First publ. in: The Journal of Comparative Neurology ; 335 (1993), 3. - pp. 413–424

Monoclonal Antibody Labels Olfactory and Visual Pathways in *Drosophila* and *Apis* Brains

GERD BICKER, SABINE KREISSL, AND ALOIS HOFBAUER
Institut für Neurobiologie, Freie Universität Berlin, W-1000 Berlin 33, Germany (G.B., S.K.), and Theodor-Boveri-Institut für Biowissenschaften der Universität Würzburg, Lehrstuhl für Genetik, W-8700 Würzburg, Germany (A.H.)

ABSTRACT

We employed a monoclonal antibody raised against *Drosophila* brain homogenate for a comparative immunocytochemical analysis of visual and olfactory pathways in brains of two insect species. On Western blots of *Drosophila* and *Apis* nervous tissue, antibody fb45 recognized an antigen with an apparent molecular weight higher than 180 kD. Application of the antibody to sections of *Drosophila* and *Apis* brain stained certain interneurons which conspicuously fasciculate in common tracts or neuropilar compartments. Both in *Drosophila* and in *Apis,* the antigen was also expressed on the perineural sheath and granular cell compartments in the majority of neuronal cell bodies.

The antibody stained monopolar cells in the visual system of both species, and in *Apis* those fibers of the anterior superior optic tract which link the medulla with the mushroom bodies. In *Drosophila,* bundles of Kenyon cells of the mushroom bodies were stained. In worker bees and drones, the relay neurons of the median and lateral antennoglomerular tracts were labelled.

Since the recognition of the antigen does not require fixation, the antibody can be employed to label selectively living neurons in dissociated cell culture. This opens up the possibility for future functional studies on the role of the antigen in vitro.

Key words: mushroom bodies, antennal lobes, monopolar cells, fasciculation, cell culture

The introduction of immunological techniques in neuroanatomy has resulted in a better understanding of connectivity and molecular organisation of the nervous system (Reichardt, '84). An important goal of the immunological approach to the nervous system has been to develop antibodies which distinguish between different subsets of cells. The application of the hybridoma technique made it possible to generate cell-specific monoclonal antibodies by immunization with homogenates of nervous systems.

A study of neuronal diversity by the hybridoma technique appeared especially promising in the anatomically less complex and clearly segmented nervous systems of annelids and arthropods. Zipser and McKay ('81) generated monoclonal antibodies (MABs) which recognized subsets of cells in the leech nervous system by immunization with segmental ganglia. MABs have also been employed as markers of glycoproteins on the surface of specific axon bundles during development of the leech nervous system (McKay et al., '83). Similar studies with MABs directed against the insect nervous system of embryonic grasshoppers (Kotrla and Goodman, '84; Bastiani et al., '87) have revealed cell-surface antigens transiently expressed only on small subsets of neurons or fasciculating axons.

The fruit fly *Drosophila* is particularly suited to the analysis of neuronal diversity because a combination of recombinant DNA and hybridoma techniques can be used to identify the gene that encodes the antigen (Fujita et al., '82; Zipursky et al., '85). A hybridoma library against head homogenate has been prepared by Hofbauer ('87) and screened immunocytochemically for binding to specific cell types in the visual system (Buchner et al., '88; Hofbauer and Buchner, '89).

Many classic anatomical studies have lead to a thorough understanding of the functional neuroanatomy of another insect, the worker honeybee (review, Mobbs, '85). However, investigation of the cell biological properties of its constituent neurons has so far been confined mainly to histochemical and immunocytochemical studies of properties related to neurotransmission. The chemical neuroanatomy of classical transmitter systems in the brain of the honeybee has rapidly expanded since histofluorescence in conjunction with high performance liquid chromatography (HPLC)

methods were applied to detect the distribution of biogenic amines (Mercer et al., '83). Meanwhile, identified aminergic interneurons have been discovered and stained intracellularly (Rehder et al., '87). Amino acid transmitters such as gamma-amino butyric acid (GABA) (Bicker et al., '85; Schäfer and Bicker, '86) and glutamate (Bicker et al., '88) were localized in subsets of inter- and motoneurons by using polyclonal antisera specific for the fixed antigen. The organization of cholinergic pathways has been revealed by acetylcholinesterase histochemistry combined with immunocytochemical localization of nicotinic receptors (Kreissl and Bicker, '89) and by autoradiographic mapping of α-bungarotoxin binding (Scheidler et al., '90). A comprehensive picture of transmitter pathways (Bicker et al., '87) is therefore gradually emerging in the insect brain, which has been the subject of a behavioral pharmacological approach to proboscis extension learning (Bicker and Menzel, '89; Braun and Bicker, '92). Several transmitter markers have also been used for the identification of cellular phenotypes in primary cell cultures of the pupal honeybee brain (Kreissl and Bicker, '92). However, since immunocytochemical stainings for neurotransmitters require fixation of the antigen, they are of limited value for cell identification in physiological studies. Progress in further in vitro studies of honeybee neurons will thus depend on the development of additional markers which do not impede cellular viability.

Here we report that a monoclonal antibody derived from the above-mentioned hybridoma library against *Drosophila* head homogenate (Hofbauer, '87) cross-reacts with nervous tissue of the bee brain. The antibody stains monopolar neurons in the visual systems of both adult *Drosophila* and *Apis* species. In addition, it labels Kenyon cells of the *Drosophila* mushroom bodies and deutocerebral projection neurons of antennoglomerular tracts in *Apis*. This provides new insights into the anatomical organisation of olfactory pathways. The antibody is also a useful tool for the identification of neurons in cell culture because it recognizes surface markers on identified neurons of the olfactory and visual system. Since this recognition of the surface marker does not depend on fixation, the antibody can be employed to selectively label living neurons in dissociated cell culture.

MATERIALS AND METHODS

Generation of monoclonal antibody fb45

The generation and screening of monoclonal antibodies has been described by Hofbauer ('87). Briefly, heads of *Drosophila melanogaster* or brains removed from freeze-dried heads were homogenized prior to intraperitoneal injection into mice. Hybridoma supernatants were assayed for mouse Ig chains by enzyme-linked immunosorbent assay (ELISA). The positive supernatants were directly screened by immunocytochemistry for selective staining in the brain of adult wildtype *Drosophila*. This paper describes the immunocytochemical staining properties of MAB fb45, an antibody of IgM isotype.

Immunoblotting

Samples of *Drosophila* heads and *Apis* brain tissue were homogenized and separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Laemmli, '70). After electrophoresis they were transferred to nitrocellulose. The nitrocellulose filters were incubated with antibody fb45 and the bound antibody was visualized with second alkaline phosphatase conjugated anti-mouse IgM + IgG antibody (Dianova) using a Vectastain Kit (Vector Laboratories, Inc., Burlingame, CA) as substrate. Control lanes carrying material from both *Drosophila* and *Apis* showed no bands when challenged only with second anti-mouse antibody.

Immunocytochemistry

Drosophila. Adult flies of the wildtype "Berlin" were fixed in freshly prepared phosphate-buffered (0.07 M) 4% paraformaldehyde (pH 7.2). The heads were dissected in ice cold fixative, the proboscis and the ventral air sacs removed, and fixed for 2–3 hours at 4°C. The heads were stored overnight in 25% sucrose, frozen in melting nitrogen (Buchner et al., '86), and sectioned at 10 μm on a cryostat. Sections were incubated with hybridoma supernatant, diluted 1:2 in phosphate-buffered saline (PBS) (0.9% NaCl/0.1 M sodium phosphate buffer: 9/1). The immunocytochemical staining was performed according to the biotin-avidin technique (Vector Laboratories Inc., Burlingame) with 3,3'-diaminobenzidine (30 mg in 50 ml Tris-buffered saline + 50 μl 30% H_2O_2) as the chromogen.

Apis. A fixative composed of 4% paraformaldehyde dissolved in sodium phosphate buffer (0.1 M, pH = 7.2) was injected into the abdomen of live bees. The brains were subsequently dissected out of the head capsule and post-fixed for 45 minutes in the same fixative. After rinsing in PBS, the tissue was dehydrated in increasing grades of ethanol and then infiltrated with paraffin. Serial sections, 10 μm thick, were cut, deparaffinized, and rehydrated. The sections were incubated overnight at 4°C with the monoclonal antibody fb45 diluted 1:100 in PBS containing 0.1% Triton X-100. Subsequently, the sections were rinsed in PBS and exposed to horseradish peroxidase (HRP)-labelled goat anti-mouse antiserum (Nordic Immunological) diluted 1:100 in PBS for 1 hour at room temperature. The sections were then washed in PBS and treated with 3,3'diaminobenzidine (DAB, Sigma, 25 mg/100 ml) and H_2O_2 (0.01%). Subsequently, they were dehydrated, cleared in xylene, and mounted in Entellan (Merck).

