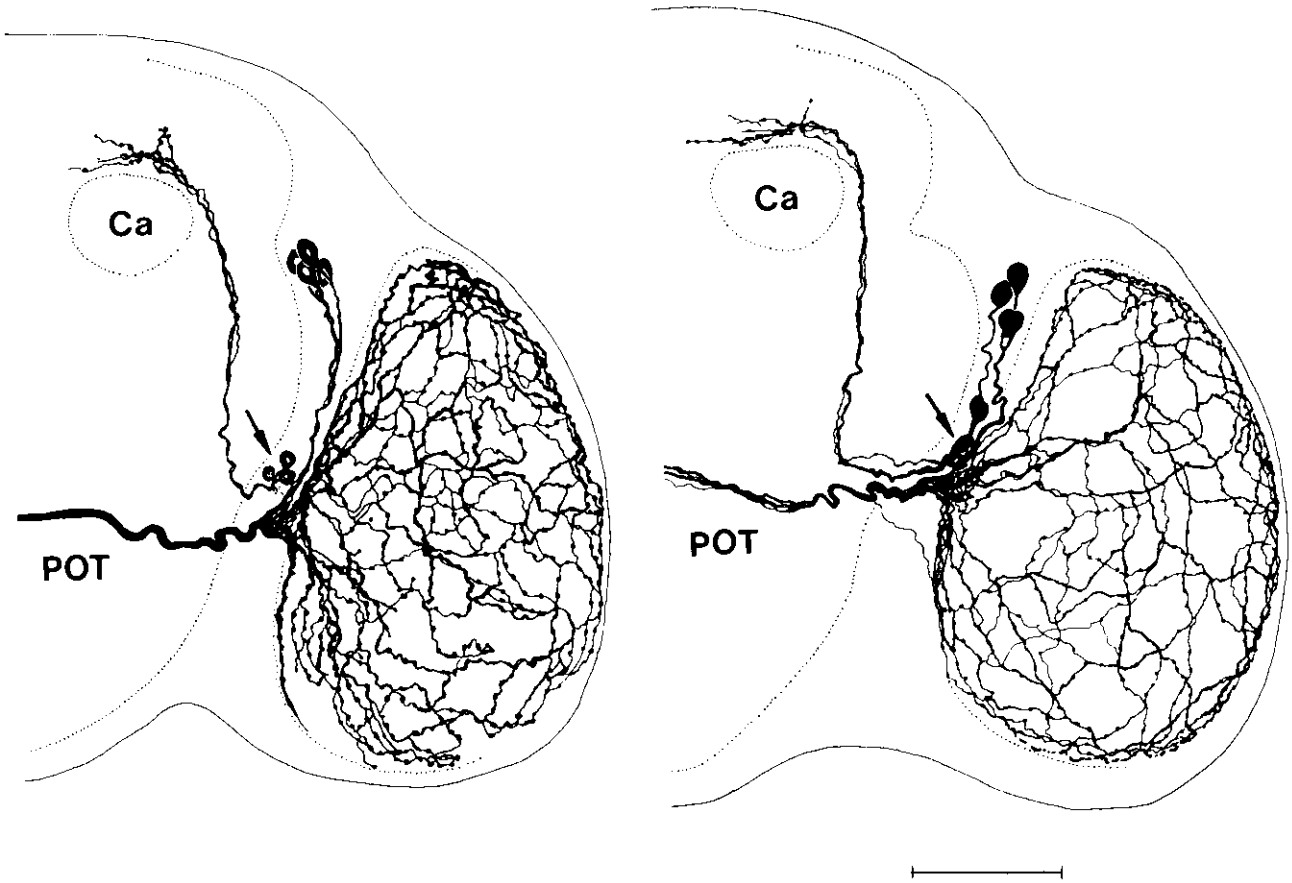

Fig. 2. Frontal reconstructions of the PDFMe neurons and their branching pattern at the right base of the medulla of two different flies. In both animals, immunolabelled fibers projecting to the calyces (Ca) originate from the small PDFMe neurons (arrow) and are clearly distinct from fibers in the posterior optic tract (POT). Scale bar = 100 μm.

Small optic lobes (sol KS58). The number and location of PDH-immunoreactive cell bodies in the brain and abdominal ganglia of *sol* mutants is identical to that seen in the wild-type. The pattern of immunostained arborizations also closely resembles that of the wild-type. *Sol* mutants, however, have a smaller arborization field in the medulla as might be expected given the smaller size of the medulla (Table 1). Occasionally, immunoreactive fibers leave the POT in the median protocerebrum and project dorsally toward the calyces of the mushroom bodies (Fig. 5B, right panel) which was never observed in wild-type flies.

Sine oculis (so). Immunostaining of *so* flies is similar to that seen in *sol* mutants, except for a further reduced arborization field in the medulla (Figs. 5C, 6). More often than in *sol*, PDH-immunoreactive fibers leave the POT and project to the dorsal protocerebrum toward the calyces.

Small optic lobes;sine oculis (sol;so). The arborizations in the medulla are strongly reduced and could often not be distinguished from the intensely immunoreactive ramifications at the base of the medulla (Figs. 5D, 6). Many additional processes, derived from the POT, invade the superior median protocerebrum and the lateral horn (Figs. 5D, 6, 7). The tracts running toward the calyces appear to contain more immunoreactive fibers than do those in the wild-type. Furthermore, many immunostained fibers leave the POT in the posterior median protocerebrum and project dorsally. On their way to the dorsal protocerebrum, the fibers tend to fasciculate and form small tracts (Fig. 7A). In spite of a considerable interindividual variability in the pathways of immunoreactive fibers toward the dorsal protocerebrum (Fig. 7), these processes never invade the central complex or the mushroom bodies. Whenever fibers approach these neuropils, they either turn back or deviate along the outer surface of the neuropil (Fig. 7). Despite the abnormalities in their arborization pattern, the number and location of the PDH-immunoreactive somata is not altered in the *sol;so* mutants, and four large and four small PDFMe somata are visible (Figs. 6, 7B,C).

