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# Cell-specific immuno-probes for the brain of normal and mutant *Drosophila melanogaster*

## I. Wildtype visual system

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**Summary.** We have screened antibodies for immunocytochemical staining in the optic lobes of the brain of *Drosophila melanogaster*. Seven polyclonal antisera and five monoclonal antibodies are described that selectively and reproducibly stain individual cells and/or produce characteristic staining patterns in the neuropile. Such antisera are useful for the cellular characterization of molecular and structural brain defects in visual mutants. In the wildtype visual system we can at present separately stain the following: the entire complement of columnar "T1" neurons; a small set of presumptive serotonergic neurons; some 3000 cells that contain and synthesize γ-amino butyric acid (GABA); and three groups of cells that bind antibodies to $Ca^{2+}$-binding proteins. In addition, small groups of hitherto unknown tangential cells that send fine arborizations into specific strata of the medulla, and two patterns of characteristic layers in the visual neuropile have been identified by use of monoclonal antibodies generated following immunization of mice with homogenates of the brain of *Drosophila melanogaster*.

**Key words:** Neurogenetics – Visual system – Neurotransmitters – Serotonin – GABA – $Ca^{2+}$-binding proteins – Monoclonal antibodies – *Drosophila melanogaster* (Insecta)

The brain of invertebrates provides a suitable model in neuroscience because most of its neurons can be reliably identified and localized on the basis of position, morphology, function and, as we discuss presently, biochemical diversity.

Various neuroanatomical methods have been used for the identification of cells in insect brains (see review by Strausfeld 1983). Immunocytochemistry has greatly extended the number of neurons that can be stained reproducibly in each individual animal. This technique has been utilized to localize putative neurotransmitters or their synthetic enzymes in various insects including flies (Duve et al. 1983; Nässel et al. 1983; 1987; Nässel and Elekes 1984; Meyer et al. 1986; Datum et al. 1986; for reviews see Nässel 1987; Hardie 1988), and more recently also in *Drosophila melanogaster* (White and Vallés 1985; Buchner et al. 1986; Vallés and White 1986, 1988; White et al. 1986; Budnik et al. 1986; Budnik and White 1988; Gorczyca and Hall 1987). The analysis of neurotransmitters used by identified neurons is of general significance for the investigation of the relation of structure and pharmacology of the brain to its function.

Reproducible cell-specific staining in the brain of *Drosophila melanogaster* is of particular importance in view of the large number of neurological mutants that have been isolated in recent years (see reviews by Hall 1982; Fischbach and Heisenberg 1985). Most of these mutants have been obtained by screening for gross structural or behavioural defects. However, using the immunocytochemical techniques described below, it should be possible to specify brain defects in several of the neurological mutants at the level of individual cells or even in terms of their molecular composition. Here, we report 12 different antibody probes that identify individual cells or that produce highly specific staining patterns in the optic lobes of wildtype *Drosophila melanogaster*. In preliminary studies, some of these probes have been used to characterize defects at the cellular level in the optic lobes of mutants, an aspect that will be dealt with in a subsequent paper (Part II of this series).

### Materials and methods

Adult *Drosophila melanogaster*, 3–10 days old, of the wildtype strain "Berlin" were used.

*Immunocytochemistry.* Details of the technique used for immunostaining brain sections of *Drosophila* have been described previously (Buchner et al. 1986). Briefly, flies were fixed in aldehyde fixatives (as specified below) after removal of the proboscis and air sacks ventral to the brain. They were washed overnight in 25% sucrose solution, frozen and sectioned at 10 μm on a cryostat microtome. For staining with primary rabbit antisera (dilution 1:1000 unless indicated differently), we followed the procedures recommended for the peroxidase-anti-peroxidase technique (Dako Corp. Santa Barbara, USA). With sheep serum (di-

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lution 1:2000) or mouse monoclonal supernatant (dilution 1:2), we used the biotin-avidin technique (Vector Laboratories Inc. Burlingame, USA). In both cases 3,3′-diaminobenzidine (30 mg in 50 ml Tris-buffered saline + 50 µl 30% $H_2O_2$) was employed as the chromogen.

The T1-cell-specific antiserum (HHA09) was obtained from a rabbit immunized with the 11 amino acid peptide "hydra head activator" (HHA). There is, however, no evidence that the antigen recognized in the T1 cell is related to HHA. Rather, test stainings with two further antisera (HHA10, 12/1) and two monoclonal antibodies (B74, F77) raised against HHA (courtesy H.C. Schaller) gave no indication of selective binding to T1 cells. For the stainings with HHA09 serum or rabbit-anti-serotonin (5-HT) serum (Immuno-Nuclear Corp. Stillwater, USA (INC)) we used a 3 h fixation in phosphate-buffered 4% paraformaldehyde. For the γ-amino butyric acid (GABA) antibody (rabbit-anti-GABA, (INC)) the best results were obtained with 1 h fixation in phosphate-buffered 4% paraformaldehyde, 0.5% glutaraldehyde, and primary antibody at 1:2000 dilution. For the glutamic acid decarboxylase (GAD) antibody (sheep-anti-rat brain GAD, the characteristics of which have been described elsewhere [Oertel et al. 1981]), we used 2 h fixation in phosphate-buffered 4% paraformaldehyde.

Sections incubated with pre-immune serum instead of specific first antibody displayed staining in the retina, fat body, and muscle, but only weak homogeneous background in brain tissue. Omission of primary antiserum eliminated all detectable staining indicating that endogenous peroxidase was absent or negligible. Specificity of the GABA staining was further tested by a preabsorption experiment. GABA-bovine serum albumin (BSA) conjugate was obtained by following the regime for the production of GABA antigen (Storm-Mathisen et al. 1983). We conjugated 100 µmol GABA to 12 mg BSA in sodium phosphate buffer (2 ml, 0.1 M, pH 7.4) by adding 100 µmol glutaraldehyde (Sigma, grade I). By this procedure one obtains, after thorough dialysis, approximately 0.4 µmol amino acid bound per mg BSA (Storm-Mathisen et al. 1983). We used the same protocol but either omitted GABA or replaced it with glycine or glutamate to produce control BSA and conjugates of glycine-BSA and glutamate-BSA, respectively. To 1 ml GABA antibody solution (dilution 1:2000), we added a constant amount of BSA containing 0, 0.1, 1, 10 or 100 µg conjugated GABA, or 10 µg conjugated glycine, or 10 µg conjugated glutamate. This was achieved by the appropriate mixing of amino acid-BSA and control BSA. To exclude any variations in experimental parameters, immunocytochemical experiments with these preabsorbed antisera and with the unmodified antiserum were always carried out simultaneously. Whereas preabsorption with BSA alone caused some reduction in both specific staining and background, no further reduction was observed using 0.1 µg conjugated GABA and 10 µg conjugated glycine or glutamate. No specific staining was obtained using 10 µg or 100 µg conjugated GABA. With 1 µg conjugated GABA, the most prominent features of the GABA staining (e.g. the difference between the ellipsoid and fan-shaped body in the central brain) were faintly visible.

Five antisera against $Ca^{2+}$-binding proteins were tested in the present study: (i) a serum against chicken muscle calbindin D-28K (CB28), (ii) a serum against rat muscle parvalbumin (PV), (iii) a serum against carp muscle PV, (iv) a serum against sarcoplasmic $Ca^{2+}$-binding protein I (SCP I), and (v) a serum against SCP II. The production and characterization of the first three antisera have been described elsewhere (Pironi and Gosselin 1974; Roth et al. 1982; Heizmann and Celio 1987). SCP I and SCP II were isolated from muscle of *Amphioxus* (*Branchiostoma lanceolatum*) (Kohler et al. 1978; Takagi et al. 1986). For the production of polyclonal antisera, rabbits were injected subcutaneously with 0.2 mg of either SCP I or SCP II dissolved in 0.75 ml 0.145 M NaCl, emulsified in 0.75 ml Freund's complete adjuvant. The injection was repeated twice at two-week intervals. The immunoglobulin fraction was isolated using standard procedures. The anti-SCP II antibodies were affinity purified by passage over a column of Sepharose 4B-conjugated SCP II. Antibodies against SCP I cross-react with SCP II and vice versa in an indistinguishable way in immunodiffusion, affinity chromatography and Western blotting. However, in these tests they do not cross-react with other structurally related $Ca^{2+}$-binding proteins such as troponin C from skeletal muscle of rabbit or calmodulin from bovine brain (cf. Cox 1986). All five antisera were used at a dilution of 1:1000 on frozen sections of *D. melanogaster* heads fixed for between 1 and 8 h in 4% paraformaldehyde.

The generation and screening of monoclonal antibodies will be described in detail elsewhere (Hofbauer, in preparation). Briefly, heads of *Drosophila melanogaster*, or brains removed from freeze-dried heads, were homogenized prior to i.p. injection into mice. Hybridoma supernatants were assayed for mouse immunoglobulin chains by enzyme-linked immunosorption assay (ELISA). The positive supernatants were directly screened by the immunocytochemical procedure described above for selective staining in brain of adult *D. melanogaster*.