Frozen sections

To detect differences in the binding properties of MAB fb45 due to different histological protocols for *Apis* and *Drosophila,* frozen sections were also prepared from *Apis* brains and processed for immunofluorescence. Dissected brains were fixed overnight in a solution of 10% formalin containing 1% $CaCl_2$ dissolved in sodium phosphate buffer (0.1 M, pH 7.4), washed in PBS and stored in 30% sucrose with 1% gum arabicum added. The tissue was blotted dry, and quickly frozen in Tissue Tek II (Miles) for sectioning. Serial sections (16 μm) were cut in a cryostat and mounted on chrome alum-coated slides. Subsequently, they were processed for immunofluorescence with the MAB fb 45 (diluted 1:100 in PBS) detected by an fluorescein isothiocyanate (FITC)-coupled anti-mouse antibody (Dianova 1:100 in PBS).

Primary cell culture of *Apis* neurons

The cell culture of dissociated *Apis* neurons has been described in detail by Kreissl and Bicker ('92). Brains of worker honeybee pupae were dissected three days before adult eclosion. The pigmented retina was cleaved from the brain and the perineural sheath encapsulating the cerebral ganglion was removed in hypertonic medium. Then optic and antennal lobes were separated from the remaining

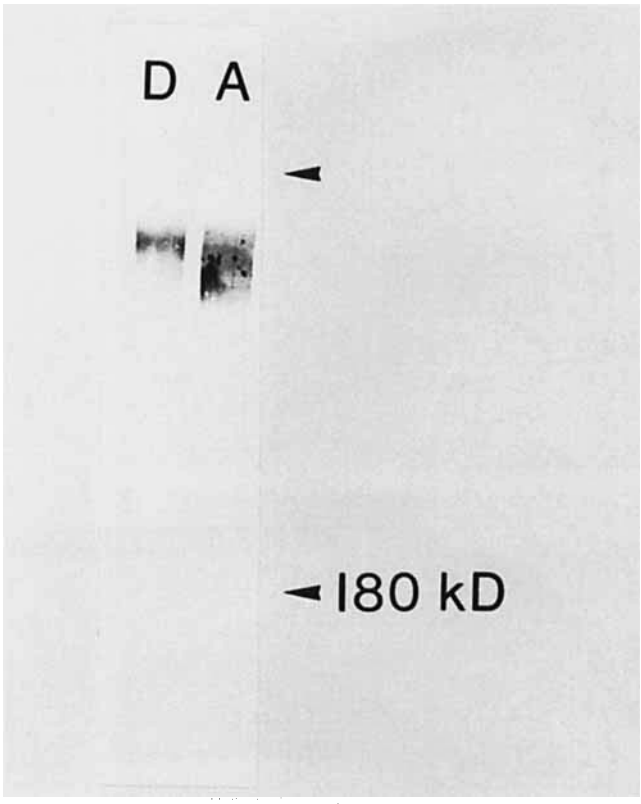

Fig. 1. Western blot with MAB fb45. Immunoblots of (D) *Drosophila* head and (A) *Apis* brain homogenate on 5% sodium dodecyl sulfate (SDS) gels. The upper arrowhead indicates the start of the running gel lane. Lower arrowhead indicates the position of molecular weight marker of 180 kD.

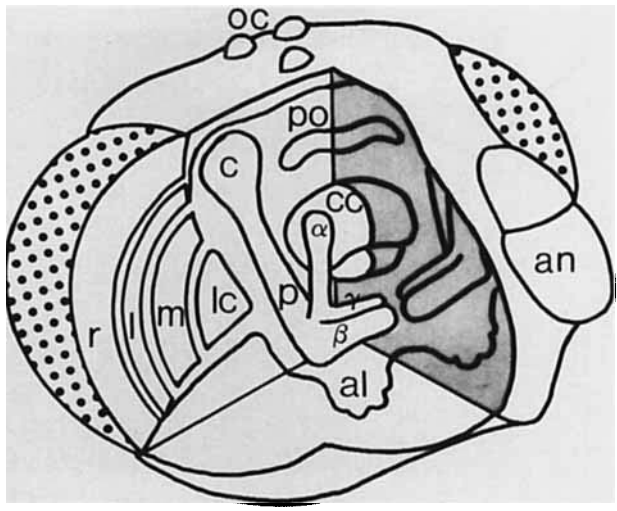

Fig. 2. Schematic drawing of *Drosophila* head. The right anterior head capsule and the anterior half of the right visual system are removed to show the position of the optic lobes with lamina (l), medulla (m), and lobula complex (lc), and the mushroom body system with the calyx (c), peduncle (p), alpha- (α), beta- (β), and gamma- (γ) lobe in the brain. al, antennal lobe; an, antenna; cc, central complex; oc, ocelli; po, pons; r, retina. The figure is based on and adapted from an artist's drawing in Heisenberg ('81).

cerebral tissue, pooled separately in cold, divalent cation-free honeybee saline, dissociated by gentle trituration with a 100 μl plastic pipette tip in an Eppendorf tube under sterile conditions, and plated in culture medium (Kreissl and Bicker, '92).

Immunocytochemistry with monoclonal antibody fb45. For immunocytochemical identification of living neurons in vitro, cultures of dissociated optic lobes were incubated with hybridoma supernatant 1:1 in culture medium for 6 hours at room temperature. After six washes, antibody binding was detected with a FITC-coupled goat-anti-mouse antibody at a dilution of 1:20 (Dianova). Neurons were photographed with a Zeiss Axiovert microscope using phase contrast optics and fluorescence optics.

RESULTS

Immunoblots

The molecular specificity of the monoclonal antibody was investigated on Western blots. Homogenates of *Drosophila* head and dissected brains of *Apis* were run on SDS gels according to the method of Laemmli ('70). Because the immunoreactive material does not easily enter the running gel, we employed a 5% SDS gel in which we let the front migrate out of the gel in order to obtain a better resolution of high molecular weight components. As shown in Figure 1, MAB fb45 recognized an antigen of similar size in both *Drosophila* and *Apis* tissue with an apparent molecular weight much higher than 180 kD. No other immunoreactive low molecular weight bands were detected on 5–12% linear gradient gels.

Drosophila

The description of immunoreactivity in the brain of *Drosophila* is based on sections cut in the horizontal plane. Figure 2 shows the position of the main neuropile areas. When applied to frozen sections, MAB fb45 binds to the perineural sheath and to a granular cellular substructure in most, if not all cell bodies of the brain, resulting in a weak overall staining of the cell body rind. In addition, some preparations showed staining of the tracheal system.

Central brain

Apart from this general staining pattern, there is characteristic and distinct immunoreactivity expressed in structures of the central brain and optic lobes. The most prominent staining in the central brain occurs in a substructure of the mushroom body system (Figs. 2, 3). In each of the two calyces, four distinctly labelled areas, mainly neuropile, were seen. In well stained preparations, adjacent cell bodies were also stained (Fig. 3c). Stained fiber tract-like structures originate from these labelled areas and fuse with one another after entering the peduncle (Fig. 3b). The labelled structures could be followed throughout the extension of the peduncle, the α-lobe (data not shown) and the β-lobe (Fig. 3a).

