Octopamine-Like Immunoreactivity in the Brain and Subesophageal Ganglion of the Honeybee

SABINE KREISSL, STEFAN EICHMÜLLER, GERD BICKER, JÜRGEN RAPUS, AND MANFRED ECKERT

Institut für Neurobiologie, FU-Berlin, 14195 Berlin, Federal Republic of Germany (S.K., S.E., G.B.); Friedrich Schiller Universität Jena, Sektion Tierphysiologie, 07743 Jena, Federal Republic of Germany (J.R., M.E.)

ABSTRACT

The organization of putative octopaminergic pathways in the brain and subesophageal ganglion of the honeybee was investigated with a well-defined polyclonal antiserum against octopamine. Five prominent groups of just over 100 immunoreactive (IR) somata were found in the cerebral ganglion: Neurosecretory cells in the pars intercerebralis innervating the corpora cardiaca via NCC I, one cluster mediodorsal to the antennal lobe, one scattered on both sides of the midline of the protocerebrum, one between the lateral protocerebral lobes and the dorsal lobes, and a single soma on either side of the central body. With the exception of the pedunculi and β-lobes of the mushroom bodies, varicose immunoreactive fibers penetrate all parts of the cerebral ganglion. Strong labelling was found in the central complex and the protocerebral bridge. Fine networks of labelled processes invade the antennal lobes, the calyces and a small part of the α-lobes of the mushroom bodies, the protocerebrum, and all three optic ganglia.

In the subesophageal ganglion, one labelled cell body was found in the lateral soma layer of the mandibular segment. Each of the three neuromeres contains a group of six to ten somata in the ventral median parts. Most of the ventral median cells send their neurites dorsally through the midline tracts, whereas the neurites of a few cells follow the ventral cell body neurite tracts.

Octopamine-IR was demonstrated in all neuropils that contain pathways for proboscis extension learning in honeybees. Because octopaminergic mechanisms seem to be involved in the behavioral plasticity of the proboscis extension reflex, our study provides anatomical data on the neurochemical organization of an appetitive learning paradigm.

Key words: neurotransmitter, mushroom body, antennal lobe, ventral median cells, insect nervous system

The biogenic amine octopamine appears to play manifold roles as a neurotransmitter, neuromodulator, and neurohormone in arthropods (see, e.g., David and Coulon, 1985; Evans, 1985, 1993; Agricola, 1988). Although octopamine has been the most widely studied biogenic amine in physiological investigations of insect motor systems, little is known of the physiology and anatomical organization of octopaminergic neurotransmission in the insect brain. So far, anatomical data on the distribution of octopamine-like-immunoreactive (OA-IR) cell bodies in the supraesophageal ganglion have been provided only for locusts (Konings et al., 1988) and crickets (Spörhase-Eichmann et al., 1992). In the locust brain, immunoreactive fibers were found in the midbrain, circumesophageal connectives, and optic lobes, some of which originated from a group of seven OA-IR cells in the protocerebrum (Konings et al., 1988). The brain receives additional OA-IR innervation from intersegmentally projecting dorsal unpaired median (DUM) neurons of the subesophageal ganglion (Bräunig, 1991). The studies in locusts and crickets did not reveal any OA-IR in neurohemal organs associated with the cerebral ganglion (Konings et al., 1988; Spörhase-Eichmann et al., 1992).

In the brain of the honeybee, octopamine has been detected by high-pressure liquid chromatography (HPLC) analysis with electrochemical detection (Mercer et al., 1983; Harris and Woodring, 1992). In this insect, several approaches have tried to elucidate its physiological functions. Iontophoretic application of octopamine enhances light evoked potentials in the mushroom bodies and reduces potentials evoked by antennal stimulation (Mercer and

Erber, 1983). A visually controlled antennal reflex is enhanced after the application of octopamine and an octopaminergic receptor ligand over the brain (Erber et al., 1991, 1993). Other investigations have addressed the question of whether octopaminergic mechanisms might be involved in the behavioral plasticity of the so-called proboscis extension reflex (Kuwabara, 1957; Menzel et al., 1974; Bitterman et al., 1983). The results of many behavioral pharmacological studies implicate an octopaminergic influence on associative and nonassociative processes of proboscis extension learning (Mercer and Menzel, 1982; Menzel et al., 1988, 1991; Braun and Bicker, 1992; Hammer et al., 1993).