## Results

A representation of a horizontal section (Fig. 1) that cuts through the head of *Drosophila melanogaster* at a level ventral to the oesophageal canal shows the outlines of the four optic neuropiles (lamina, medulla, lobula and lobula plate) and the suboesophageal ganglion. Neuronal cell bodies are located in the cellular rind surrounding the neuropile masses.

### *T1-cell-specific staining*

Selective staining with the HHA09 antiserum is found only in the lamina and in the distal layer of the medulla (Fig. 2a), and in a few cells in the antennae (not shown). The camera

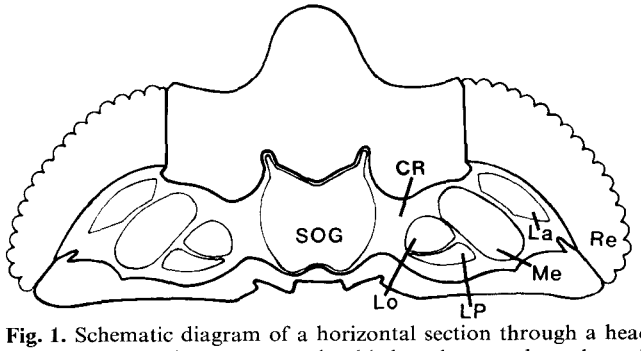


**Fig. 1.** Schematic diagram of a horizontal section through a head of *Drosophila melanogaster* at a level below the oesophageal canal. The outlines of the retina (*Re*) and the brain with optic neuropiles: lamina (*La*); medulla (*Me*); lobula (*Lo*); lobula plate (*LP*), and the suboesophageal ganglion (*SOG*) embedded in the cell body rind (*CR*) are shown

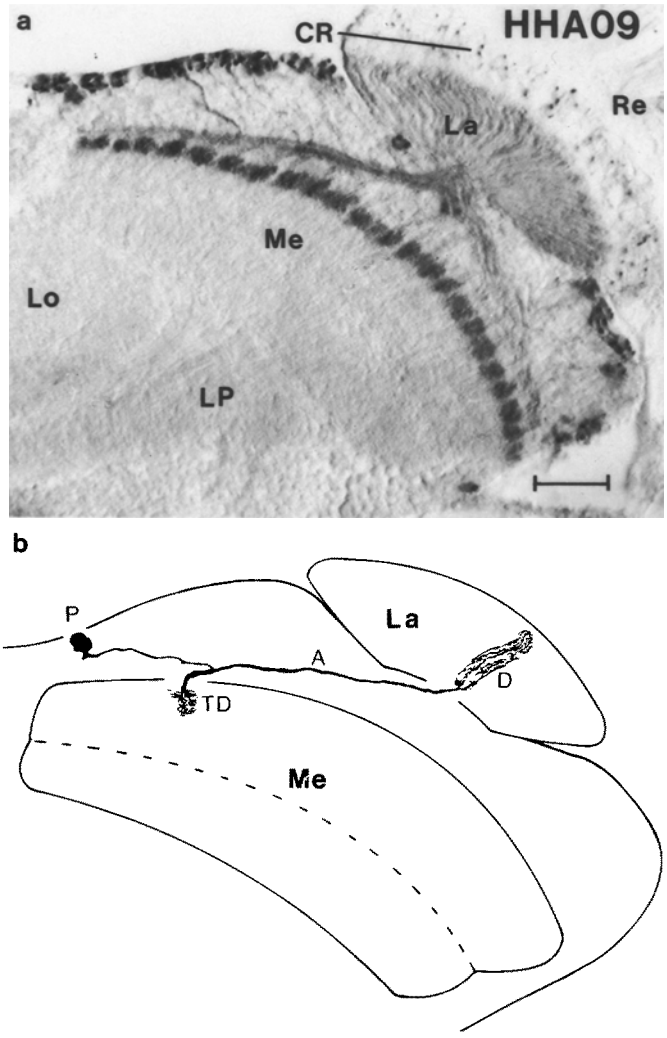


**Fig. 2a, b.** Right optic lobe stained with HHA09 antiserum (horizontal section) (**a**). In comparison with the drawing in **b** of a Golgi-impregnated T1 cell (Fischbach 1983), it is clear that the perikaryon (*P*), axon (*A*) and telodendritic arborizations (*TD*) are stained in each column. Low levels of antigen in the dendritic branching (*D*) may cause the diffuse "background" staining of the entire lamina neuropile. Note the non-homogeneous staining within each perikaryon and the small spots in the lamina cell body rind (*CR*). Abbreviations as in Fig. 1. ×490. Scale bar: 20 μm

lucida drawing of a Golgi-impregnated single "T1" neuron (Fischbach 1983) has been inserted into the outlines of the lamina and medulla for comparison (Fig. 2b). A single T1 cell is known to be present in each of 700 or so columns (neuro-ommatidia) of each optic lobe of *D. melanogaster*. The staining of individual T1 cell bodies, neurites, and axons in the outer optic chiasma, and of the complement of terminal arborizations, one in each column of the medulla, can readily be seen. The "baskets" of dendritic arborizations in the lamina are not resolved as separate, stained entities. However, the diffuse staining of the entire lamina neuropile may result from low antigen concentrations in T1 dendrites. A large number of small, stained dots are recognizable in the cellular rind of the lamina. Their structural correlates are unknown. A comparison of the stained T1 cell bodies with those stained by antibodies against 5HT, GABA or GAD (Figs. 3–5) indicates that the HHA09 serum stains a small number of patches within each perikaryon.

In order to establish some properties of the T1-cell-specific antigen, several different conditions of tissue fixation were employed. The staining with the HHA09 antiserum is abolished when cryostat sections prepared as described above are postfixed for 15 min in 2% glutaraldehyde. It is preserved, however, if fresh frozen sections are fixed for 10 min in 95% ethanol. Good staining is obtained when flies are fixed for 3–12 h in periodate-lysine-paraformaldehyde fixative (McLean and Nakane 1974). Furthermore, two hours of washing of the antibody-loaded sections with 8 M urea prior to the DAB reaction does not substantially reduce the staining. This suggests that antibody-antigen binding is of high avidity.

*Serotonin-like immunoreactivity*

There are only about 100 neurons in the entire *Drosophila* brain that stain with the serotonin (5-HT) antiserum. The essential features of this staining in the visual system are very similar to those described for *Calliphora* (Nässel and Klemm 1983; Nässel and Elekes 1984; Nässel et al. 1985). A cluster of about 10–15 stained perikarya is located at the central rim of each medulla at the dorso-ventral level of the oesophageal canal. About four perikarya are found more dorsally, and three are found more ventrally between the medulla and the mid-brain neuropile. Stained fibres belong to one of two distinct categories. A few relatively thick, smooth axons provide interconnections between widely separated brain regions, whereas numerous very thin fibres showing characteristic varicose thickenings branch extensively in all neuropiles. The distribution of the varicose arborizations is characteristic for each neuropile. Branching of these presumed 5-HT release sites in the lamina is confined mainly, but not exclusively (cf. Fig. 3e–h), to the monopolar cell body layer (Fig. 3a). Horizontal sections of the medulla reveal three layers of dense arborizations: (i) a well-defined distal double layer that, in tangential sections, displays a "chicken-wire" pattern of fibres reflecting the columnar organization of the medulla; (ii) a thin middle layer; and (iii) a fairly diffuse, broad layer that invades the outer half of the proximal medulla. In the lobula, two broad diffuse layers form a medial and an anterior layer of arborizations. Arborizations in the lobula plate are comparatively sparse and not clearly layered.

Because the network of arborizations is so dense and most fibres are so thin, we have not succeeded in reconstructing the entire extension of individual neurons in *D. melanogaster* (cf. Nässel (1987) for larger species of Diptera). It is thus impossible to allocate certain arborizations unequivocally to specific axons and cell bodies. Nevertheless, the number and position of stained cell bodies, the course of the major axons, and the pattern of arborizations in each neuromere are highly characteristic; any major deviations from normal are immediately recognizable (cf. Part II). However, the course of an axon is not necessarily invariant in wildtype flies, as illustrated in Fig. 3e–h. The axons projecting to the lamina in this particular fly, instead of passing the medulla centrally before entering the first chiasma, penetrate the second chiasma along the proximal surface of medulla and pass the medulla laterally to reach the first chiasma. This variation has been observed in both hemispheres in only one out of some 30 flies.