The location of the cells and their extension into the peduncle and the α- and β-lobes suggests that the immunostaining belongs to Kenyon cells forming the median peduncle (Heisenberg, '80). Two main types and several subtypes of Kenyon cells are known (Strausfeld, '76), but they cannot be correlated with the distribution of the labelled cells into four grape-like aggregates. Rather, it is tempting

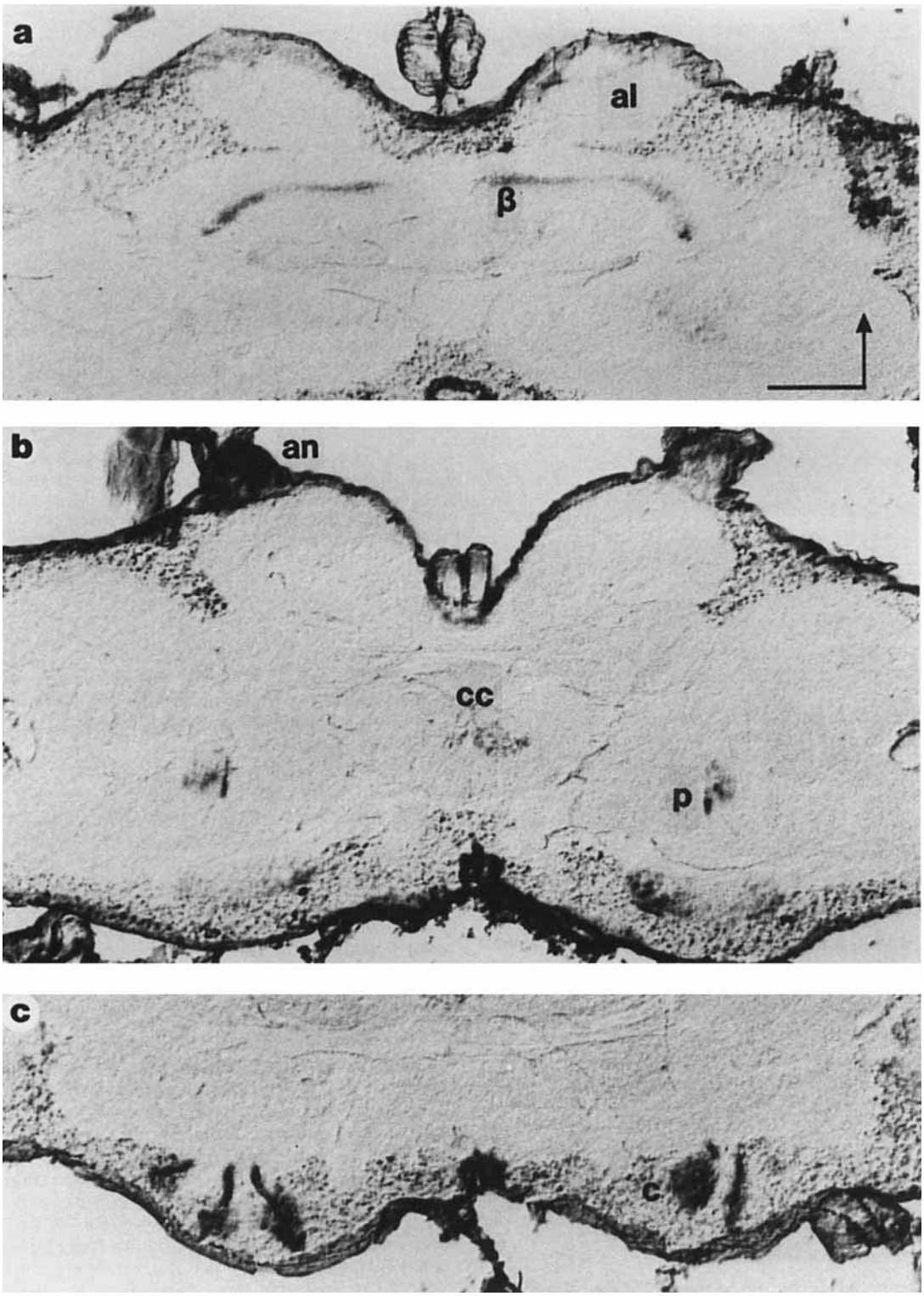

Fig. 3. **a–c:** Oblique horizontal cryostat sections through the central brain of *Drosophila*. Section a is more ventral than section c. The label identifies grape-like aggregations of cells in the cell body rind of the calyces (c). From there, structures can be followed through the peduncle (p) into the beta lobe (β). (The labelled α lobe is not shown in this figure.) Label is also present in the antennal nerves (an), but not in the antennal lobes (al). cc, central complex. Arrow points towards anterior. Scale bar: 50 μm for a–c.

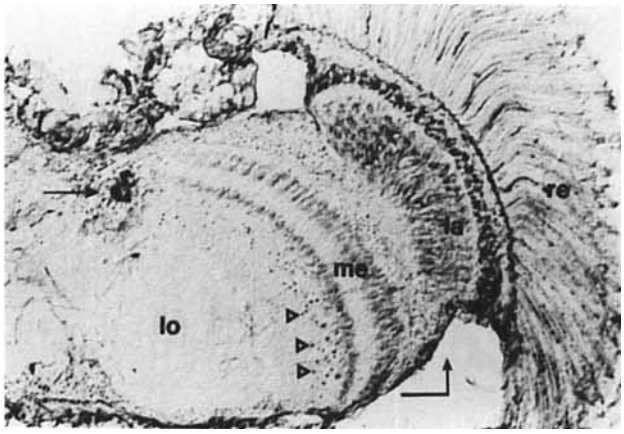

Fig. 4. Optic lobes of *Drosophila*. Horizontal cryostat section through the visual system of *Drosophila*. MAB fb45 binds to the cell body layer of the lamina between the retina (re) and lamina neuropile (la), to the lamina neuropile itself, and to two discrete layers of the medulla (me). In addition, there are fine dots in the medulla (arrowheads) which could be part of the arborisations of the labelled cells anterior to the medulla (arrow). lo, lobula. Arrow indicates anterior. Scale bar: 20 μm.

to correlate these aggregations and their common projections into the peduncle with a common developmental fate, as is suggested by the observation of Ito and Hotta ('92), that four neuroblasts are associated with the developing Kenyon cells.

In addition to the described immunoreactivity of the mushroom body system, a few homogeneously labelled somata were present in the caudal cell body rind of the central brain. The projection pattern of these cells is not known. Strong labelling was also observed in the antennal nerve up to its entrance into the brain (Fig. 3b). The immunoreactivity of the nerve decreases rapidly upon entering the antennal lobe. Only occasionally was some faint staining associated with the antennal glomeruli observed.

Optic lobes

In the optic lobes, a prominent staining could be detected in the cell body layer of the lamina, between the fenestrated layer of the retina and the lamina neuropile (Fig. 4). This type of labelling had a different appearance than the overall staining of the cell body rind of the brain. Instead of expressing small granular structures in the soma, the whole cell except for the nucleus was homogeneously labelled. Additional staining was found throughout the neuropiles of lamina and of the second optic ganglion, the medulla. The staining of the medulla was restricted to two layers, a broad distal layer covering about 0–20%, and a thinner more proximal layer at about 38–42%, when the proximal border of the neuropil is taken as 0% and the distal border as 100% medullar depth. The labelled medulla layers corresponded to the layers 1, 2, and 5 as defined by Fischbach and Dittrich, '89. The pattern of stained cell bodies and neuropil layers (Fig. 4) corresponds well to the Golgi-stained monopolar cells L1 and L2 (Fischbach and Dittrich, '89), suggesting that these cells are the main components of the structure stained by fb45.

A few more homogeneously labelled cell bodies were found anterior to the medulla, near the border between optic lobes and central brain (Fig. 4, arrow). This area contains the somata of a very heterogeneous population of neurons including several types of tangential cells of the medulla. The innervation of the medulla by immunoreactive tangential fibers would explain the small stained structures in the middle of the neuropile (Fig. 4, arrowheads).

Apis

Initially, we applied the antibody to frozen sections of *Apis* brains in order to match the histological staining procedure in *Drosophila*. Since the antibody showed identical staining patterns on paraffin serial sections, thus allowing a more convenient anatomical tracing of immunoreactive neurons, the following description is based on immunostaining of paraffin-embedded tissue. Immunoreactivity was restricted to the perineural sheath of the nervous system and to the membranes including homogeneous cytoplasmic staining of identified populations of central projection neurons. As in *Drosophila*, almost all neurons expressed granular immunoreactive material in their cell bodies. Staining of particular cell types described here refers only to the immunoreactive cell-surface and homogeneous cytoplasmic label.

Olfactory pathways

In *Apis*, sensory afferents of the antennal nerve project in four major tracts (T1–T4) into the antennal lobe (Suzuki, '75; Mobbs, '85). A quantitative ultrastructural study (Gascuel and Masson, '91) demonstrated that, as in many other insects, synaptic integration between sensory afferents, local interneurons and projection neurons is mainly confined to the spherical glomeruli which surround the central coarse neuropile. The anatomical pathways of the output relay neurons in bees are, however, slightly different than in flies (Strausfeld, '76; Mobbs, '85). The principal output of the antennal lobes is via the median and lateral antennoglomerular tracts into the calycal lips of the mushroom bodies and into the lateral protocerebral neuropile (Mobbs, '85) (Fig. 5). Stainings with cobalt (Mobbs, '85; Arnold et al., '85) and immunocytochemical investigations (Schäfer and Bicker, '86) have revealed additional but less prominent outputs such as, for example, the mediolateral antennoglomerular tract or a small tract interconnecting the two antennal lobes.