Some *sol;so* mutants have an eye remnant on one side of the head. The optic lobe of this side consists of lamina, medulla, and lobula complex and its size is a function of the size of the eye remnant (Power, '43). The eyeless side has an optic lobe typical of eyeless *sol;so* mutants. We used such flies to investigate the influence of the unilateral presence of an optic lobe remnant on the pattern of PDHLI. These flies show a very dense meshwork of immunolabelled fibers in the medulla ipsilateral to the eye remnant, both in the distal layer and in the serpentine layer (Figs. 5E, 7D,E). Both layers are connected by immunoreactive fibers. In addition, a few PDH-immunoreactive fibers invade the lamina (Fig. 7D). Apparently, these fibers do not terminate in the lamina but loop back into the medulla (Fig. 7D). Considerably fewer fibers derived from the POT invade the superior median protocerebum as compared to the number

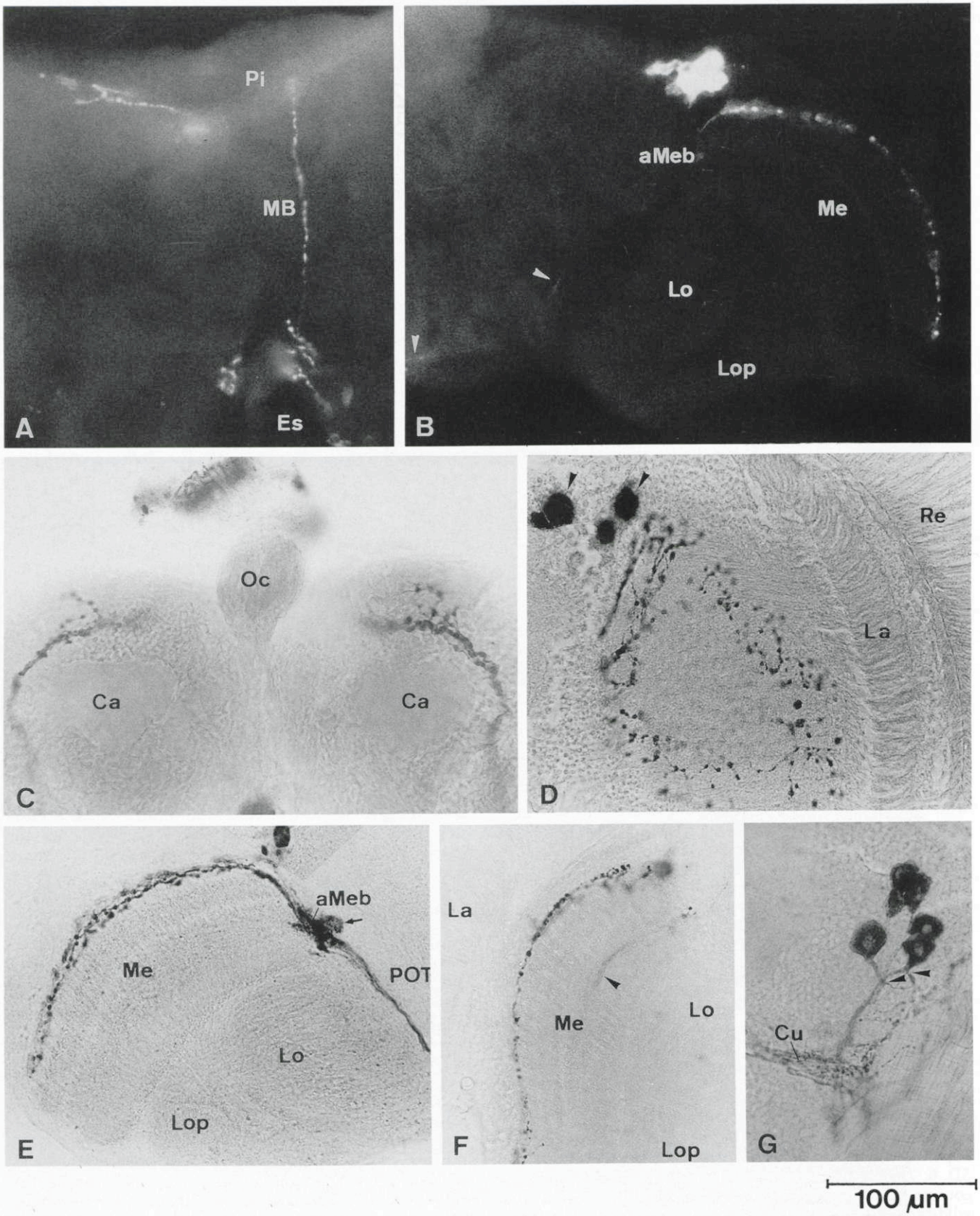
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Figure 3