*GABA- and GAD-containing cells*

The inhibitory transmitter GABA is known to be synthesized by the enzyme GAD, which is found exclusively in

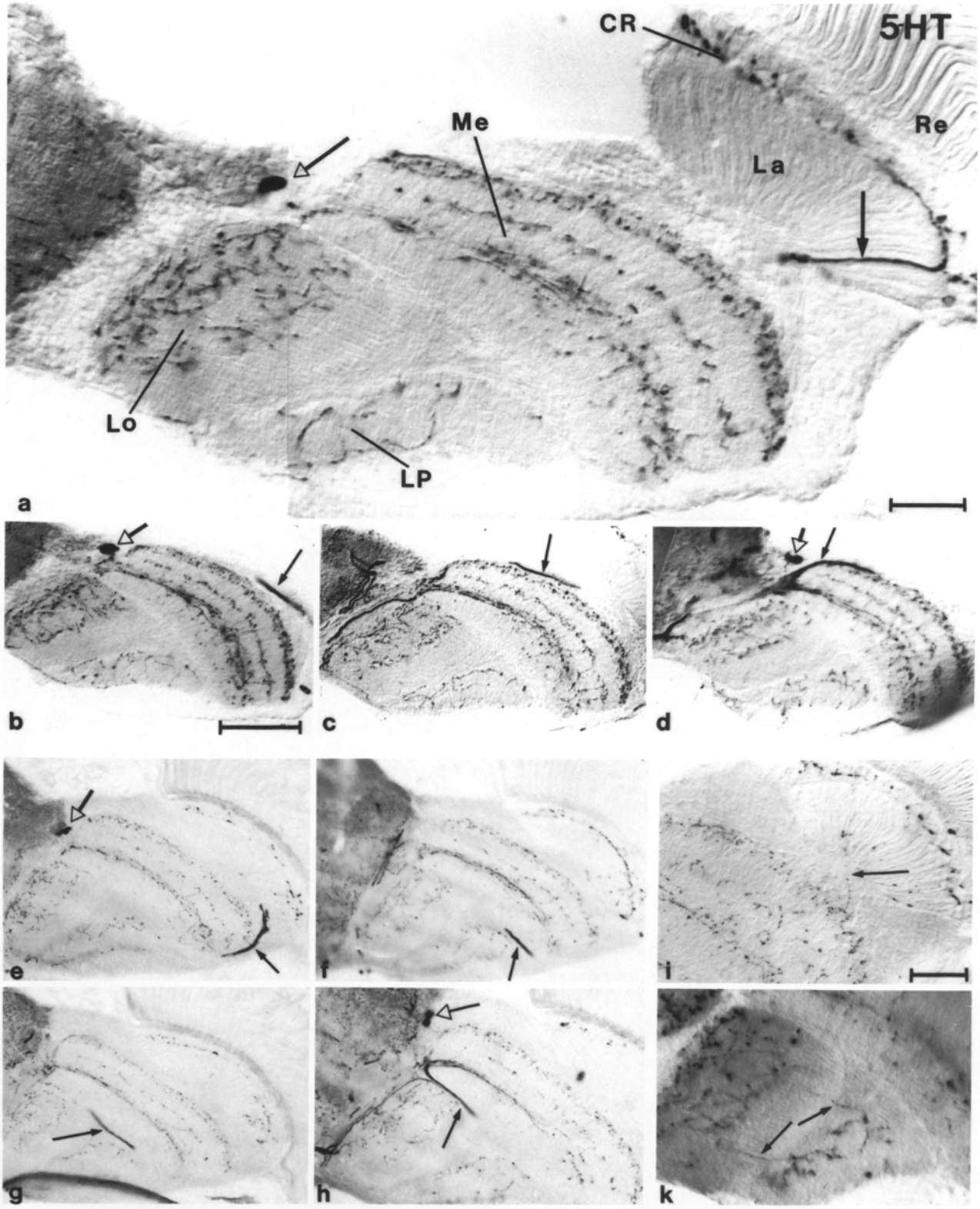


**Fig. 3a–d.** Horizontal serial sections of the right optic lobe stained with serotonin (5-HT) antiserum. Note the stained arborizations in the lamina cell body rind (*CR*), the axons connecting these arborizations to the medulla and mid-brain (*filled arrows*), small clusters of cell bodies adjacent to the medulla (*open arrows*), and the various patterns of arborizations in the optic lobe neuropiles (see text). Occasionally, the lamina-medulla connection projects laterally around the medulla neuropile (**e–h**) instead of passing it centrally (**a–d**, *filled arrows*). Note the fine branchings apparently interconnecting lamina and medulla (*i*), or lobula and lobula plate (*k*). ×600 (a), ×280 (b–h), ×500 (i, k). Scale bars: 20 μm (a), 50 μm (b–h), 20 μm (i, k)

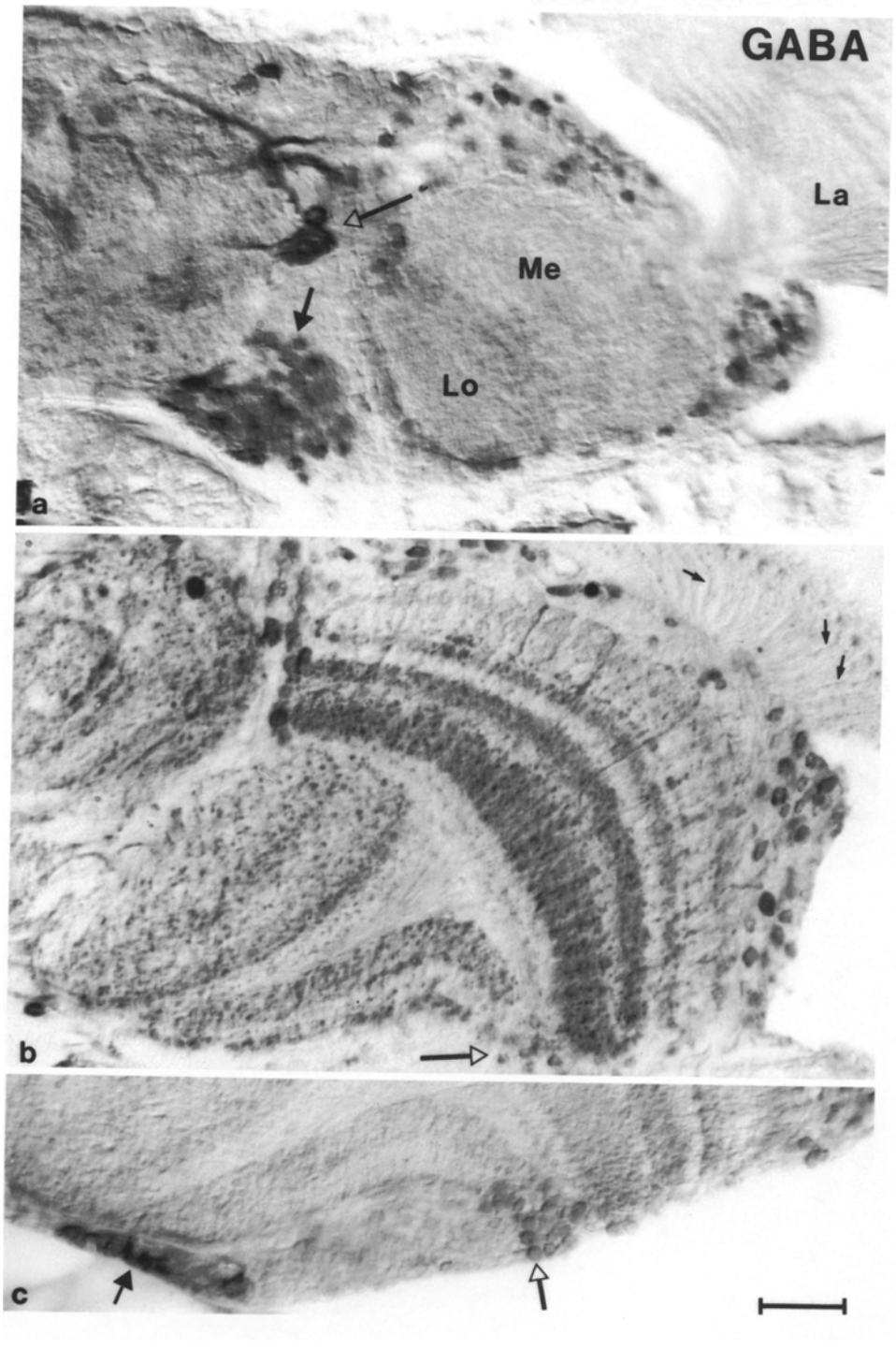


**Fig. 4a–c.** Staining obtained with an antiserum against γ-aminobutyric acid (*GABA*) characterized by (i) the dispersed perikarya in the medulla rind, (ii) two clusters of perikarya adjacent to the dorsal crest of the lobula (*open* and *filled arrows* in **a**), (iii) two caudal clusters adjacent to the distal and proximal rim of the lobula (*open* and *filled arrows*, respectively, in **b** and **c**), (iv) fine arborizations in the lamina cartridges (*small arrows* in **b**), (v) five layers with characteristic positions and widths in the medulla, and (vi) two layers each in the lobula and the lobula plate. Note that the most proximal layer of the medulla and the most posterior layer of the lobula are almost devoid of staining. ×600. Scale bar: 20 μm