In order to advance our understanding of the anatomical organization of the octopaminergic system in the brain and subesophageal ganglion, we used a polyclonal antiserum against octopamine coupled to a carrier protein, which was described by Eckert et al. (1992), on paraffin and vibratome sections. Thus, we provide the first mapping of octopamine-IR neurons in parts of the central nervous system of the honeybee which contain the reflex pathways for proboscis extension. The present study shows that the pattern of OA-IR is completely different than the previously reported distribution of serotonin-IR (Schürmann and Klemm, 1984; Schäfer and Bicker, 1986; Rehder et al., 1987) and dopamine-IR (Schäfer and Rehder, 1989). In addition, we compare the pattern of OA-IR to autoradiographic studies of octopamine binding sites in the brain of the bee (Erber et al., 1991).

MATERIALS AND METHODS

Immunocytochemical staining was performed on paraffin or vibratome sections of the brains of 160 honeybees. In the majority of experiments, foraging worker bees were captured near the hive and immobilized by cooling at 4°C. Animals were either immediately perfused with fixative and brains were dissected out of the prefixed head, or brains were dissected out of the cooled head capsule and subsequently immersed in the fixative. In three independent experiments, animals were captured and kept without feeding at 12°C until dissection on the following day. Brains were fixed either at room temperature 4–6 hours or at 4°C overnight (6% glutaraldehyde and 1% acetic acid in saturated picric acid). After dehydration and embedding in Paraplast overnight (60°C), either horizontal, sagittal, or frontal serial sections (12 μm) were cut and mounted on polylysine coated slides.

Fig. 1. Summary of octopamine-immunoreactive (OA-IR) groups of somata in the cerebral and subesophageal ganglion in a frontal view. Numbers indicate the distance from the frontal surface of the brain. **a:** OA-IR in the anterior part of the brain. The first soma cluster is positioned within the protocerebral median neurosecretory cell bodies. Group 2, containing eight to ten somata, lies mediodorsal to the antennal lobes scattered on both sides of the anterioventral midline of the protocerebrum. Group 3 lies medial to each antennal lobe. **b:** OA-IR in the posterior part of the brain and the subesophageal ganglion. One soma is located on either side of the most posterior part of the central body upper division and is indicated as group 4. Group 5 is positioned between the lateral protocerebral lobes and the dorsal lobes of the deutocerebrum of each hemiganglion. There is one OA-IR soma in the subesophageal ganglion on both sides in the most anterior lateral region of the soma rind (group 6). Group 7 comprises three cell clusters (six to ten somata each) in the ventral median parts of the three neuromeres of the subesophageal ganglion.

After rehydration through a graded ethanol series, the sections were washed in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.6, containing 0.3 M NaCl) to remove fixative. To saturate double bonds, sections were incubated for 30 minutes in an 1% sodium borohydride solution in 0.1 M Tris-HCl at room temperature. After three rinses (10 minutes each) in TBS, sections were incubated in either 5% normal goat serum or the blocking solution of a Vectastain ABC kit diluted in SST (0.1 M Tris-HCl, pH 7.6, containing 0.3 M NaCl and 0.1% Triton X-100) for 1 hour to reduce unspecific binding of the primary antibody and to permeabilize the tissue. We applied the antioctopamine antiserum diluted 1:800 in SST containing 0.1% Triton X-100 and 1% normal goat serum.