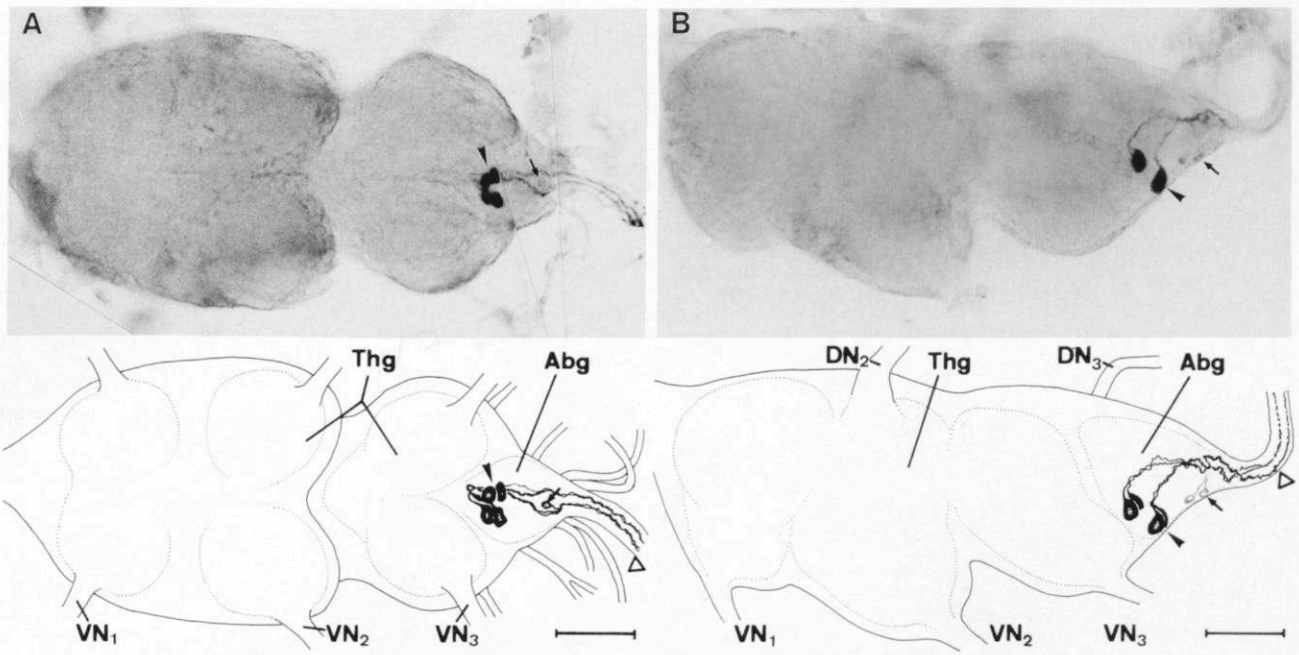

Fig. 4. Photographs and reconstructions of two wholemount preparations of the thoracic nervous system showing PDH-immunoreactive neurons in the ventral midline of the abdominal ganglia (Abg). **A:** Ventral view. **B:** Lateral view. Four large (arrowheads) and 1–2 small (arrows) PDH-immunoreactive somata can be seen. Fibers project from these cells via the median abdominal nerve trunk (open arrowheads) into the abdomen. Thg, thoracic ganglia; DN2–3, dorsal nerves; VN_{1-3}, ventral nerves innervating the legs. Scale bars = 100 μm.

of these seen in eyeless flies (Figs. 5E, 7D,E). This allows one to follow the path of single fibers in the median protocerebrum. Most neurites project dorsally to the calyces. Here they either fasciculate with other fibers or terminate close to the arborizations of the small PDFMe neurons. Some fibers project to the midline of the brain, fasciculate with others coming from the calyces, and run in the median bundle toward the esophageal foramen. In one preparation, a fiber projected dorsofrontally to the central complex. It looped around the central body by first running upward on the backside of the central body and then projecting downward in front of it (fiber 1 in Fig. 7E). The fiber continued ventrofrontally until it fasciculated with a PDH-immunoreactive neurite in the median bundle and projected toward the esophageal foramen. Another fiber ran laterofrontally and terminated in the lateral frontal protocerebrum (fiber 2, Fig. 7E). On its way, it approached the calyx of the mushroom body, turned subsequently and continued laterally.

Disconnected (disco 1). Neither flies exhibiting the connected or the unconnected phenotype (Table 1) of *disco* flies (Steller et al., '87) exhibit immunostaining in the PDFMe neurons (Figs. 6, 8A). As in the wild-type and in the other mutants, the PDFCa neurons near the calyces are still present (Fig. 8C), and occasionally the PDFPi neurons in the pars intercerebralis are also immunostained. Most flies show immunostaining in fibers of the median bundle (Fig. 8B) and around the esophageal foramen (Fig. 8D). As observed in some wild-type brains, the lamina exhibits a higher background staining in many *disco* flies and some animals show PDHLI in the nuclei of the photoreceptor cells of the retina (not shown). The neurons in the abdominal ganglia of *disco* flies always exhibit strong PDHLI (Fig. 8A).

per^0. PDH-immunostaining of the *per^0*-mutants was indistinguishable from that of the wild-type (not shown).

Fig. 3. Vibratome sections (A,B,E–G) wholemount (C), and paraffin section (D) showing details of the PDH-immunoreactive neurons in the brain of *D. melanogaster*. Preparations are either labelled by (A) Texas Red- or (B) fluorescein isothiocyanate (FITC)-conjugated secondary antibody or (C–G) immunostained by the peroxidase-antiperoxidase (PAP) technique. **A:** Frontal vibratome section showing a PDH-immunoreactive fiber in the median bundle (MB) in the right hemisphere, immunoreactive fibers running toward the MB in the left hemisphere, and some arborizations around the esophageal foramen (Es). Pi, Pars intercerebralis. **B:** Horizontal section of the right optic lobe at the level of the esophagus. Fibers from the somata of three large PDFMe neurons run to the anterior base of the medulla (aMeb) and invade the distalmost layer of the medulla (Me). Arrowheads point to immunoreactive fibers in the POT. Lo, lobula; Lop, lobula plate. **C:** Frontal view of the dorsal posterior brain showing varicose arborizations of the small PDFMe neurons dorsal to the calyces (Ca). Oc, ocellar tract with ocelli. **D:** Mounting of two consecutive frontal sections, showing the tangential network of varicose PDH-immunoreactive arborization in the distal medulla. Arrowheads point to the four large PDFMe somata. Lamina (La) and retina (Re) are without immunoreactive fibers. **E:** Horizontal vibratome section at the level of the esophagus showing dense fibrous immunostaining at the anterior base of the medulla (aMeb), a small PDFMe soma (arrow), and the origin of the POT. **F:** Horizontal vibratome section through the medulla showing PDH-immunoreactive fibers in the serpentine layer (arrowhead). **G:** Frontal-saggital vibratome section showing six instead of the normally four large PDFMe somata with axons entering Cucatti's bundle (Cu). Arrowheads point to possible side branches to the ipsilateral medulla.