In the brain of the worker bee, the monoclonal antibody fb45 selectively labelled the median and lateral antennoglomerular tracts (Figs. 6a, 7a,b), the corresponding cell bodies of the relay neurons in the ventrolateral and dorsal soma rind of the antennal lobe (Fig. 6b,c) and the glomeruli (Fig. 6c,d). We counted 462 ± 83 (SD, n = 8) somata per antennal lobe with cell-surface staining. The distribution of immunoreactive material in cell bodies is illustrated in a sagittal section through the dorsal soma rind (Fig. 6b). All cells contained immunoreactive granular cell compartments but only the cell bodies of the relay neurons express the immunoreactive granules, homogeneous cytoplasmic, and immunoreactive cell-surface staining (Fig. 6b). The diameter of the immunoreactive granules was measured on projected slides of specimens photographed through a 100 × oil immersion objective. Depending on cell type and size, the grana diameters ranged from 1 to 2 μm. The dendrites of the relay neurons which arborize in the glomeruli were immunoreactive (Fig. 6d). Immunoreactivity appeared denser in the cortical layer of the glomeruli than in the central core (Fig. 6c,d).

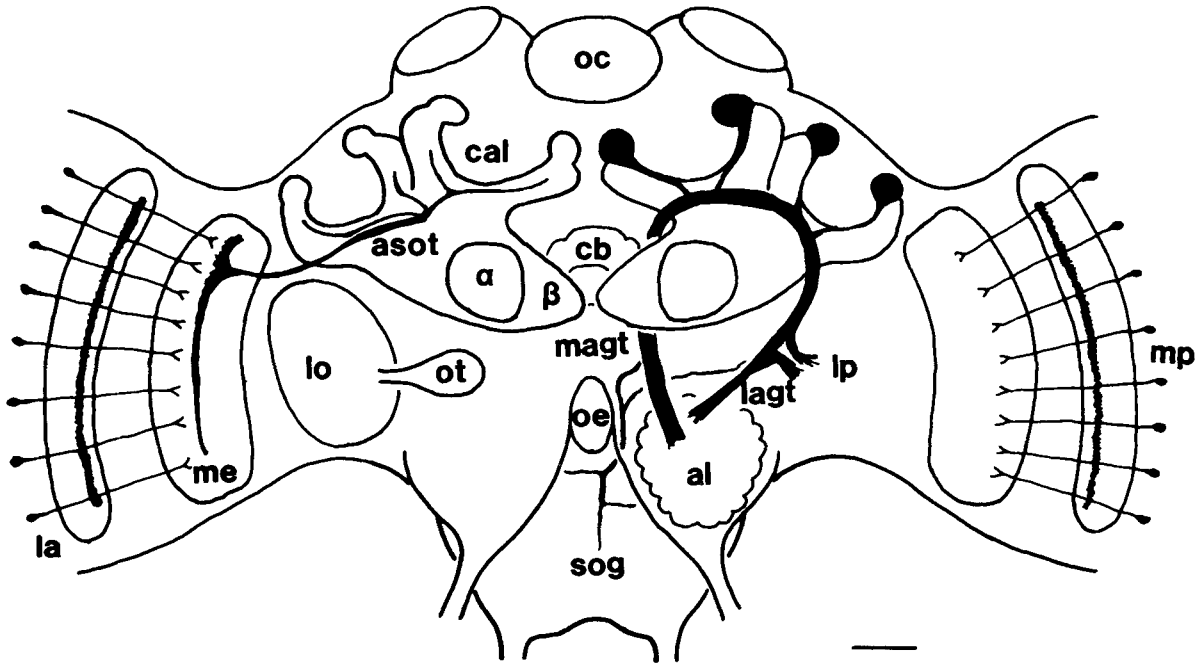

Fig. 5. Frontal view of *Apis* brain. Schematic drawing of immunoreactive fiber tracts. Immunoreactivity was found in the antennal lobe (al), which sends relay neurons via the median antennoglomerular tract (magt) and the lateral antennoglomerular tract (lagt) into the calyces (cal) of the mushroom bodies and into the lateral protocerebral neuropile area (lp). Immunoreactive fibers with arborizations in the suboesophageal ganglion (sog) approach the magt and lagt. The optic lobes comprise lamina (la), medulla (me), lobula (lo), and optic tubercle (ot). Immunoreactivity was found in certain monopolar cells (mp) and in fibers of the anterior superior optic tract (asot). α, α-lobe; β, β-lobe; cb, central body; lp, lateral protocerebrum; oe, oesophagus. Scale bar: 100 μm.

No label was found in the antennal sensory afferents projecting into the antennal or dorsal lobe, or in the mediolateral antennoglomerular tracts. Glomeruli, occurring singly or in groups can be identified (Arnold et al., '85; Flanagan and Mercer, '89) based on their innervation by four different branches of the antennal sensory nerve (Suzuki, '75). Branch T1, for example, innervates a group of glomeruli in the dorsal and anterior region of the antennal lobe. We traced immunoreactive fibers from their glomerular exit in serial sections (Fig. 6d) and found that projection fibers of T1 glomeruli converged in the lateral antennoglomerular tract (Figs. 6a,d). Unfortunately, the high density of immunoreactive fibers in the antennal lobe makes it impossible to determine whether the lateral antennoglomerular tract collects the projections of all T1 glomeruli. The schematic drawing (Fig. 6a) shows the lateral antennoglomerular tract exiting the antennal lobe posteriorly below the bifurcation of main branch of the median antennoglomerular tract. The immunocytochemical staining resolved one root of the median antennoglomerular tract originating from the ventrally placed T3 glomeruli region and another root originating from the medial T2 region, respectively.

The olfactory system of the honeybee and of many other insects displays a high degree of sexual dimorphism. In addition to the ordinary glomeruli, the antennal lobe of the drone contains four macroglomerular complexes (Arnold et al., '85) which are thought to be involved in sensory information processing of queen pheromones. We found immunoreactivity in both categories of glomeruli, but it appears less intense in the macroglomerular complexes (Fig. 6e). This is a consistent feature which can be ascribed to the lower density of neurite arborisations in the macroglomerular complexes. Projections of the median and lateral antennoglomerular tracts into the mushroom bodies and lateral protocerebral neuropile of the drone were also stained by the antibody.

Projection neurons of the median and lateral antennoglomerular tract eventually terminate in or near the calyces of the mushroom bodies and projection areas of the lateral protocerebral neuropile area (Mobbs, '85). The known protocerebral projections of the antennoglomerular tracts including the lip regions of mushroom body calyces and the lateral protocerebral lobes also carried the surface label (Figs. 5 and 7). Figure 7a and b show the immunoreactive fiber tracts and immunoreactive arborisations in the lateral protocerebral neuropile area. Intracellular Lucifer Yellow injections (Homberg, '84) and cobalt stainings (Mobbs, '85; Arnold et al., '85; Bicker et al., '87) have revealed a main target area of the projection neurons in the lip neuropile of the calyces. The immunoreactive arborizations of the projection neurons clearly delineated the lip neuropile of the calyces (Fig. 7c). However, the intensity of immunostaining in the calyx was consistently lower than the staining of processes in the antennal lobe or the antennoglomerular tracts.

Weak immunolabelling in the basal ring, which receives projections from the tritocerebrum and suboesophageal ganglion (Mobbs, '85), is unaccounted for, but might be due to collateral branches of antennoglomerular tract fibers. We could identify additional immunoreactive input from the optic ganglia via the anterior superior optic tract entering the mushroom bodies through the outer ring tract (Mobbs, '85). The collaterals of these fibers displayed only faint reactivity in a segment of the collar neuropile.