The primary antiserum was applied over night in a humidity chamber at room temperature and unbound serum was subsequently removed by three rinses with SST, 10 minutes each. Specific binding of the antiserum was detected either by the peroxidase technique by incubation in goat anti-rabbit IgG (Sigma, St. Louis, MO; 1:40 in SST, 1 hour) followed by three washes in SST and incubation with rabbit peroxidase-antiperoxidase complex (Dakopatts, Copenhagen, Denmark), diluted 1:100 in SST for 1 hour.

Abbreviations

α	α-lobe of the mushroom body
al	antennal lobe
AN	antennal nerve
ant	anterior
β	β-lobe of the mushroom body
br	basal ring of the mushroom body calyx
c	c-layer of the lamina
ca	corpora allata
cb	central body
cb(l)	central body, lower division
cb(u)	central body, upper division
cc	corpora cardiaca
co	collar of the mushroom body calyx
d	dorsal
dl	dorsal lobe
gl	glomerulus
la	lamina
lcal	lateral calyx of the mushroom body
LN	labial nerve
lo	lobula
lp	lateral protocerebrum
mb	mushroom body
mcal	median calyx of the mushroom body
MdN	mandibular nerve
me	medulla
med	median
MNC	median neurosecretory cells
MVT	median ventral tract
MxMT	maxillary midline tract
NCC I	nervus corporis cardiaca I
NCC II	nervus corporis cardiaca II
no	noduli of the central body complex
och	outer optic chiasm
oes	esophagus
ot	optic tubercle
ped	pedunculus of the mushroom body
po	pons of the mushroom body complex (protocerebral bridge)
post	posterior
pot	posterior optic tract
rn	recurrent nerve
sog	subesophageal ganglion
v	ventral
vnc	ventral nerve cord