GABA-ergic neurons. Antiserum raised against GABA and a serum against rat GAD lead to very similar patterns of staining in the visual system of *D. melanogaster* (Figs. 4 and 5, respectively). This indicates that both types of staining are specific. The number of GABA-immunoreactive perikarya has been counted from a complete series of 22 sections through both optic lobes. A clear distinction between stained and unstained cells is not always possible because staining intensities varying from very dark to barely detectable are found in a single section. The cell counts give a minimum estimate of about 1500 perikarya in that part of the cell body rind that can be assigned to one optic lobe. About 900 of these cell bodies are dispersed in the distal medulla rind. Although it is impossible at present to match the stained elements with Golgi-impregnated cells, several features of the staining are highly characteristic and should help in assessing any abnormality in mutants: (i) The density of stained perikarya in the outer medulla is rather uniform, whereas stained perikarya at the posterior rim of the second chiasma and near the lobula plate are clustered (Figs. 4c, 5c). (ii) A characteristic stained structure at the central rim of the medulla coincident with the dorso-ventral level of the giant commissure (open arrow; Figs. 4a, 5a) may represent either a tight cell cluster or a giant perikaryon. (iii) The distribution of staining in all visual neuropiles is highly characteristic and very similar

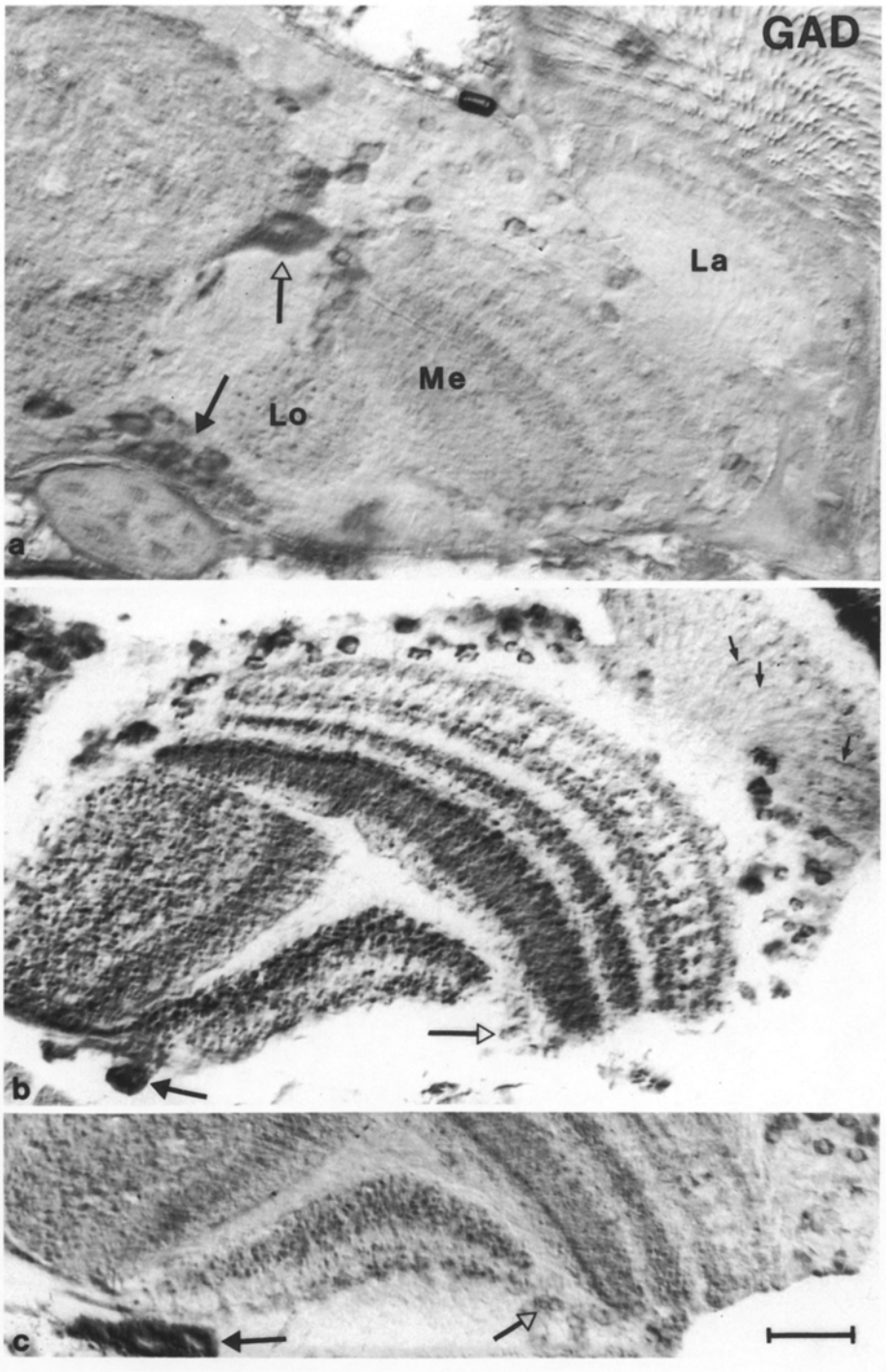


**Fig. 5.** Staining with an antiserum against glutamic acid decarboxylase (*GAD*). All characteristic features described for GABA staining (Fig. 4) are reproduced with GAD antiserum. × 600. Scale: 20 μm

for GABA and GAD antibodies. There is no indication in *D. melanogaster* of specific staining in the retina. The lamina shows very little staining: in the best preparations a thin fibre is faintly recognizable in each column (Figs. 4b, 5b). Five immunoreactive layers with characteristic positions, widths and densities are stained in the medulla. The columnar organization of these layers is clearly recognizable. Four stained tangential layers can be identified within the diffusely stained neuropile of the lobula and lobula plate. A thin layer lines the posterior surface of the neuropile of the lobula plate, and a broad layer extends across its anterior half. A columnar organization of this staining is not discernible. The most posterior layer of the lobular neuropile shows little staining, but an adjacent layer stains strongly both with GABA and GAD antibodies. A second faint layer extends through the medial neuropile of the lobula.

### *Cells stained by antisera raised against $Ca^{2+}$-binding proteins*

Highly specific $Ca^{2+}$-binding proteins are likely to be involved in controlling intraneuronal levels of free $Ca^{2+}$; they play a crucial role in a variety of functions. Several of these proteins have been found in subpopulations of vertebrate brain cells, notably parvalbumin (PV) (Braun et al. 1985;