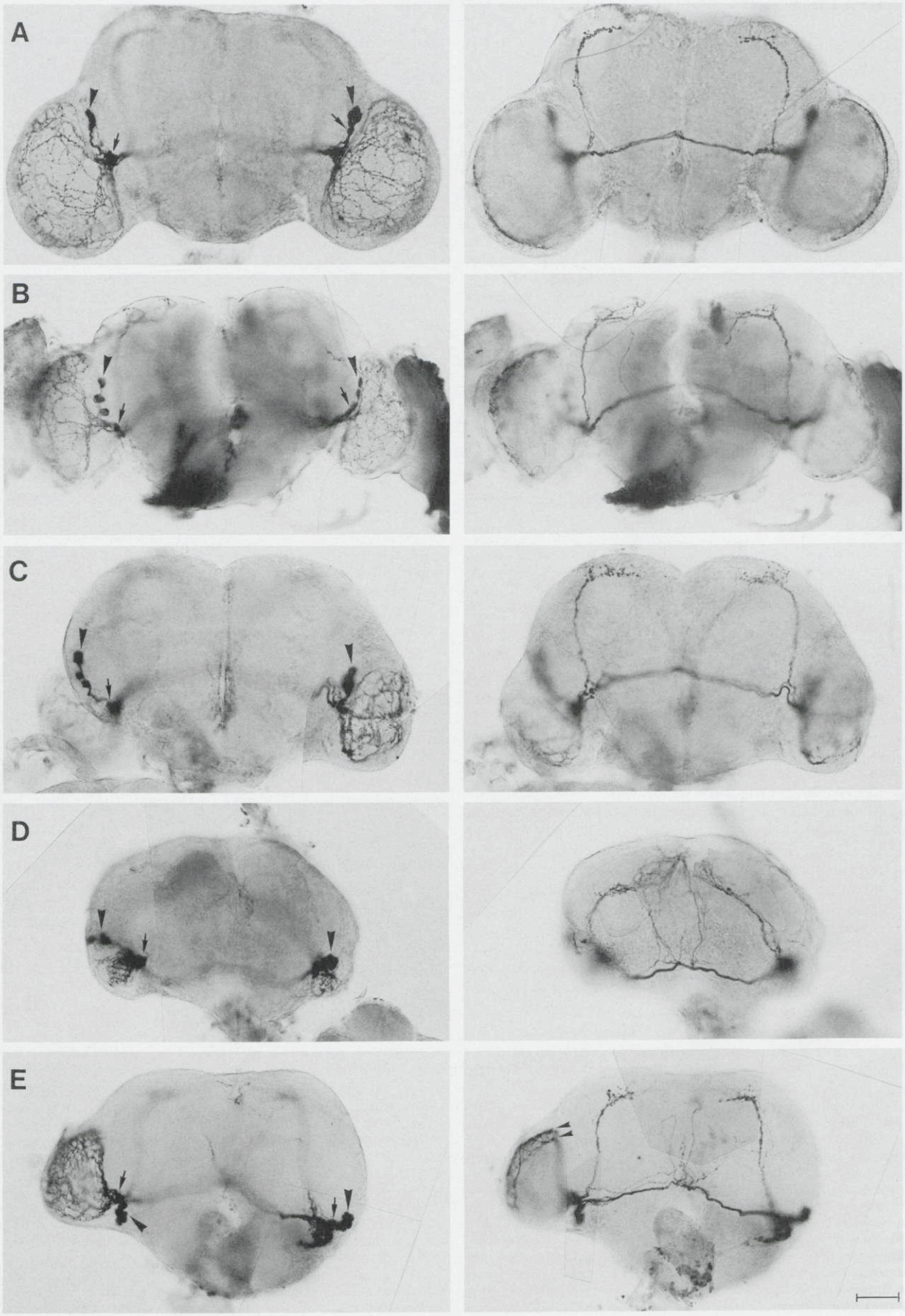

Figure 5

DISCUSSION

Two groups of neurons, the PDFMe neurons at the base of the medulla and a cell group in the abdominal ganglion, show intense PDHLI in the nervous system of *D. melanogaster*. The high affinity to the β-PDH antiserum strongly suggests that these neurons contain peptides with close similarity to the crustacean β-PDH. In addition, small groups of PDH-immunoreactive neurons near the calyces (PDFCa neurons) and in the pars intercerebralis (PDFPi neurons) show considerably weaker immunostaining and were predominantly found in paraffin sections. This might be explained by better penetration of antisera into the 10 μm paraffin sections as compared to the 30 μm vibratome sections. The PDFCa and PDFPi neurons, therefore, either contain only small amounts of PDH-like peptides or molecules with lower affinity to the antiserum. We assume that the PDH-immunoreactive fibers in the median bundle and around the esophageal foramen are parts of the PDFCa neurons, because in *disco* mutants, where the PDFMe neurons are missing, the PDFCa somata, fibers in the median bundle, and terminals around the esophagus are still immunostained.

PDFMe neurons. The PDFMe neurons are the most prominent PDH-immunoreactive neurons in the brain of *D. melanogaster*. In each hemisphere, a group of four small and four large somata could be distinguished. This is in accordance with results in the blowfly *P. terraenovae* (Nässel et al., '91). In orthopteroid insects, the number of PDFMe cells is higher (9–17 cells) and, as in flies, their somata are of different sizes (Homberg et al., '91a). The PDFMe neurons of orthopteroids appear to be morphologically heterogenous and individual neurons innervate different areas in the midbrain. This seems to be also true for *D. melanogaster*. Here we have evidence that the four small PDFMe neurons send axons to the calyces, whereas the four large PDFMe neurons have tangential arborizations in the medulla and connect both medullae via the POT. In orthopteroid insects, more regions of the midbrain are invaded by immunostained fibers than in *P. terraenovae* and *D. melanogaster*. This might be due to the higher number of PDFMe neurons which form several subpopulations with individual branching patterns in the brain.

The four large PDFMe neurons of *D. melanogaster* are medulla tangential neurons as defined by Fischbach and Dittrich ('89). Their cell bodies lie anterior to the medulla; their dendrites and/or axonal terminals extend tangentially throughout the entire retinotopic map of the medulla, and their axons connect both medullae via the POT. As judged by light microscopic criteria, the varicose arborizations in the distal medulla appear to be presynaptic, while the faintly immunostained processed in the serpentine layer might be dendritic. Both ramifications seem to originate from the large PDFMe neurons. The origin of the arborizations at the anterior base of the medulla is not clear. These processes might be part of the four small or of all eight PDFMe neurons.