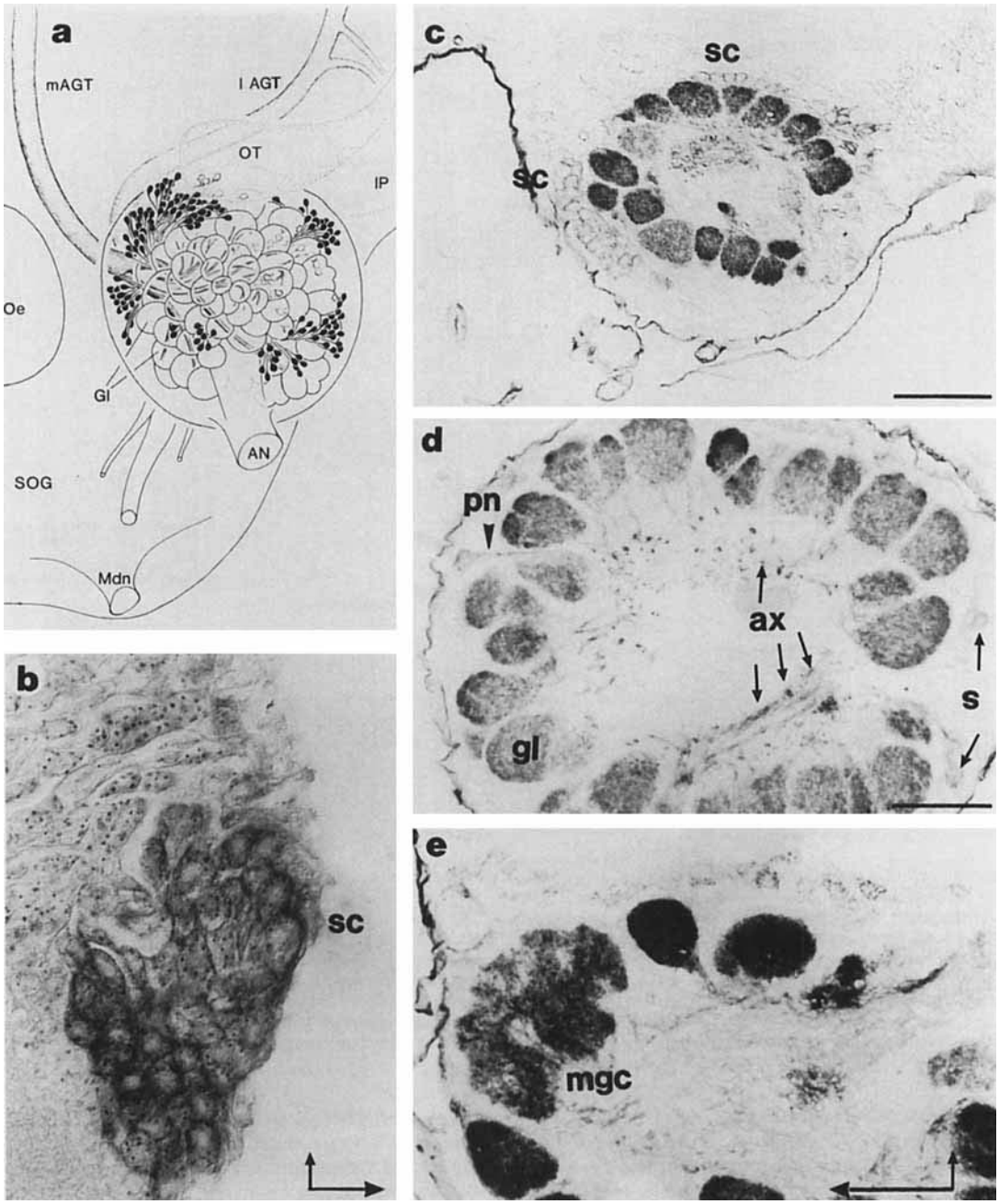

Fig. 6. Immunoreactivity in the chemosensory system. **a:** Schematic drawing of the antennal lobe relay neurons showing soma position and the projection pattern into the median (mAGT) and lateral antennoglomerular tract (lAGT). AN, antennal nerve; Mdn, mandible nerve; Gl, glomeruli; lP, lateral protocerebrum; Oe, oesophagus; OT, optic tubercle; SOG, suboesophageal ganglion. **b:** Immunoreactivity in the dorsal soma rind of the antennal lobe. Immunoreactive soma cluster (sc) of the relay neurons originating in T1 glomeruli. The relay neurons express immunoreactive surface, homogeneous cytoplasmic, and granular staining. Other cell bodies in the vicinity contain immunoreactive granules within the clear cytoplasm but no surface label. Small arrow indicates dorsal, large arrow indicates anterior. **c:** Frontal section of the antennal lobe showing the immunoreactivity in two clusters of somata (sc), in the glomeruli and the central neuropile. **d:** Frontal section of the antennal lobe. The position of a labelled primary neurite (pn) including its cell body is shown. Other labelled somata (s) are among unlabelled cell bodies. The dendrites of the relay neurons arborise in the glomeruli (gl). Axon bundles (ax) converging in the antennoglomerular tracts are shown in cross section. **e:** Sagittal section through the antennal lobe of a drone. Immunoreactivity in macroglomerular complex (mgc) GC I and ordinary glomeruli. Small arrow points towards dorsal, large arrow indicates anterior. Scale bars: 50 μm for b, d, and e; 100 μm for c.

Unlike the stained columns of Kenyon cells in the mushroom bodies of *Drosophila,* the Kenyon cells of *Apis* carried no immunoreactive epitope on their cell surface. There was, however, a clear distinction between two groups of Kenyon cells with respect to the distribution of immunoreactive grana in the cell bodies. Kenyon cell somata in the core of the calyces expressed the immunoreactive grana (Fig. 7c), whereas a second population of Kenyon cell somata lining the calycal cups was completely devoid of immunoreactivity (Fig. 7c).

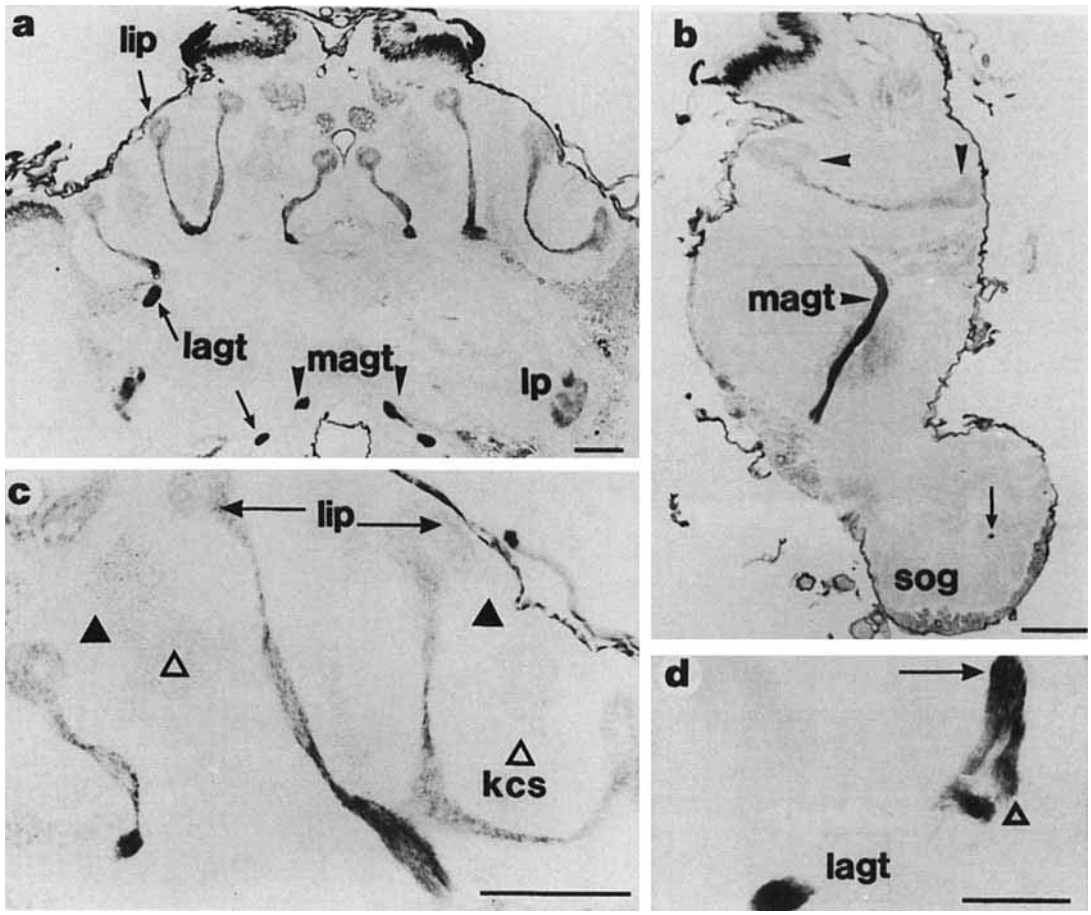

Fig. 7. Frontal section of immunoreactive projections of antennoglomerular tracts. **a:** Median antennoglomerular tract (magt) and lateral antennoglomerular tract (lagt) project into the lip neuropile of the calyces and the lateral protocerebral neuropile (lp). **b:** Sagittal section through the brain and suboesophageal ganglion. The section partly follows the course of the median antennoglomerular tract (magt). Arrowheads indicate immunoreactivity in the calyces. Arrow indicates immunoreactive neurites in the suboesophageal ganglion (sog). **c:** Immunoreactivity in mushroom bodies. A central population of Kenyon cell somata (kcs) contains immunoreactive grana in the cell bodies. The open triangles indicate the central population, whereas the closed triangles are surrounded by Kenyon cell somata that are not immunoreactive. Terminal arborisations of antennoglomerular tract fibers delineate the lip neuropile of the two calyces. **d:** Lateral protocerebrum. Fibers of the lateral antennoglomerular tract (lagt) fasciculate with descending fibers of the median antennoglomerular tract (arrow). Lagt and magt collaterals leave the common tract at separate exits (triangle) into the lateral protocerebrum. Scale bars: 100 μm.