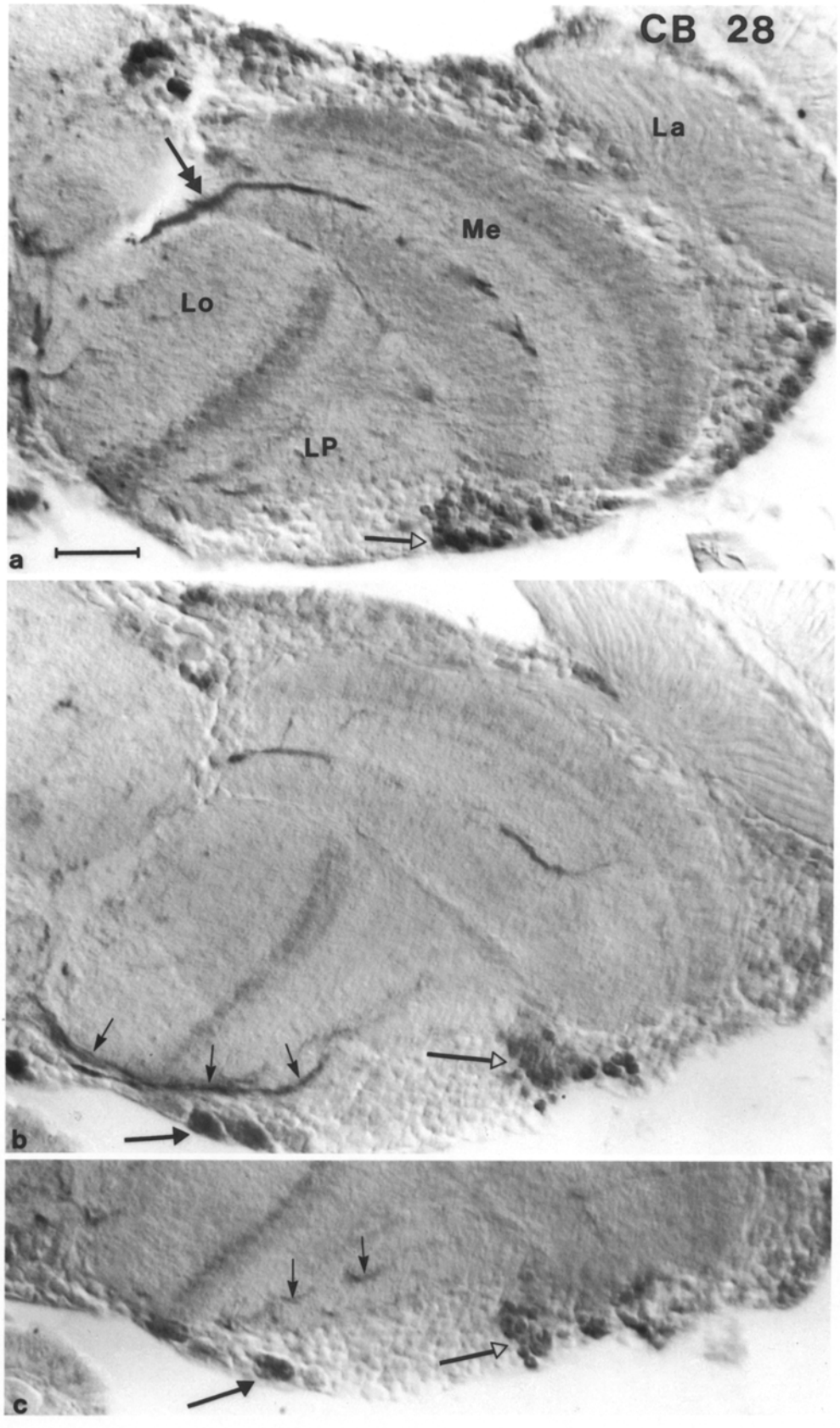


**Fig. 6.** Optic lobe stained with an antiserum against chicken calbindin 28 K (*CB28*). Reproducible and characteristic features include (i) dispersed cell bodies in the frontal cellular rind between the optic lobes and midbrain, (ii) two clusters of stained perikarya posterior to the distal and proximal rim of the lobula plate (*open* and *filled arrows*, respectively), (iii) at least one large-diameter tangential neuron in the medulla (*double-head arrow* in **a**), (iv) several tangential neurons in the lobula plate (*small arrows*), and (*v*) a layer in the centre of the posterior half of the lobula. × 560. Scale bar: 20 μm

Stichel et al. 1986; see reviews by Heizmann and Berchtold 1987; Heizmann and Celio 1987) and calbindin D 28k (CB28) (Jande et al. 1981; Baimbridge et al. 1982; Garcia-Segura et al. 1984). Specific antibodies against the two proteins stain different groups of neurones in the rat, zebra finch, cat, monkey and man (Heizmann and Celio 1987).

In *D. melanogaster*, antisera against chicken CB28 and rat or carp PV stain a large number of perikarya in the

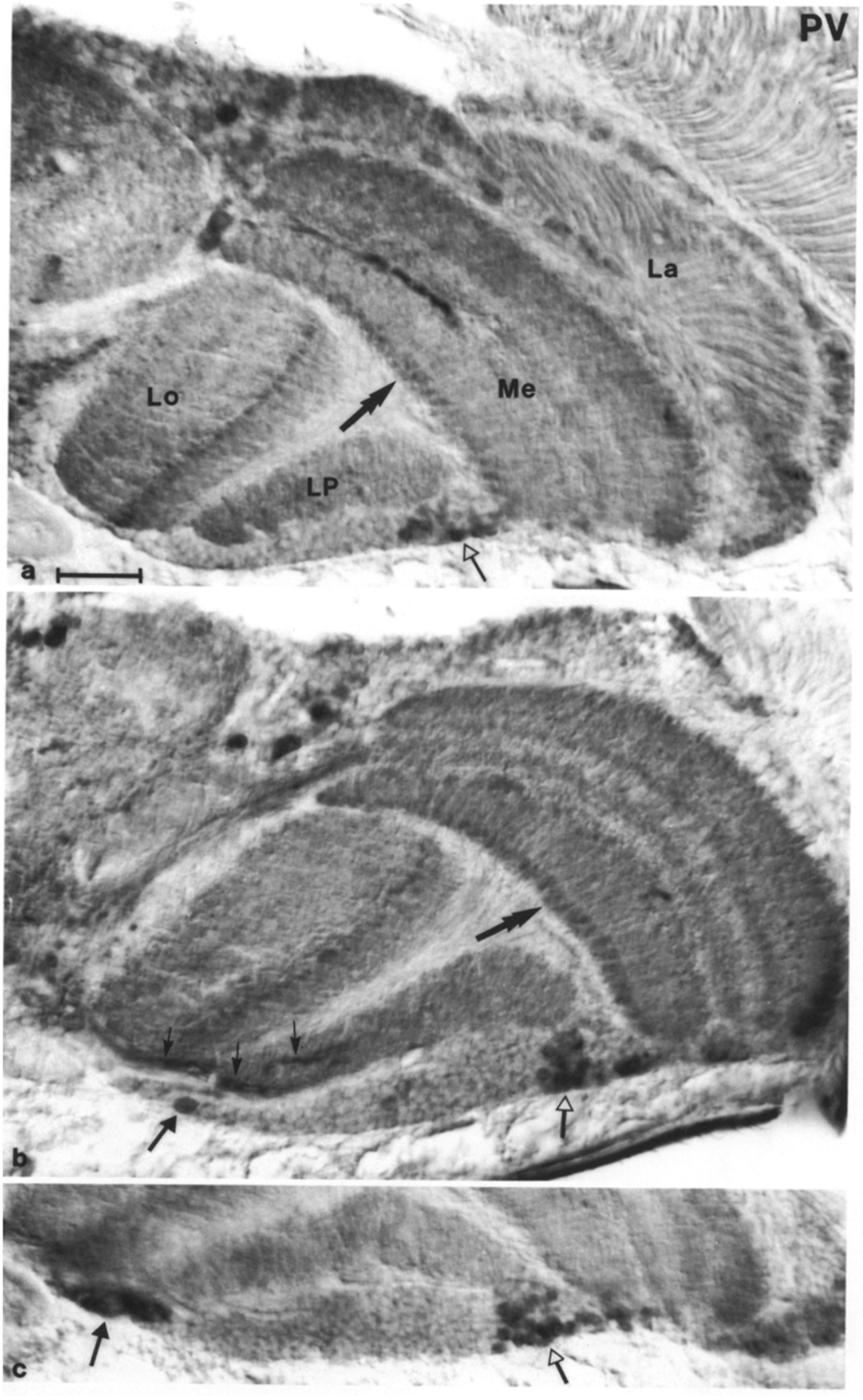


**Fig. 7.** Staining with an antiserum against carp II parvalbumin (*PV*). The characteristic features described for CB28 (Fig. 6) are found with this antiserum with one exception: a proximal layer of the medulla is clearly stained (*double-head arrow*). × 550. Scale bar: 20 μm

optic lobes; clustering is found in the cortex caudal to the medulla-lobula plate interface (Figs. 6, 7). Some differences in the staining patterns should be noted, however. The most proximal layer of the medulla neuropile stains reproducibly with carp PV but not with rat PV or CB28 antiserum. Even more pronounced differences are found for certain groups of muscles, some of which stain with PV antiserum, some with CB28 antiserum, and a third group with SCP II antiserum (cf. below). As with GABA/GAD antisera, the binding appears to be graded. Furthermore, the clustering of stained perikarya near the lobula plate displays some similarity with GABA/GAD-stained cells. The pattern of