Since a significant proportion of the axons in the POT are of contralateral origin (Fischbach and Dittrich, '89), the PDH-immunoreactive tangential arborizations in the distal medulla could be derived from either the ipsilateral or the contralateral PDFMe neurons. Some preparations suggest that the latter case is true (Fig. 1A). Consequently, the putatively dendritic trees in the serpentine layer might originate from the ipsilateral large PDFMe neurons. Giant medulla tangentials of *Musca domestica* show exactly this branching pattern (Strausfeld, '76, plate 7.14).

Nevertheless, more studies are needed to confirm this proposed arborization scheme of the PDFMe neurons. Electron microscopic studies will help to decide whether the terminals in the two medulla layers are pre- or postsynaptic. Studies of gynandromorphs which carry the phenotype of the wild-type on one side of the brain and the *disconnected* phenotype (without PDFMe neurons) on the other side could be helpful in the analysis of the shape of the PDFMe neurons.

Immunostaining in the mutant "sol;so." PDH-immunoreactive fibers in wild-type *D. melanogaster* project via the POT to the contralateral medulla, whereas this connection between the medullae of both sides is altered in *sol;so* mutants. In these mutants, many PDH-immunoreactive fibers leave the POT in the median posterior protocerebrum and often arborize in or close to regions where other PDH-immunoreactive neurons have terminals. These fibers project from the POT either dorsally to the calyces close to the terminals of the small PDFMe neurons, or they follow the axons of the PDFCa neurons and end around the esophagus. It is tempting to speculate that the changed arborizations of the four large PDFMe neurons is caused by the lack of their target neurons in the medulla. In *sol;so*, all columnar neurons of the optic lobe die during embryogenesis (Fischbach and Technau, '84). As a consequence, the tangential PDFMe neurons cannot establish normal contacts with columnar neurons in the distal medulla and might instead make alternative connections in the central brain, which is not affected by the mutation. PDH-immunoreactive fibers in the POT might not connect both medullae as in the wild-type but follow the POT for a short distance and then leave it towards the dorsal protocerebrum. Some fibers turn back to the POT as can be seen from loops leaving the POT. It is even conceivable that fibers first run in the POT to the contralateral optic lobe and then turn back in the POT after failing to make adequate contacts. This could explain why the POT in most *sol;so* mutants contains at least as much immunoreactive fibers as it does in the wild-type. Additional evidence for compensatory innervation of the median protocerebrum in *sol;so* mutants comes from the staining pattern in mutants with one medulla present. In these flies, the number of PDH-immunoreactive fibers which leave the POT in the median protocerebrum is significantly reduced in comparison to animals which lack both medullae. In addition, the medulla remnant of these animals shows a very dense innervation by PDH-immunoreactive fibers (Fig. 7D,C).

PDHLI in the mutant disconnected. In *disco* mutants, the PDFMe neurons are not immunostained, whereas the PDFCa neurons, the PDFPi neurons, and the cell group in the abdominal ganglia show normal immunoreactivity. The absence of immunostaining in the PDFMe neurons may

Fig. 5. Wholemount preparations showing PDHLI in a wild-type brain (**A**) and in the brains of the mutants *small optic lobes* (**B**) *sine oculis* (**C**) and *small optic lobes;sine oculis* (**D,E**). D shows an eyeless fly and E a fly with unilateral eye remnant. The left panels show frontal views. Arrowheads point to the large PDFMe somata, arrows to the small ones. The right panels show views from the back side of the brains. Arrowheads in the right panel of E point to PDHLI in the distalmost layer and in the serpentine layer of the medulla. Both layers are connected by PDH-immunoreactive fibers. For further details see text. Scale bar = 100 μm for all panels.