Descending median and ascending lateral antennoglomerular tract fibers form a closed loop in the lateral protocerebrum (Fig. 5) with different exits into the lateral protocerebral neuropile area. The separate routes of the median and lateral tract fibers into the lateral protocerebral neuropile were also clearly revealed by our immunocytochemical stainings (Fig. 7d).

Suboesophageal ganglion and thoracic ganglia

In both the worker bee and the drone, we found immunoreactive fibers not only in the preoral neuropiles of the brain but also in the suboesophageal ganglion and in the thoracic ganglia. As shown in the schematic drawing of Figure 5, arborisations of these fibers approach the median antennoglomerular tract near the oesophagus at several locations. Reconstruction of the immunoreactive fibers in serial sections also revealed arborisations in the vicinity of the lateral antennoglomerular tract but we could never confirm a discrete link. The main fiber pathway runs close to the oesophagus into the suboesophageal ganglion, forming a connection between the deutocerebrum, tritocerebrum, and the three neuromeres of the suboesophageal ganglion. The principal immunoreactive fiber trunk joins the median ventral tract in the suboesophageal ganglion, extends lateral arborisations into all three neuromeres and finally exits through the cervical connective (Fig. 8a). The immunoreactive neurites were followed into the thoracic ganglia (Fig. 8b) but we could not resolve their origin.

Optic lobes

Similar to the staining in *Drosophila,* certain monopolar cells of the lamina are immunoreactive in the bee (Figs. 5, 9a). From counts of the clustered cell bodies in the fenestrated layer under Nomarski optics, we estimate that approximately 25% of the monopolar cells expressed immunoreactivity on their surface. Both labelled and unlabelled monopolar cell somata are visible in a frontal section through the fenestrated layer (Fig. 9b). A horizontal section showed the staining of somata, fine neurites in the distal neuropile of the lamina and extensive lateral arborisations in the external plexiform layer C, which is a narrow stratum nearest to the outer chiasma (Fig. 9a). The axonal

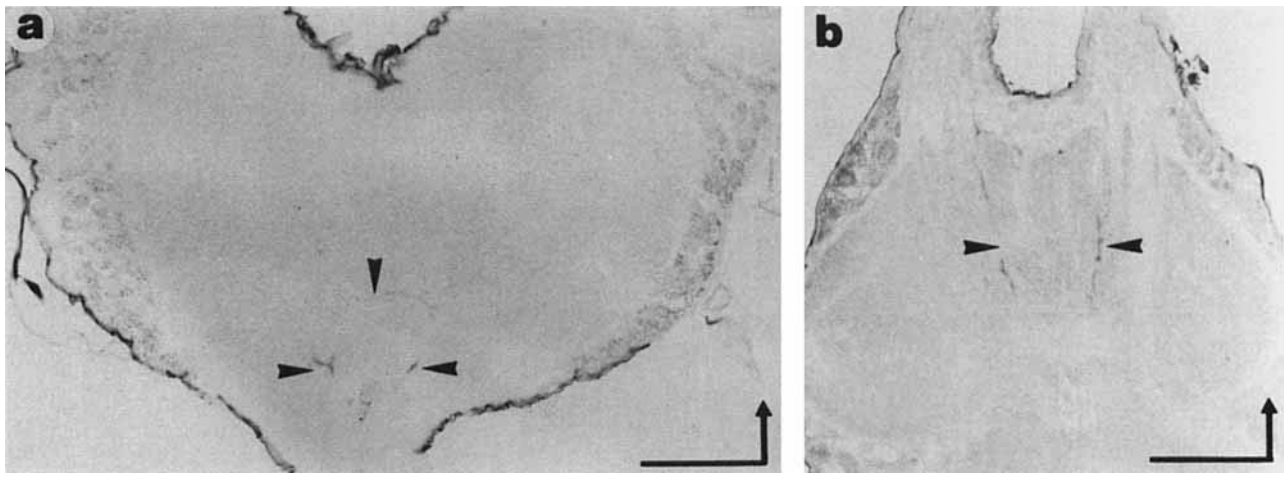

Fig. 8. Suboesophageal and thoracic ganglia. **a:** Horizontal section through the suboesophageal ganglion. Arrowheads indicate immunoreactive fibers with projections into the cervical connective. **b:** Horizontal section through the mesothoracic neuromere. Arrowheads indicate immunoreactive fibers connecting to the other thoracic neuromeres. Arrow indicates anterior. Scale bars: 100 μm.

projections of the monopolar cells could be traced through the outer chiasma as shown in horizontal section (Fig. 9a). The terminal processes penetrate deep into the proximal regions of medullar stratum 2 (Ribi and Scheel, '81), suggesting that the labelled neurons are L1 monopolar cells (Ribi, '75). According to Ribi ('75), other monopolar cell types terminating in more distal parts of layer 2 lack lateral arborisations in the external plexiform layer C of the lamina. However, the light microscopical resolution did not enable us to exclude other medullar intrinsic sources of immunoreactivity in stratum 2.

The terminal arborisations of the monopolar cells are approached from the proximal side of the medulla by processes of immunoreactive neurons (Fig. 9a) which converge into the anterior superior optic tract. This tract contains a heterogeneous population of neurons projecting from the lobula and the medulla to the mushroom bodies (Fig. 6). Only the fibers which leave the medulla through the median exit showed immunoreactivity. The lobula fibers were not stained.

Identification of living cells in primary culture

Neurons from specific regions of the pupal brain such as the antennal lobe, mushroom bodies, or optic lobes can be grown in dissociated cell culture, and MAB fb45 has been employed for the identification of olfactory relay neurons in vitro (Kreissl and Bicker, '92). Because the histological staining pattern on sections is rather independent of fixation conditions, we tested whether the antibody would recognize the cell surface epitope without prior fixation. Here we compare the staining properties of the antibody with and without fixation of the cell cultures. In cell cultures fixed with paraformaldehyde, immunoreactive grana like those seen in sectioned tissue were found in many neurons (Fig. 10a). However, we also detected single neurons expressing the immunoreactivity on their entire surface. Since the cell culture of Figure 10a was obtained from dissociated antennal lobes, we conclude that these neurons represent the olfactory relay neurons.

It was also possible to label the cell surface of certain neurons in unfixed cultures of the antennal and optic lobes by indirect immunofluorescence with MAB fb 45. In cell cultures obtained from the optic lobes, less than 4% of the

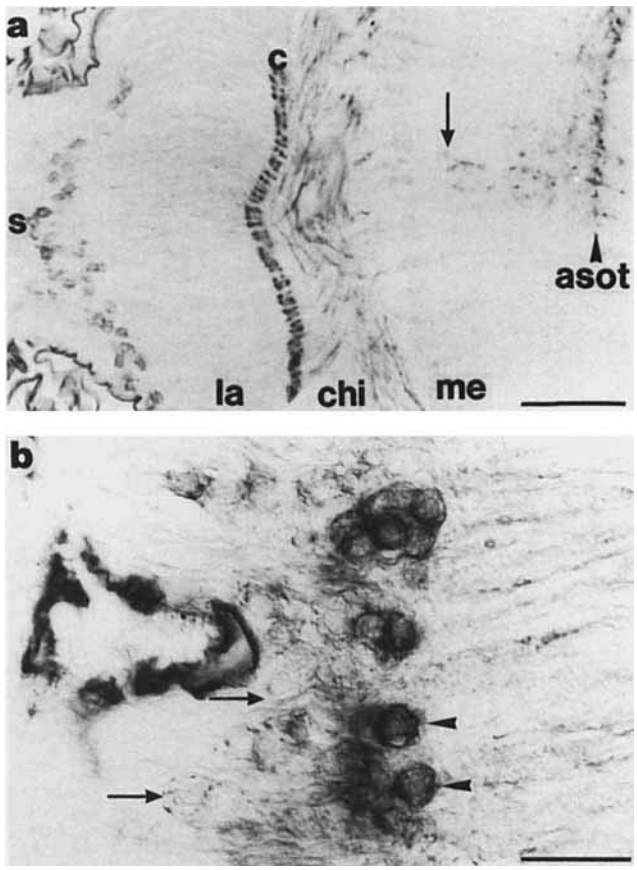

Fig. 9. Optic lobes. **a:** Horizontal section through optic ganglia. Immunoreactive somata (s) of monopolar cells give rise to lateral arborisations in the external plexiform layer c (c) of the lamina (la). Axons project through the outer chiasma (chi) and terminate in the medulla (me). Terminals of monopolar cells are approached by immunoreactive neurites (arrow) from interneurons exiting the medulla through the anterior superior optic tract (asot). **b:** Frontal section through the fenestrated layer. Monopolar cell somata with surface label (arrowheads) are shown together with unstained monopolar cell somata (arrows) in the vicinity of a trachea. Scale bars: 50 μm in a, 20 μm in b.