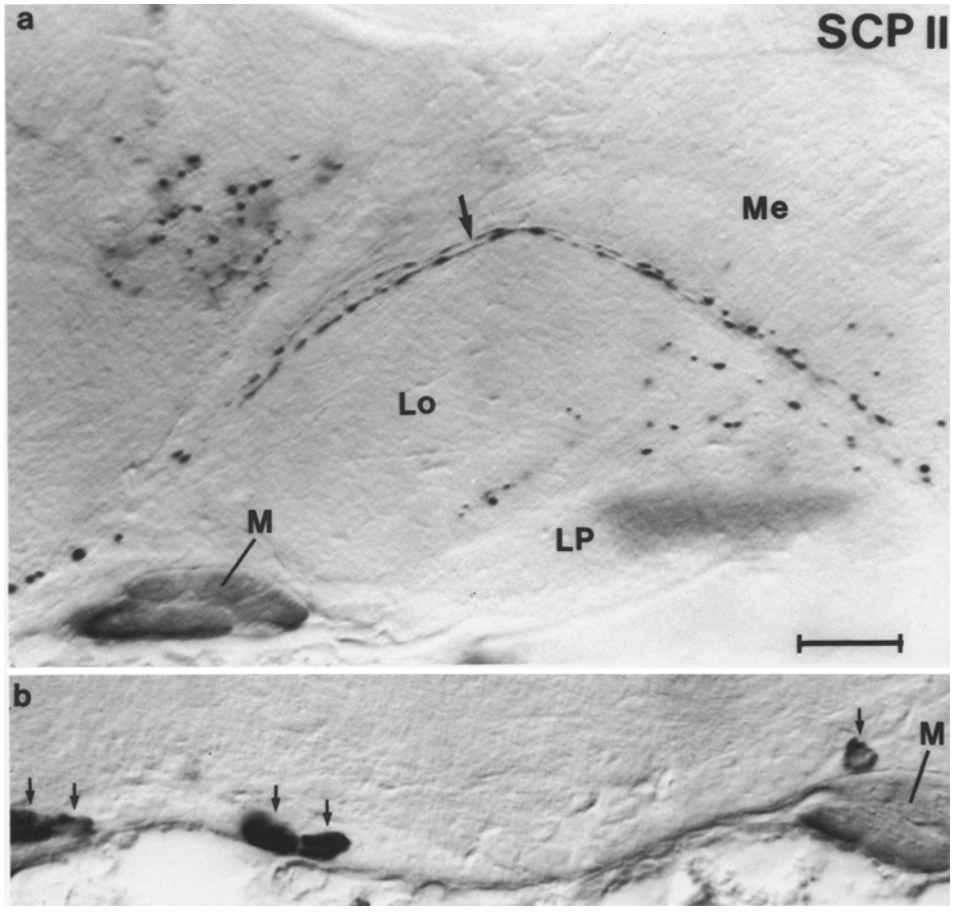


**Fig. 8a, b.** A few varicose axons (*arrow* in **a**) and their presumed arborizations as well as a small number of perikarya (*arrows* in **b**) are stained by the antiserum against sarcoplasmic calcium-binding protein II (*SCPII*). Note the stained retractor of rostrum muscle (*M*). ×600. Scale bar: 20 μm

stained tangential neuropile layers is, however, clearly different from that of the GABA/GAD staining (compare with Figs. 4, 5). The characteristic lobula layer of Figs. 6 and 7 lies more anteriorly, and no lobula plate layers can be observed. A large medulla tangential fibre, which may be identical to the Golgi MT4 neuron described by Fischbach (1983), is stained with antisera against PV and CB28 but not with antisera against GABA or GAD.

The staining obtained in the brain of *D. melanogaster* using a serum against sarcoplasmic $Ca^{2+}$-binding protein II (SCP II) is totally different from that obtained with CB28 and PV antisera. Only some 10–20 neurons are densely stained in the entire brain (Fig. 8). Also, in contrast to the smooth axon staining with CB28 and PV sera, SCP II-stained fibres are very thin and varicose (arrow in Fig. 8a). The spots not connected by thin fibres are interpreted as varicose thickenings of arborizations running obliquely through the plane of sectioning. In addition, this serum strongly stains certain head muscles of *D. melanogaster* (e.g., the retractor of rostrum, Fig. 8); these are at most only weakly stained with either CB28 or PV sera. The SCP I antiserum does not bind to *D. melanogaster* brain sections under the prevalent experimental conditions in spite of mutual cross-reactivity of SCP I and II antisera in biochemical assays.

*Staining patterns obtained with monoclonal antibodies*

Extensive screening of IgG-positive hybridoma clones has identified a large number of supernatants that selectively and reproducibly stain various brain structures (Hofbauer, in preparation). Here we report five monoclonal antibodies that stain components of the visual system; these antibodies seem most appropriate for probing the integrity of the optic lobes of structural brain mutants at the level of individual cells or at the level of neuropile layering. Three antibodies (*ca8/3*, *ab47/7*, *ab43/2*) selectively stain small numbers of cell bodies and characteristic networks in horizontal strata of the medulla and lamina. Two antibodies (*ab49/9*, *ca51/2*) stain neuropile masses in a manner similar to monoclonal antibodies described by Fujita et al. (1982), but with a characteristic preference for specific layers in each neuropile. All five antibodies stain various central brain elements, a subject not further considered here.

The three cell-specific antibodies share the ability to stain approximately a dozen perikarya in the medulla rind at a location adjacent to the anterior border of the medulla neuropile (Fig. 9). A few more stained cells are found in the dorsal and ventral cell body rind, between the optic lobes and the neuropile of the brain proper. In the medulla, the three antibodies stain structures presumed to be arborizations within a tangential stratum that includes a certain depth of the serpentine layer, the latter usually associated only with axons of medullary tangential cells. Staining may also include some of the neuropile proximally adjacent to it. Antibodies *ca8/3* and *ab47/7* also stain arborizations within the most distal medulla neuropile and in a layer between the outer surface of the lamina neuropile and the lamina cell-body layer. These two antibodies differ in their staining intensity in the two layers of the medulla. Whereas

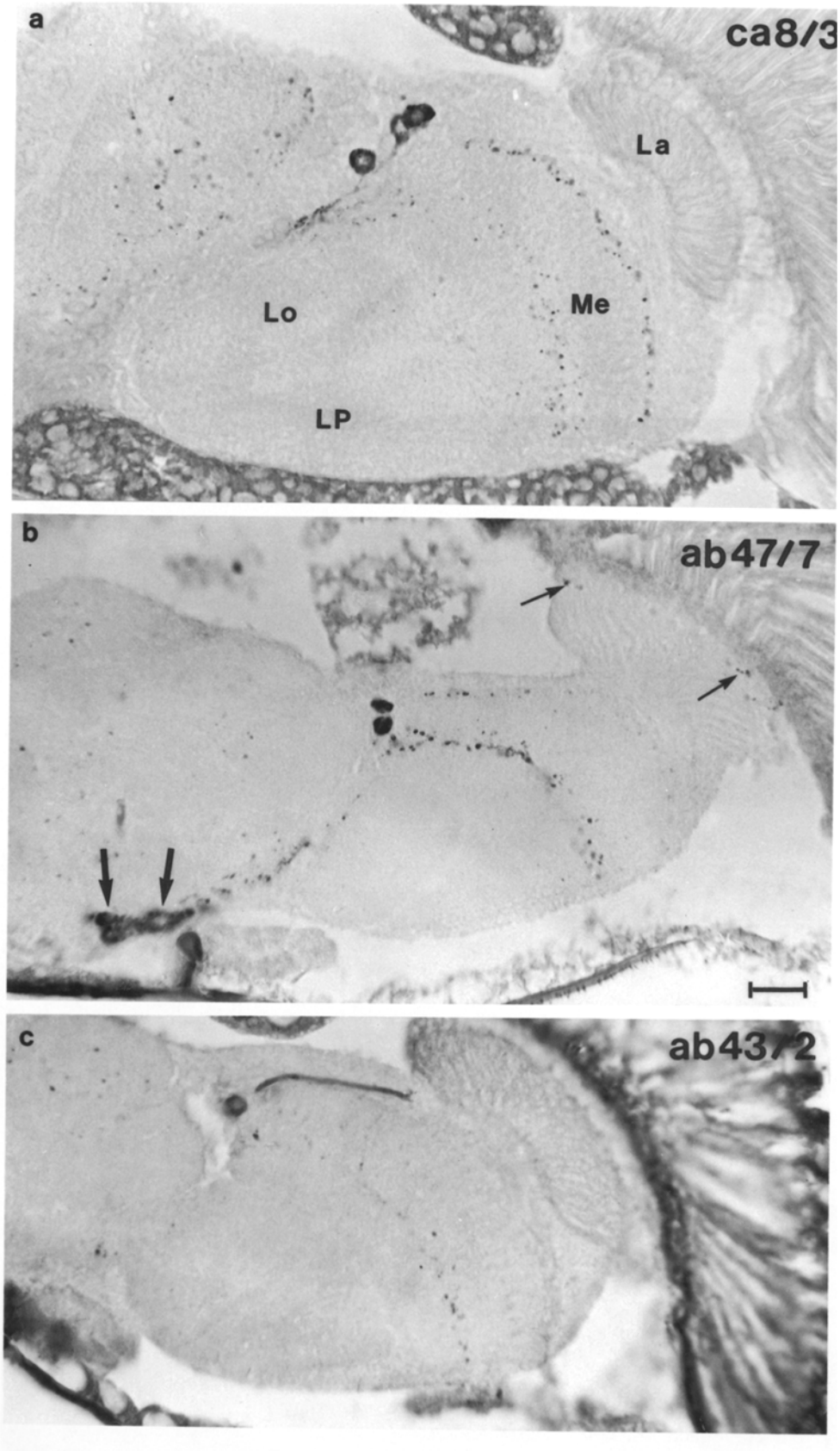


**Fig. 9a, b.** Staining of presumed tangential neurons in the medulla with three monoclonal antibodies (*ca8/3*, *ab47/7*, *ab43/2*). Axons in **a** and **b** either do not bind the antibody or are too thin to be recognized. The strata of immunostained spots may represent varicosities connected by thin neurites. Using *ca8/3* and *ab47/7*, some sections have stained spots also in a distal stratum of the lamina (*small arrows* in **b**). A set of cell bodies of the central brain is stained with *ab47/7* (*large arrows* in **b**) but not with *ca8/3*. With *ab43/2* an axon apparently connecting the lamina and medulla is stained. These differences indicate that different epitopes are recognized by all three antibodies. × 400. Scale bar: 20 μm

*ca8/3* selects the distal layer, the medial layer stains more prominently with *ab47/7*. In contrast, antibody *ab43/2* stains only a single layer of the medulla. Furthermore, in one preparation, a stained fibre running along the outer surface of the medulla neuropile can be followed through the first chiasma into the lamina neuropile (Fig. 9c). So far, we have not been able to localize arborizations in the lamina with this antibody.