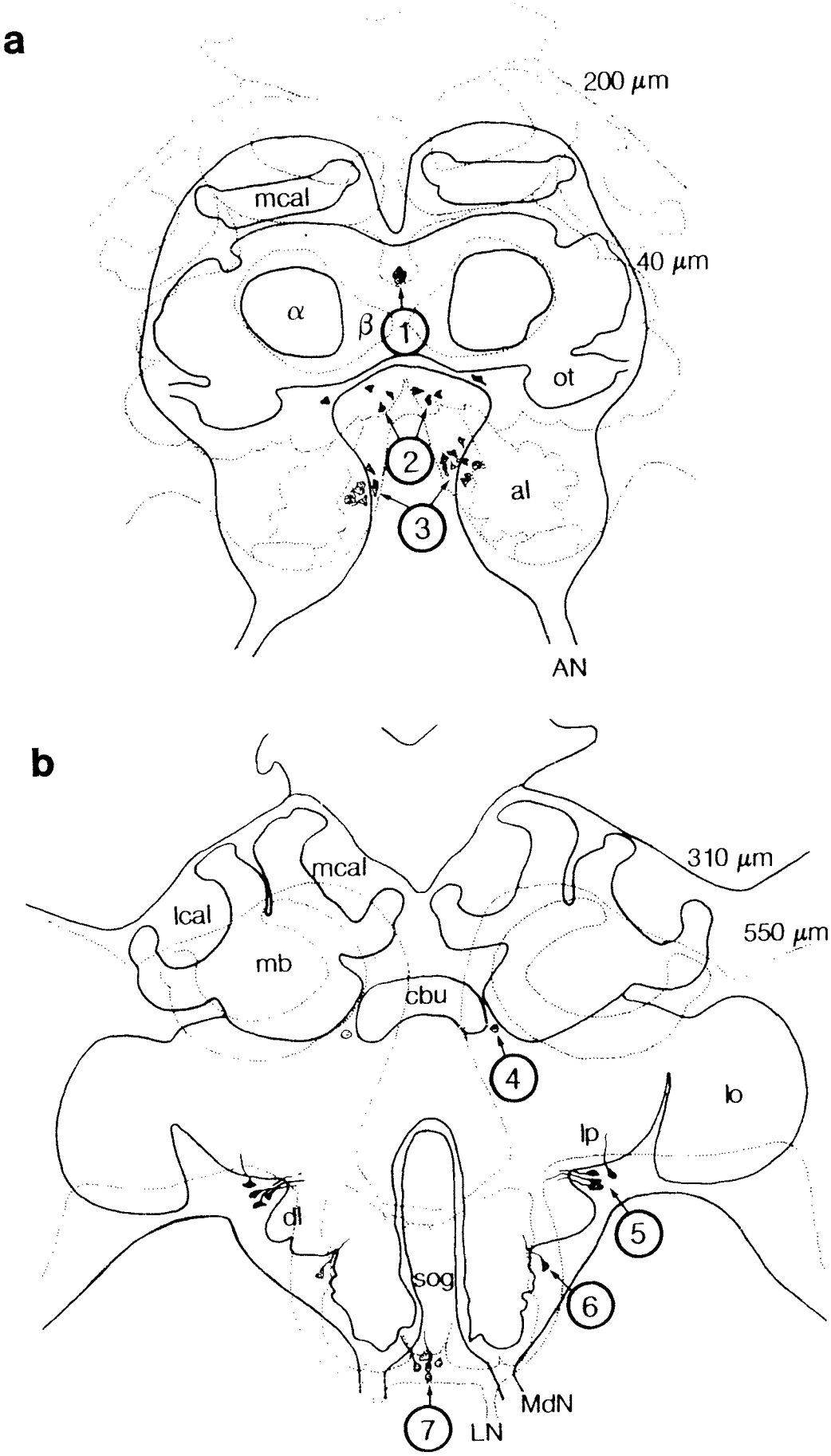

a
200 μm
mcal
40 μm
α
β
1
ot
2
al
3
AN
b
310 μm
mcal
lcal
550 μm
mb
cbu
4
lo
lp
dl
5
sog
6
MdN
7
LN