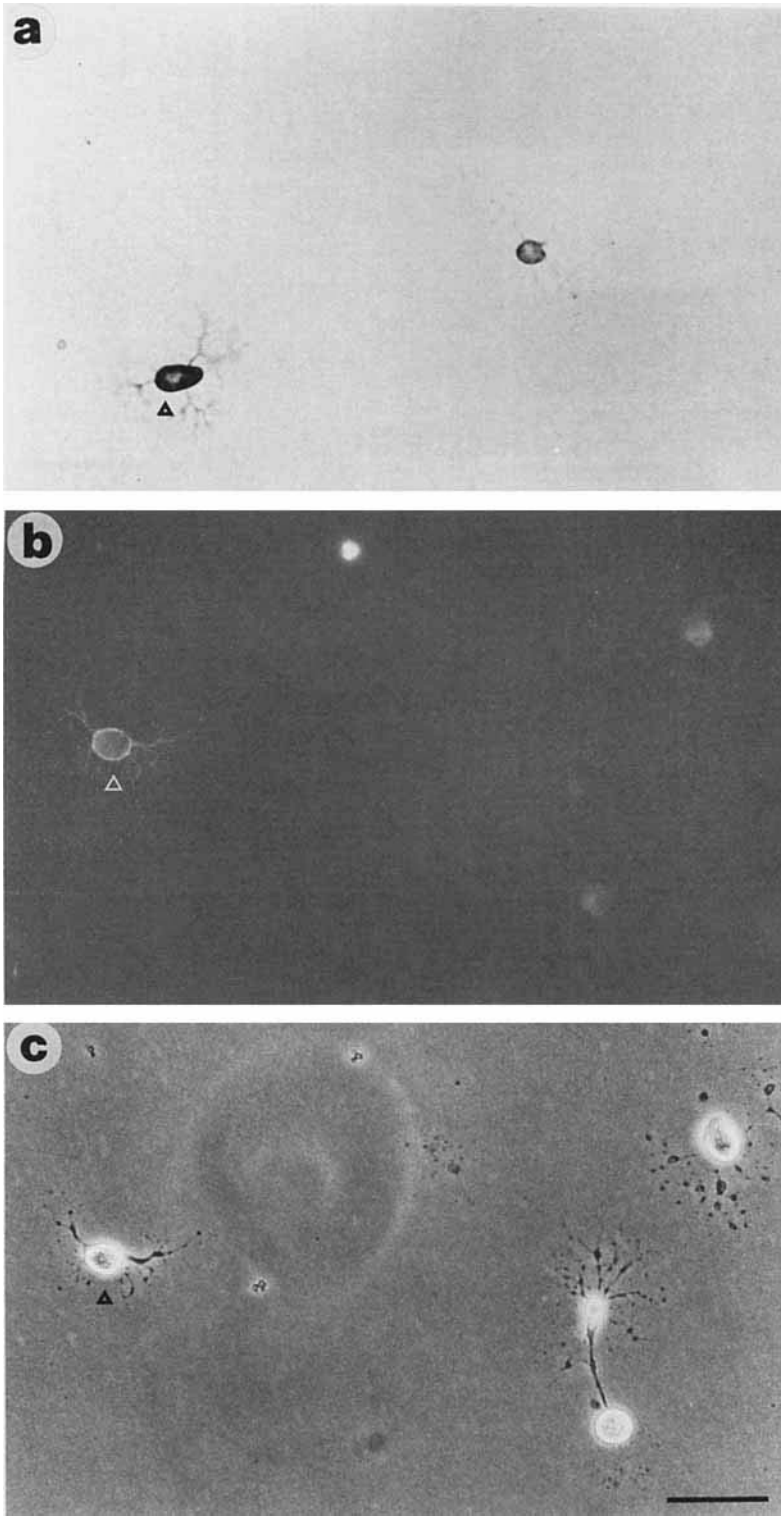

Fig. 10. Primary cell cultures of *Apis* neurons. **a:** Dissociated neurons of antennal lobe grown in culture and fixed in paraformaldehyde. Peroxidase staining reveals immunoreactivity on the surface of the neuron and stained grana in the cell body (triangle). The other neuron shows no surface label but immunoreactive grana in the soma. **b:** Indirect immunofluorescence of living optic lobe neurons grown in culture. Immunofluorescence is evident on the surface of the neuron (triangle). **c:** Phase contrast micrograph of the optic lobe neurons shown above (triangle) revealing the other two unlabelled neurons. The phase contrast micrograph of the living neurons was photographed after the immunofluorescence staining procedure of b. Scale bar: 50 μm for a–c.

neurite-bearing cells were stained. These are most likely the L1 monopolar cells. As it did on sections, the antibody labelled the entire surface including the outgrown neurites, but no immunoreactive grana were observed in the living cells. An immunofluorescent neuron together with two unlabelled neurons is shown in Figure 10b.

DISCUSSION

Monoclonal antibody fb45 recognized on Western blots of *Drosophila* and *Apis* tissue a single band of low electrophoretic mobility (apparent molecular weight > 180 kD), suggesting a common antigen in both species. We did not

characterize the biochemical properties of the antigen any further, but it is commonly found that material not entering SDS gels readily is either of high molecular weight or/and heavily glycosylated. Application of the antibody to sections of *Drosophila* and *Apis* brain demonstrated immunoreactive grana of variable density in the majority of neuronal cell bodies with the exception of certain Kenyon cells in *Apis*. The light microscopical resolution of our immunocytochemical study did not enable us to assign the immunoreactive grana to a specific cell organelle. In both *Drosophila* and *Apis*, the antigen was also expressed on the perineural sheath and on the surface of particular neurons which conspicuously fasciculate in common tracts or neuropilar compartments. The combined results of immunoblotting and immunocytochemistry suggest that the antibody exhibits molecular and anatomical specificity.

Fasciculation

The majority of neurons both in *Drosophila* and *Apis* contain the antigen in granular cell compartments but expression of the immunoreactivity on the cell surface is restricted to a limited number of neurons. Since the Western blot resolved only a single high molecular weight band, we suggest that the epitope is part of a molecule which is concentrated in the immunoreactive grana and expressed in extracellular form only by a specific set of interneurons. However, in the absence of electron microscopic data, it is not clear whether the epitope is part of the cell surface or of a molecule secreted into the extracellular matrix.

Relay neurons of the median and lateral antennoglomerular tracts of *Apis* share common projection areas in the lips of the mushroom body calyces and lateral protocerebrum (Figs. 5–7). The segment between mushroom body neuropile and lateral protocerebrum of the antennoglomerular tract is thus formed by a mixture of ascending lateral antennoglomerular tract fibers and descending fibers of the median antennoglomerular tract (Fig. 5). Thus, selective cellular interactions of the relay neurons are likely to play an important role during the formation of the tract in neuronal development, but may also be required for stabilizing the mature structure in the adult. The immunocytochemical staining pattern of the adult worker bee does not yet allow the ascription of a specific role to the fb45 antigen in the formation and maintanance of the antennoglomerular tracts. However, a detailed investigation of fb45 immunoreactivity during the development of the antennoglomerular tracts in the pupa is in preparation (Eichmüller and Rybak, personal communication). In *Drosophila*, the antennoglomerular tract was not stained, but the antigen was expressed in bundles of Kenyon cells. The staining pattern in the mushroom bodies of *Drosophila* (Fig. 3) provides another example for the expression of the antigen on fasciculating cells.

We obtained evidence at the light microscopical level that stained monopolar cells, which most likely represent the L1 type (Ribi, '75), contact immunoreactive neurons of the anterior superior optic tract in the medulla of the bee (Fig. 9a). These new anatomical data suggest that the two types of visual interneurons are linked in a common neural pathway.

Here we showed that monopolar cells, and in a previous study (Kreissl and Bicker, '92), antennoglomerular tract neurons, express the fb45 antigen in primary cultures of pupal honeybee brain. Indirect immunofluorescence revealed a surface label on somata and neurites of living neurons (Fig. 10). The selective binding of MAB fb45 to living neurons opens up the possibility for future functional studies on the role of the antigen during formation of neurites.