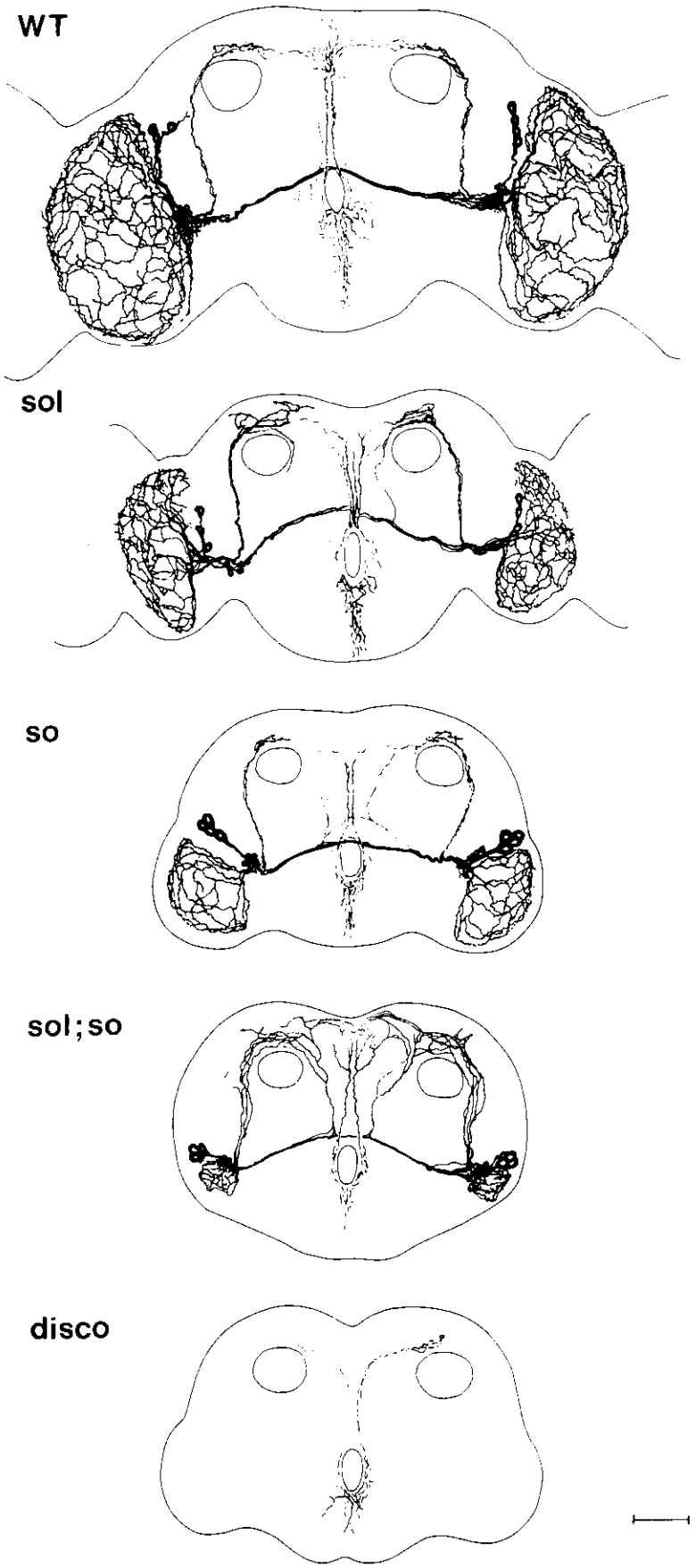

Fig. 6. Frontal reconstructions of the PDHLI in the wild-type brain (WT) and in the mutants *small optic lobes* (sol), *sine oculis* (so), *small optic lobes;sine oculis* (sol;so), and *disconnected* (disco). The reconstruction in *disco* was made from paraffin sections; all others were made from wholemount preparations. For explanations see text. Scale bar = 100 μm.

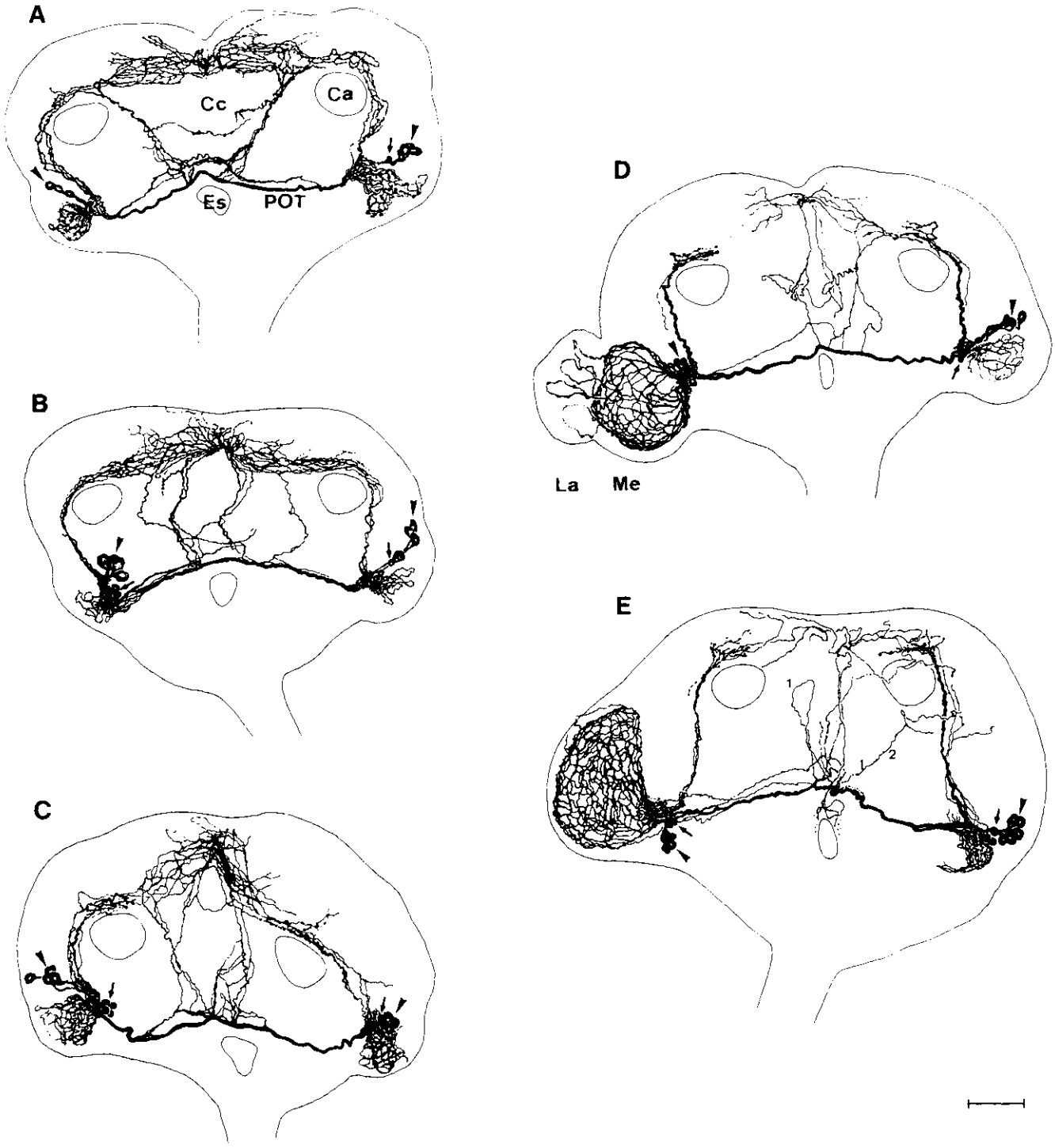

Fig. 7. Reconstructions of PDHLI in the brains of different *sol;so* mutants. **A–C:** Completely eyeless flies. **D,E:** Flies with unilateral eye remnants showing dense PDHLI in the underlying medulla (Me) and PDH-immunoreactive fibers invading the lamina (La in D). In E, the lamina was torn off during preparation. The PDH-immunoreactive fibers in the median bundle are omitted in all preparations except in E. Arrowheads point to the large, and arrows, to the small PDFMe somata. In spite of the interindividual variability, the PDH-immunoreactive fibers never invade the central complex (Cc) or the calyces (Ca) of the mushroom bodies. The fibers which seem to cross these neuropils are actually behind or in front of them. The pathways of fibers 1 and 2 in E are described in the text. Es, esophageal foramen; POT, posterior optic tract. Scale bar = 100 μm.