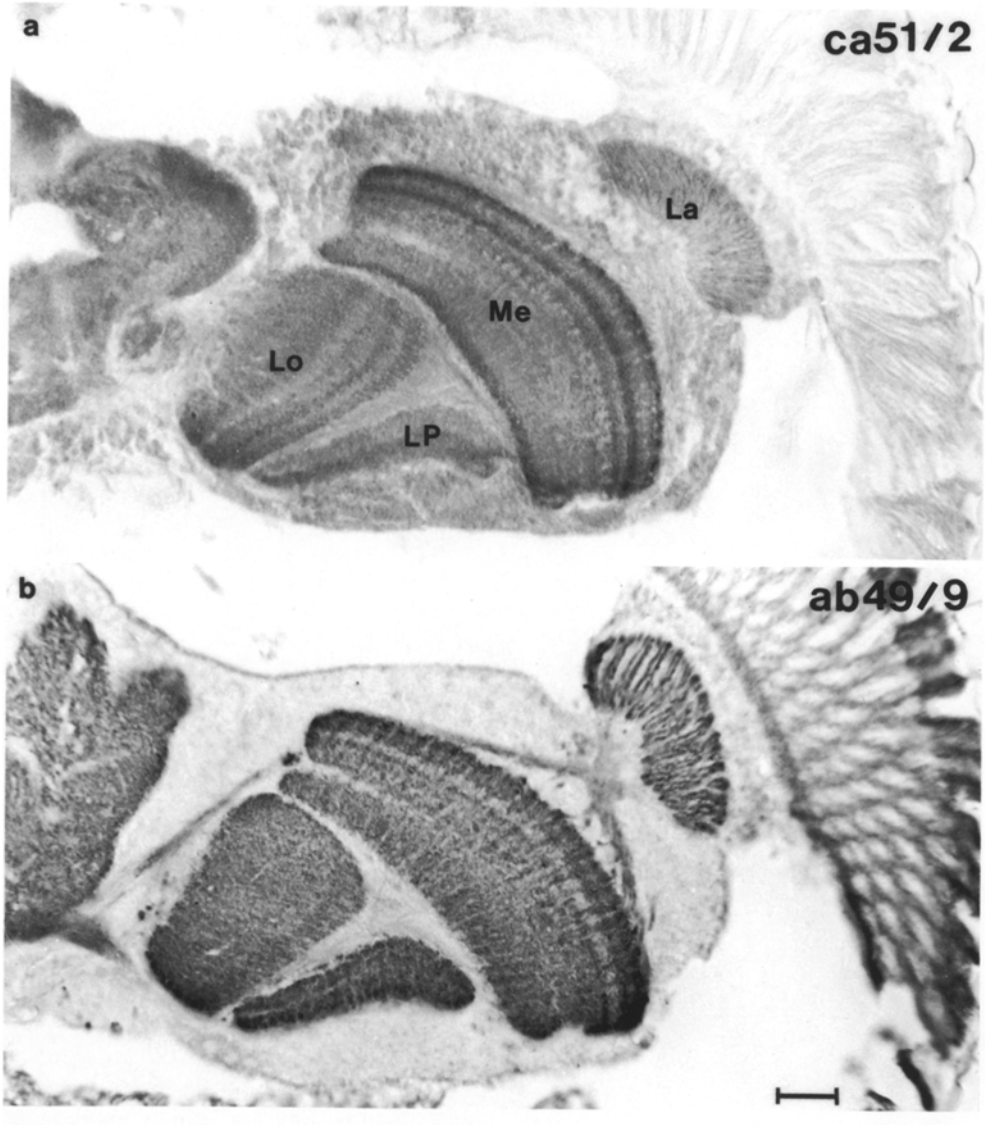


**Fig. 10.** Two different patterns of tangential layers obtained with two monoclonal antibodies (*ca51/2* (**a**), *ab49/9* (**b**)). The width and position of these layers are highly characteristic for each neuropile of wildtype flies such that any major deviation from this pattern in structural brain mutants should be recognizable. × 360. Scale bar: 20 µm

Antibodies *ca51/2* and *ab49/9* stain all neuropile regions throughout the brain of *D. melanogaster* (Fig. 10). In the optic lobes, however, each antibody reveals a different but characteristic pattern of layering in the medulla and the lobula complex.

## Discussion

We have at present no information concerning the identity of the T1-specific antigen recognized by the HHA09 serum. The serum may bind to formaldehyde- or ethanol-modified epitopes of HHA not recognized by the other HHA-antibodies tested; it may recognize a peptide contaminant generated in the original synthesis of HHA; or the T1 antigen could bind to a serum subfraction unrelated to the immunization procedure. The high avidity of the binding suggests, however, that the staining is based on antibody recognition of homologous antigens or closely related epitopes.

We have not tested the specificity of the 5-HT antibody binding because this has already been performed using 5-HT antisera from many sources (including INC) for insects (Klemm and Sundler 1983; Nässel and Klemm 1983). The binding in *Drosophila* shows sufficient similarity to that in *Calliphora* to infer that the stained cells contain serotonin.

For the GABA staining, we have relied on two independent tests of specificity. The preabsorption experiments demonstrate that the main features of the staining in Fig. 4 are generated by serum fractions recognizing the GABA epitope of GABA-BSA conjugates. Although double staining of the same sections was not carried out, it is clear that anti-GABA and anti-GAD antibodies mostly stain identical structures in the *Drosophila* brain. Artefacts that might arise as a result of endogenous peroxidase or unspecific binding of second or third antibody have been excluded by appropriate controls. Thus, we conclude that staining with GABA antiserum is specific for GABA and that *Drosophila* brain GAD is so similar to rat brain GAD that antibodies against the latter crossreact with the *Drosophila* enzyme. The last conclusion is also supported by immunoblotting of *Drosophila* brain homogenate with the GAD antiserum after sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (S. Buchner and M. Buderath, unpublished). These experiments have demonstrated GAD-

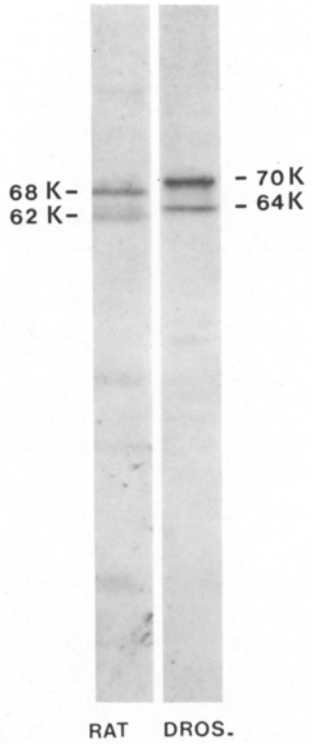


**Fig. 11.** Immunoblots of rat brain extract and *Drosophila melanogaster* brain homogenate using the anti-rat brain GAD antiserum. After electrophoresis in 11% SDS gel, the proteins were electrophoretically transferred to nitrocellulose and stained with GAD antiserum at 1:1000 dilution using the "proto-blot" system (Atlanta GmbH, Heidelberg)

serum-specific staining of two polypeptides at 70000 and 64000 daltons for *Drosophila melanogaster* and at 68000 and 62000 daltons for rat brain (Fig. 11) in close proximity to the published values for various mammals (66000 and 60000 daltons; Kaufman et al. 1986). Antibody crossreactivity for enzymes from two species as distant as rat and fruitfly could indicate that a substantial fraction of the anti-GAD serum is directed against conserved (and therefore presumably functionally important) sites of the enzyme. Antiserum preabsorbed with glycine or glutamate reproduced the salient features of the GABA staining. To claim, however, that no component of the staining was affected would require more extensive experiments.