Olfactory pathways of *Apis*

The anatomical studies published to date (Mobbs, '85; Arnold et al., '85; Flanagan and Mercer, '89), as well as the intracellular recordings (Homberg, '84) have provided clues to the soma location of individually stained olfactory relay neurons. Using immunocytochemistry of MAB fb45, we have determined the position of the somata of relay neurons projecting through the median and lateral antennoglomerular tracts (Fig. 6). In addition, we have obtained new anatomical data about the organisation of antennal lobe exits via median and lateral antennoglomerular tracts. The glomeruli have been classified according to their sensory input from the four antennal nerve tracts innervating the antennal lobe (Mobbs, '85; Arnold et al., '85; Flanagan and Mercer, '89). Our evidence suggests segregation of antennal lobe output based on glomerular position. Although our immunocytochemistry, which simultaneously stained ca. 460 interneurons, did not allow reconstruction of single fibers, and glomeruli can only be unambiguously identified by backfilling their sensory innervation, serial sections indicate that the lateral antennoglomerular tract is mainly innervated by relay neurons from T1 glomeruli, whereas the median antennoglomerular tract is supplied mainly by the other glomeruli. At present, it is not known whether the different sensory tracts of the antennal nerve represent different input channels of chemosensory information. Even though the neuropile of the antennal lobe allows for many lateral interactions in chemosensory integration, our immunocytochemical results suggest that different sensory input may leave the antennal lobe via separate output channels, providing the anatomical substrate for information processing in distinct labelled lines.

ACKNOWLEDGMENTS

We thank Uli Müller and Matthias Bregulla for their patient help with the immunoblotting of the antigen. We also thank Christine Jaeckel, Dagmar Richter, and Sybille Schaare for technical assistance; Stefan Eichmüller and Jürgen Rybak for comments on an earlier draft of this paper; and Mary Cahill for correcting a preliminary version of the text. This work was supported by a Heisenberg Fellowship to G. Bicker and grants (Bi 262/3 and Ho 798/3) of the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

Arnold, G., C. Masson, and S. Budharugsa (1985) Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (*Apis mellifera*). Cell Tissue Res. *242*:593–605.

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

Bicker, G., and R. Menzel (1989) Chemical codes for the control of behaviour in arthropods. Nature *337*:33–39.

Bicker, G., S. Schäfer, and T. Kingan (1985) Mushroom body feedback interneurones in the honeybee show GABA-like immunoreactivity. Brain Res. *360*:394–397.

Bicker, G., S. Schäfer, and V. Rehder (1987) Chemical neuroanatomy of the honeybee brain. In R. Menzel and A. Mercer (eds): Neurobiology and Behavior of Honeybees. Berlin: Springer-Verlag, pp. 202–224.

Bicker, G., S. Schäfer, O.P. Ottersen, and J. Storm-Mathisen (1988) Glutamate-like immunoreactivity in identified neuronal populations of insect nervous systems. J. Neurosci. *8:*2108–2122.

Braun, G., and G. Bicker (1992) Habituation of an appetetive reflex in the honeybee. J. Neurophysiol. *67:*588–598.

Buchner, E., S. Buchner, G. Crawford, W.T. Mason, P.M. Salvaterra, and D.B. Satelle (1986) Choline acetyltransferase-like immunoreactivity in the brain of *Drosophila melanogaster.* Cell Tissue Res. *246:*57–62.

Buchner, E., R. Bader, S. Buchner, J. Cox, P.C. Emson, E. Flory, C.W. Heizmann, S. Hemm, A. Hofbauer, and W.H. Oertel (1988) Cell-specific immuno-probes for the brain of normal and mutant *Drosophila melanogaster* I. Wildtype visual system. Cell Tissue Res. *253:*357–370.

Fischbach, K.F., and A.P.M. Dittrich (1989) The optic lobe of *Drosophila melanogaster.* 1: A Golgi analysis of wild-type structure. Cell Tissue Res. *258:*441–475.

Flanagan, D., and A.R. Mercer (1989) An atlas and 3-D reconstruction of the antennal lobes in the worker honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) Int. J. Insect Morphol. Embryol. *18:*145–159.

Fujita, S.C., S.L. Zipursky, S. Benzer, A. Ferrus, and S.L. Shotwell (1982) Monoclonal antibodies against the *Drosophila* nervous system. Proc. Natl. Acad Sci. USA *79:*7929–7933.

Gascuel, J., and C. Masson (1991) A quantitative ultrastructural study of the honeybee antennal lobe. Tissue Cell *23:*341–355.

Heisenberg, M. (1980) Mutants of brain structure and function: What is the significance of the mushroom bodies for behavior? In O. Siddiqi, P. Babu, L.M. Hall, and J.C. Hall (eds): Development and Neurobiology of Drosophila. New York, London: Plenum Press, pp. 373–390.

Heisenberg, M. (1981) Forscher beginnen ein Gehirn zu begreifen. Bild der Wissenschaft *18:*46–59.

Hofbauer, A. (1987) Monoclonal antibodies reveal anatomical details in the Drosophila brain. In N. Elsner and O. Creutzfeldt (eds): New Frontiers in Brain Research. Stuttgart, New York: G. Thieme Verlag, p. 253.

Hofbauer, A., and E. Buchner (1989) Does *Drosophila* have seven eyes? Naturwissenschaften *76:*335–336.

Homberg, U. (1984) Processing of antennal information in extrinsic mushroom body neurons in the bee brain. J. Comp. Physiol. A. *154:*825–836.

Ito, K., and Y. Hotta (1992) Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster.* Dev. Biol. *149:*134.

Kotrla, K.J., and C.S. Goodman (1984) Transient expression of a surface antigen on a small subset of neurones during embryonic development. Nature *311:*151–153.

Kreissl, S., and G. Bicker (1989) Histochemistry of acetylcholinesterase and immunocytochemistry of an acetylcholine receptor-like antigen in the brain of the honeybee. J. Comp. Neurol. *286:*71–84.

Kreissl, S., and G. Bicker (1992) Dissociated neurons of the pupal honeybee brain in cell culture. J. Neurocytol. *21:*545–556.

Laemmli, U.V. (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature *227:*680–685.

McKay, R.D.G., S. Hockfield, J. Johansen, I. Thompson, and K. Frederiksen (1983) Surface molecules identify groups of growing axons. Science *222:*788–794.

Mercer, A., P. Mobbs, A.P. Davenport, and P.D. Evans (1983) Biogenic amines in the brain of the honeybee (*Apis mellifera*) Cell Tissue Res. *234:*655–677.

Mobbs, P.G. (1985) Brain structure. In G. Kerkut and L.I. Gilbert (eds): Comprehensive Insect Physiology Pharmacology and Biochemistry Vol. 5 Nervous Systems: Structure and Motor Function. Oxford: Pergamon Press, pp. 299–370.

Reichardt, L.F. (1984) Immunological approaches to the nervous system. Science *225:*1294–1299.

Rehder, V., G. Bicker, and M. Hammer (1987) Serotonin-immunoreactive neurons in the antennal lobes and suboesophageal ganglion of the honeybee. Cell Tissue Res. *247:*59–66.

Ribi, W.A. (1975) The neurons of the first optic ganglion of the bee (*Apis mellifera*). Adv. Anat. Embryol. Cell Biol. *50:*6–45.

Ribi, W.A., and M. Scheel (1981) The second and third optic ganglia in the worker bee. Golgi studies of neuronal elements in the medulla and lobula. Cell Tissue Res. *221:*17–43.

Schäfer, S., and G. Bicker (1986) Distribution of GABA-like immunoreactivity in the brain of the honeybee. J. Comp. Neurol. *246:*287–300.

Scheidler, A., P. Kaulen, G. Brüning, and J. Erber (1990) Quantitative autoradiographic localization of [^{125}I]α-bungarotoxin binding sites in the honeybee brain. Brain Res. *534:*332–335.

Strausfeld, N.J. (1976) Atlas of an Insect Brain. Berlin, Heidelberg, New York: Springer Verlag.

Suzuki, H. (1975) Antennal movements induced by odor and central projections of the antennal neurons in the honey bee. J. Insect Physiol. *6:*168–179.

Zipser, B., and R. McKay (1981) Monoclonal antibodies distinguish identifiable neurones in the leech. Nature *289:*549–554.

Zipursky, S.L., T.R. Venkatesh, and S. Benzer (1985) From monoclonal antibody to gene for a neuron-specific glycoprotein in *Drosophila.* Proc. Natl. Acad. Sci. USA *82:*1855–1859.