have two reasons. Either the PDFMe cells contain no or not enough PDFs, or the PDFMe neurons are missing. The latter alternative appears more likely, because *disco* mutants lack the optic pioneer neurons which have axons in the POT and serve as orientation for medulla tangential neurons (Tix et al., '89). In early pupal stage, fibers of

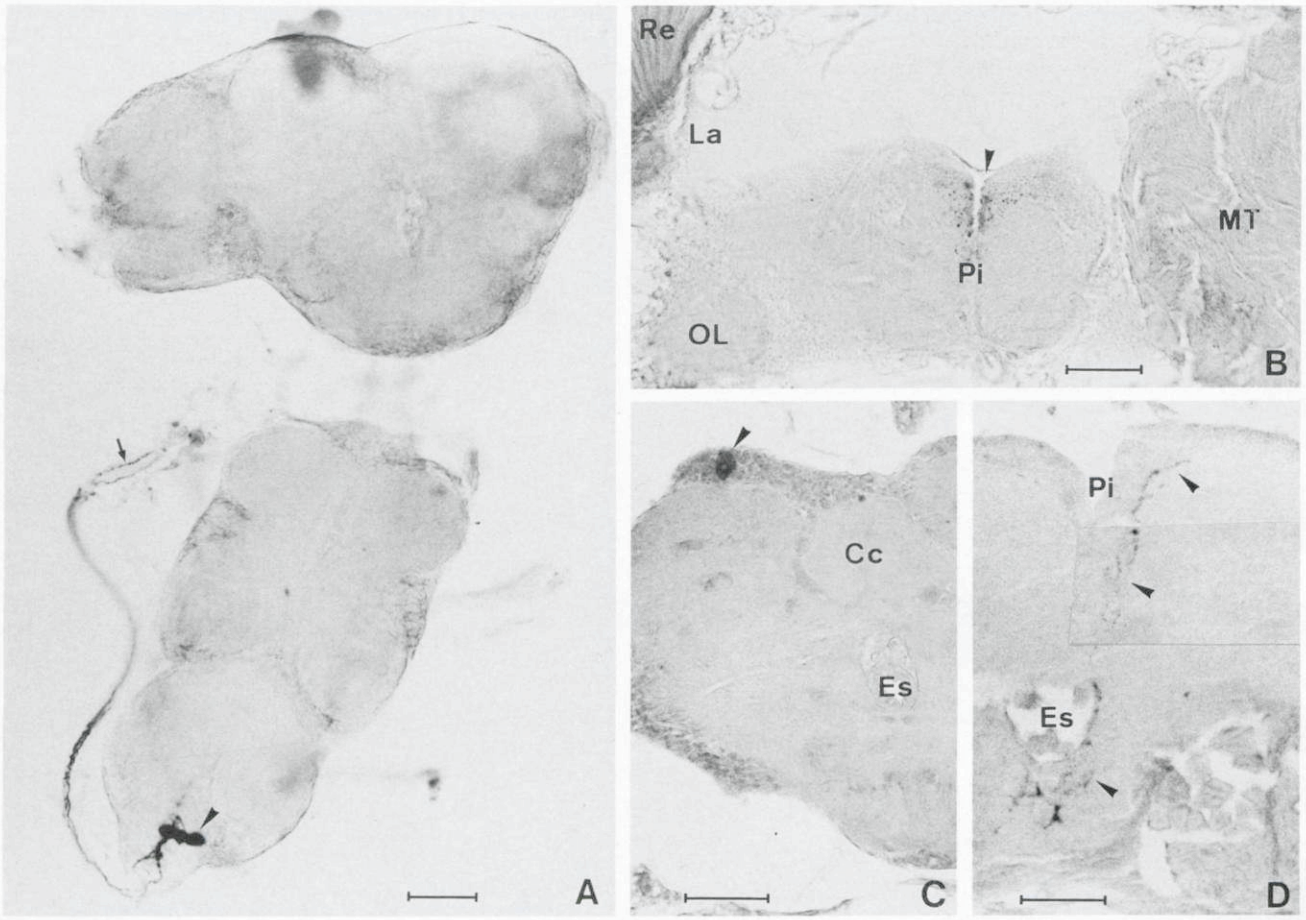

Fig. 8. PDHLI in the mutant *disco*. **A:** Wholemount of brain (left optic lobe connected, right one unconnected) and thoracic nervous system showing the large PDH-immunoreactive neurons in the abdominal ganglia (arrowhead). PDH-immunoreactive fibers of these neurons could be traced as far as 1 mm into the abdominal median nerve (arrow). **B:** Horizontal vibratome section showing PDHLI (arrowhead) in the median bundle in front of the pars intercerebralis (Pi). The left optic lobe has the connected phenotype. The lamina (La) and the disorganized other parts of the optic lobe (OL) can be distinguished. The right optic lobe has the unconnected phenotype. A mass of disorganized muscle tissue (MT) occupies the space between compound eye and central brain. Re, retina. **C:** Frontal paraffin sections showing a PDFCa cell body dorsal to the left calyx (arrowhead). Cc, Central complex; Es, esophageal foramen. **D:** Mounting of two consecutive frontal paraffin sections showing PDH-immunoreactive fibers (arrowheads) projecting from the right calyx to the pars intercerebralis and then via the median bundle to arborizations around the esophageal foramen (ES). Scale bars = 100 μm.

medulla tangentials grow centrifugally along the axons of the optic pioneer neurons toward the medulla (Tix et al., '89). Without contacting these pioneer neurons in *disco* mutants, the PDFMe neurons probably degenerate as do other medulla tangential neurons during pupal development (Steller et al., '87).