The distribution of putative GABA-ergic cell bodies and neuropile layers in the visual system of *Drosophila melanogaster* reported here disagrees with that tentatively proposed on the basis of $^3$H-GABA uptake (Campos-Ortega 1974). However, experiments using freeze-fixation, freeze-drying and dry autoradiography after injection of $^3$H-GABA and its analogues, $^3$H-muscimol and $^3$H-$\beta$-chlorophenyl-GABA, (Buchner, unpublished results) support the data of Campos-Ortega (1974). Both these uptake studies show little differential accumulation of $^3$H-GABA or its analogues in the brain of *Drosophila melanogaster* except for strong labelling of retina and lamina. Further experiments are now required to determine whether the high-affinity GABA-uptake mechanism described for insect synaptosomal fractions (Gordon et al. 1982; Breer and Heilgenberg 1985) can in fact be attributed to the presumed GABA-ergic cells stained in this study; this should be possible as suggested by earlier $^3$H-GABA uptake studies on cockroach brain slices (Frontali and Pierantoni 1973). Published figures demonstrate selective uptake in the lower division of the central complex, a structure that exhibits prominent GABA-staining in the bee brain (Schäfer and Bicker 1986), in *Musca* (Meyer et al. 1986), and in *Drosophila* (ellipsoid body, unpublished data).

We can compare the number of GABA-ergic cell bodies with the 700 or so retinotopic columns in one optic lobe. Each column contains processes from at least 50 "columnar" cell types (Fischbach 1983) such that there are at least 35000 columnar neurons in each lobe. The majority of GABA-stained cell bodies is found in the distal rind of the medulla where the perikarya of the cell types MI, DM, Tm, and TmY are located (Fischbach 1983). The minimum number of stained cell bodies counted in this region ($\sim$900) supports the speculation that at least one columnar cell type of the medulla is GABA-ergic. The majority of the remaining ($>$600) stained cell bodies is clustered near the distal rim of the lobula plate (open arrows in Fig. 4b, c), a region where the perikarya of the columnar cell types C2, C3, T2, and T3 are located (Fischbach 1983). Again, it seems likely that at least one further columnar cell type from this region is GABA-ergic.

It is interesting to compare our GABA/GAD data with those recently published for larger Diptera (Meyer et al. 1986; Datum et al. 1986). In *Drosophila melanogaster*, we find no indication of specific photoreceptor staining, contrary to reports for *Calliphora* (Datum et al. 1986). Further experiments will be required to decide whether this apparent discrepancy is a result of inter-species differences, false-negative staining in *Drosophila*, or false-positive staining in *Calliphora*. Other features, such as the distribution of stained perikarya and the position of stained layers in the visual neuropiles, are very similar in the three species. Profiles stained in the lamina of larger diptera have been associated with the C2 columnar neuron (Meyer et al. 1986; Datum et al. 1986). GABA/GAD staining in *Drosophila* is consistent with such an interpretation, because there are thin stained profiles in the lamina and stained perikarya in the region beneath the medulla where the C2 cell bodies are localized. However, other interpretations cannot be excluded by our data, e.g. that C3 cells projecting into the lamina, or other cell types originating in the lamina, may be stained (see also below). The tentative identification of GABA-staining of the centrifugal horizontal wide field neurons of the lobula plate (the CH-cells) in *Musca* (Meyer et al. 1986) is interesting in view of the selective deoxyglucose uptake by these cells during sustained stimulation by visual movement (Buchner et al. 1984). Since other movement-sensitive neurons are not labelled under the same conditions, there might be a causal relationship between GABA utilization and deoxyglucose uptake by the CH-neuron. Thus far, the CH cells have not yet been identified neuroanatomically in *Drosophila melanogaster* nor can they be discerned in either our GABA/GAD or deoxyglucose preparations. On the other hand, GABA/GAD-stained layers in the neuropile only partially overlap with layers that are labelled by deoxyglucose during visual stimulation (Buchner et al. 1984). Thus, a possible relationship between deoxyglucose mapping and GABA utilization does not appear to be a tenable hypothesis for all cells.

Finally, GABA/GAD staining can be compared with recent results concerning the distribution of choline acetyltransferase-like immunoreactivity (ChAT-LI) in the visual system of *Drosophila melanogaster* (Buchner et al. 1986; Gorczyca and Hall 1987). Since the monoclonal ChAT anti-

body did not bind reliably to perikarya, only the characteristic patterns of neuropile staining can be compared. Prominent features of the banding patterns are, for the most part, opposite for the two systems.

Striking differences concern the proximal layer of the medulla and the most posterior layer of the lobula. Both display little GABA/GAD but strong ChAT staining, whereas the reverse holds for a layer of the lobula anteriorly adjacent to the most posterior layer. A single distal structure in each column of the lamina is stained both with GABA and with ChAT antibody although the detailed patterns appear different. The staining of this structure with GAD antibody is unclear in *Drosophila*. Since Golgi studies (Fischbach 1983) have so far identified only a single species of columnar cell with such a prominent structure in the distal lamina, viz. the C2 cell, we have previously pointed out the similarity of this structure with those staining for ChAT (Buchner et al. 1986). From the work on larger Diptera (Meyer et al. 1986; Datum et al. 1986), there is now substantial evidence that the C2-cell may be GABA-ergic. The identity of the ChAT-stained lamina structure should therefore be considered as unresolved.

Our studies on *Drosophila melanogaster* have not allowed unequivocal correlations between GABA/GAD-stained structures and neurones identified by the Golgi technique (Fischbach 1983). This would require further improvements in tissue preservation. Comparative immunocytochemical staining of structural brain mutants (Part II of this investigation) may help to clarify the cellular nature of the complex arrangements of immunocytochemically stained layers in the optic lobes. Reciprocally, such data could also indicate the cellular basis of anatomical defects in the visual system of these mutants.

In the case of antisera raised against $Ca^{2+}$-binding proteins there is no direct evidence for binding specificity in the brain of *Drosophila melanogaster*. Although each of the three sera stains a different sub-set of muscle fibres (most $Ca^{2+}$-binding proteins have in fact been isolated from muscle), this does not prove that the same antibody fractions of the polyclonal sera bind to identical antigens in brain and muscle. It will be interesting to investigate whether the observed similarities of staining by antisera against CB28 and PV (such as the distribution of stained perikarya, the position of a stained layer in the lobula, and the staining of large tangential neurons in medulla and lobula plate) are a result of the co-localization of the respective antigens, a similar organization of the cells binding either CB28 or PV antisera, or cross-reactivity of the antisera under the conditions used in our investigations. With regard to the partial similarity of PV or CB28 staining with GABA/GAD distribution as described above it should be recalled that co-localization of GABA-like, PV-like and CB28-like immunoreactivity has been reported for vertebrate cerebellar Purkinje cells (Jande et al. 1981; Baimbridge et al. 1982; Garcia-Segura et al. 1984; see review Heizmann and Celio 1987). Eventually, the questions about specificity and mutual co-localization of CB28-like immunoreactivity, PV-like immunoreactivity, and GABA in brain cells of *Drosophila melanogaster*, will have to be directly investigated using preabsorption and double staining. Nevertheless, the three antisera represent a valuable extension of our collection of cell-specific stains for characterizing brain defects of mutants.

The five monoclonal supernatants (Figs. 9, 10) serve the same purpose. Apart from their selective distribution in the brain, no information on the antigenic binding sites is presently available. Without double staining it is not possible to decide whether the three antibodies of Fig. 9 stain different cell populations in the optic lobes. However, the staining pattern in the mid-brain suggests that there different epitopes are indeed recognized by the different antibodies. The position of the stained perikarya and the structure and localization of the stained arborizations suggest that these antibodies bind selectively to certain medulla tangential-cells that have not been described before. The two patterns of neuropile staining obtained with *ca51/2* and *ab49/9* monoclonal antibodies differ from all others in the present study and from those obtained with ChAT antibody (Buchner et al. 1986; Gorczyca and Hall 1987). At present, it is not possible to relate these patterns of tangential layers with arborizations of Golgi-identified columnar neurons. Such associations may become feasible if specific subsets of these layers are missing in certain visual mutants.

## Conclusion

We have described examples for three groups of selective immunocytochemical probes used to investigate the brain of *Drosophila melanogaster*. In the first group, the antigen is a well-known brain molecule (5-HT, GABA, GAD). For the second group, one may speculate that, in the brain of *D. melanogaster*, molecules exist that have structural and perhaps functional homology with the antigens used for immunization ($Ca^{2+}$-binding proteins). Regarding the third group (HHA09 and the monoclonal antibodies), we have no information concerning the molecular identity of the antigenic binding site in the section. Any molecule, however, having a distribution as selective as the T1-cell-specific antigen or the antigens binding the monoclonal antibodies demonstrated in Figs. 9 and 10, is likely to be of fundamental neurochemical importance. The antibodies may provide access to these molecules (Zipurski et al. 1984). We have generated a large number of hybridoma supernatants binding to unknown cell-specific antigens. The rarity of identical staining and the confinement of many antigens to very small subsets of neurons correspond well with the high genetic complexity of about 11000 RNA species estimated as being present in homogenates of *Drosophila* heads (Levy and Manning 1981).

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