Possible relation between PDH-immunoreactive neurons and neurosecretory cells

Previous studies in the optic lobes of Crustacea have demonstrated PDHLI in interneurons and in neurosecretory cells with terminals in the sinus gland (Dircksen et al., '87; Mangerich et al., '87; Rao and Riehm, '89; Dircksen, '92). In the cricket, *Acheta domesticus,* the moth, *Manduca sexta,* and the fly, *Musca domestica,* PDHLI was found in neurosecretory cells of the pars intercerebralis (Homberg et al., '91a,b; Zwerenz and Helfrich-Förster, unpublished results). The PDH-immunoreactive neurons in the brain of *D. melanogaster* appear to be interneurons. However, Köpf ('57), Rensing ('66), and Helfrich ('85) have shown that neurosecretory cells of similar number and size lie in the same positions as the PDFCa and the PDFMe neurons. Recent studies revealed that the neurosecretory cells anterior to the medulla are absent in *disco* mutants (Helfrich-Förster, '91). Finally, preliminary ultrastructural investigations of the PDFMe somata of *D. melanogaster* show that PDHLI is concentrated in neurosecretory granules (Dircksen and Helfrich-Förster, unpublished), which further support a neurosecretory role of these neurons. Therefore, the PDFMe neurons might indeed be identical with the described neurosecretory cells anterior to the medulla.

Physiological role of the PDFMe neurons

The PDFMe neurons show considerable similarities in all insects studied so far. In *D. melanogaster, P. terraenovae,* the beetle, *Pachymorpha sexguttata,* and in orthopteroid insects, these neurons have somata at the anterior base of the medulla. They show striking similarities in their branching pattern in the medulla, and they apparently contain

closely related peptides (Homberg et al., '91a; Nässel et al., '91; Fleissner and Fleissner, '92). Therefore, the PDFMe neurons are probably homologous neurons in these and possibly other insect species and might play a common physiological role. Circumstantial evidence suggests that the PDFMe neurons are involved in the circadian system of cockroaches and crickets (Homberg et al., '91a; Stengl and Homberg, '92). Our observations add further evidence to the possible involvement of the PDFMe neurons in the circadian timekeeping system. Behavioral studies on *so* and *sol* mutants suggest that circadian pacemaker neurons in *D. melanogaster* are tangential neurons of the medulla (Helfrich and Engelmann, '83). The PDFMe neurons are medulla tangential neurons which interconnect the medullae of the two hemispheres via the POT. In the normally rhythmic mutants, *so* and *sol*, the arborization pattern of the PDFMe neurons is surprisingly normal, in spite of the reduced size of the medulla in these mutants. In the mutant, *sol;so* the connection between the bilaterally distributed PDFMe neurons via the POT seems to be deranged because fibers project to the dorsomedian protocerebrum instead of arborizing properly in the contralateral medulla. This might explain the splitting often observed in the locomotor activity rhythm of *sol;so* mutants (Helfrich, '86). In the arrhythmic *disco* mutants, the PDFMe neurons are not immunostained and seem to be absent. The possible neurosecretory role of the PDFMe neurons adds further evidence to their participation in the circadian system because hormonal factors seem to be involved in the control of circadian locomotor activity in *D. melanogaster* (Rensing, '66; Handler and Konopka, '79). Interestingly, the anterior base of the medulla, which is densely supplied by immunoreactive processes from PDFMe neurons, appears to receive direct visual input from extraretinal photoreceptors located at the posterior edge of the compound eye (Hofbauer and Buchner, '89). These projections are still intact in *so* flies, which can be synchronized by light to circadian rhythms even when they are eyeless (Helfrich and Engelmann, '83). This suggests that these extraretinal photoreceptors are the photoreceptors of the circadian system and perhaps directly contact the PDFMe neurons at the anterior base of the medulla.

Possible identity of PDH-immunoreactive neurons with neurons containing the *period* protein

The *period* gene product appears to be critically involved in the circadian system and is widely distributed in the central nervous system of *D. melanogaster* (Hall and Kyriacou, '90). The *period* protein is expressed in photoreceptor cells of the retina, in many glial cells, and in some neuronal cells of the brain (Ewer et al., '92). The neurons containing the *period* protein lie in the posterior dorsal cortex (dorsal to the calyces) like the PDFCa neurons, and anterior to the medulla (LNs) like the PDFMe neurons. In both areas, the *period* protein-containing neurons and the PDH-immunoreactive neurons are of similar size. Recent double label experiments have investigated PDH immunoreactivity in transformant flies, which carry the gene for the bacterial enzyme β-galactosidase as a reporter of *period* gene expression (Helfrich-Förster, '93). In these flies, the PDFMe cells, indeed, express the *period* gene and appear to be a subpopulation of the *period* protein-containing LN neurons near the medulla. A full report of that study will be given elsewhere (Helfrich-Förster, '93).

The apparently normal pattern of PDHLI found in the arrhythmic per^0 mutants shows that the per^0 mutation does not prevent the expression of PDH-like peptides in the PDFMe neurons. However, it is not clear whether synthesis rate or time of release of the peptide is affected by the mutation. Assuming that the *period* protein is directly involved in the pacemaking system, the PDFs might rather be important as chemical signals from the pacemakers to the effector organs. This is in accordance with findings of Zerr et al. ('90) and Hardin et al. ('92), who showed that the *disco* mutants which lack the LNs still exhibit circadian rhythmicity in photoreceptors and glial cells. Furthermore, mosaic flies which were per^0 in the LNs and wild-type in *period* protein-containing glial cells still show some rhythmicity in locomotor activity (Ewer et al., '92). The authors concluded that the LNs are not the only pacemakers in the brain of *D. melanogaster* (Ewer et al., '92) and that they might rather be important for the pathway from the pacemakers to the effector organs (Hardin et al., '92). The same might be true for the PDFMe neurons.

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Note added in proof: Recently Nässel et al. ('93) described PDH-immunoreactivity in the nervous system of *Drosophila melanogaster*. In contrast to our findings these authors reported 6 large and only 2 small PDH-immunoreactive somata at the anterior medulla base. In a few animals, we also found 6 large PDFMe neurons (See Fig. 3G), but usually 4 large and 4 small PDFMe cells were detected.