Figure 1

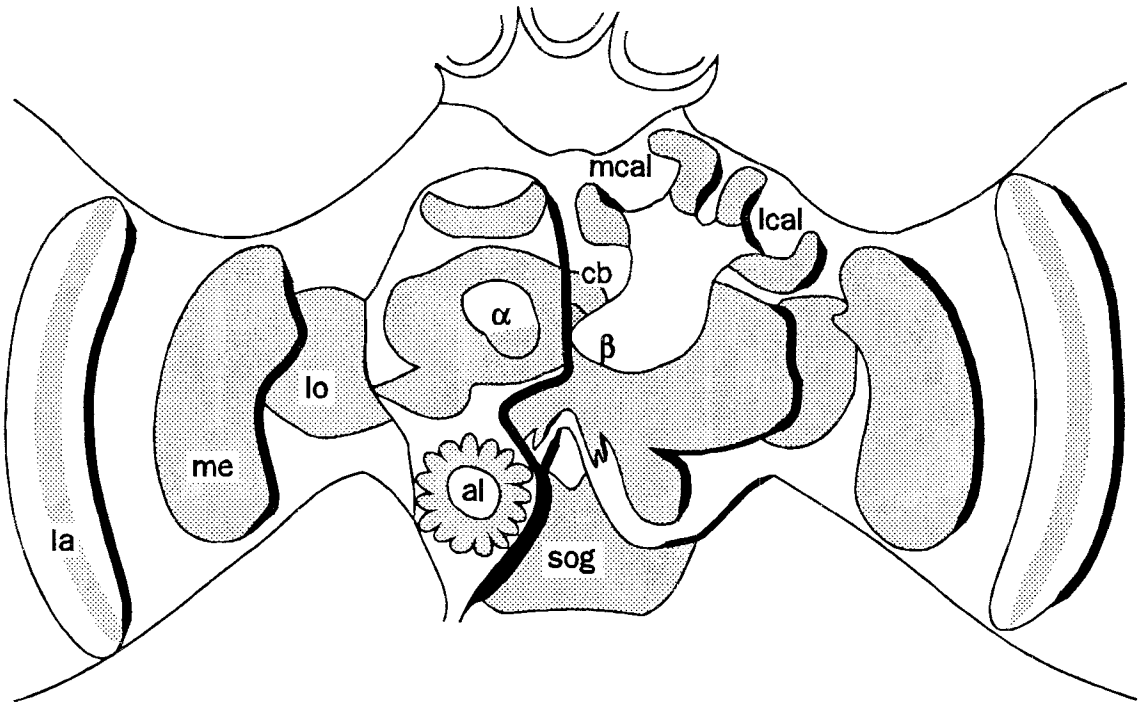


Fig. 2. Survey of neuropils containing OA-IR in the brain. Left hemisphere shows anterior, right hemisphere posterior portions of the brain. Areas containing immunoreactive fibers are shaded. In all neuropils of the brain and subesophageal ganglion, excluding the pedunculi and the β-lobes of the mushroom bodies, OA-IR was detected. In the α-lobes of the mushroom bodies, only very few labelled fibers were visible.

Alternatively, sections were treated according to the protocol provided with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using SST for the biotinylated secondary antibody and TBS for the ABC complex as dilution buffers. After washing in 0.1 M Tris-HCl (pH 7.6, 3 × 10 minutes), sections were treated with diaminobenzidine solution (0.2 mg/ml in Tris-HCl with 0.001% H_2O_2, 5–15 minutes; Sigma). Finally, sections were washed, dehydrated in an ethanol series, cleared in xylene, and mounted in Entellan.

A similar protocol was employed for vibratome sections. Fixed brains were washed in buffer, embedded in 6% agar, and 40 μm sections were cut on a vibratome. All incubations were performed on free floating sections with duration and protocol as described for paraffin sections. Sections were viewed and photographed with a Polyvar microscope (Reichert-Jung), and schematic drawings were constructed from serial sections with the aid of a camera lucida.

RESULTS

Specificity of primary antiserum

The primary antiserum was raised in rabbits using octopamine coupled to thyroglobulin via glutaraldehyde. We employed the antiserum on paraffin sections in a similar histological protocol as has been reported by Eckert et al. (1992) and Stevenson et al. (1992). Details of tests for specificity of the employed antioctopamine serum were given elsewhere (Eckert et al., 1992). Briefly, the affinity of the antiserum to the protein carrier-octopamine conjugate and its lack of cross-reactivity with dopamine, noradrenaline, and serotonin was demonstrated by dot blot analysis. This assay revealed a 1,000-fold less sensitive affinity to tyramine, the natural precursor of octopamine. The antiserum stains neurons in other arthropod species, which, based on physiological and biochemical evidence, are octopaminergic (Evans and O'Shea, 1978; Stevenson et al., 1992; Schneider et al., 1993). Control sections of honeybee nervous tissue incubated in the absence of primary antiserum showed no immunoreactivity.

Notes on staining procedure

The immunocytochemical stainings revealed a consistent pattern of immunoreactive somata and varicose fibers within the neuropil. Although we observed a certain variability in staining intensity of neuronal cell bodies and processes in different brain regions, we present a detailed report of repeatedly observed distribution of OA-IR in the cerebral and subesophageal ganglia of a hymenopteran insect.

Generally, cell bodies stained intensely, whereas the staining in primary neurites was often low. Nevertheless, clearly labeled neuritic profiles were traceable over considerable distances within the CNS. However, due to the low OA-IR in the thin primary neurites, it was possible only in some preparations to trace the connections between the labelled cell bodies and their arborizations in the neuropil.

Cell bodies in the brain

The cerebral ganglion contains five prominent groups of OA-IR somata (Fig. 1). The diameters of all OA-IR somata are in the range between 15 and 25 μm. The first soma

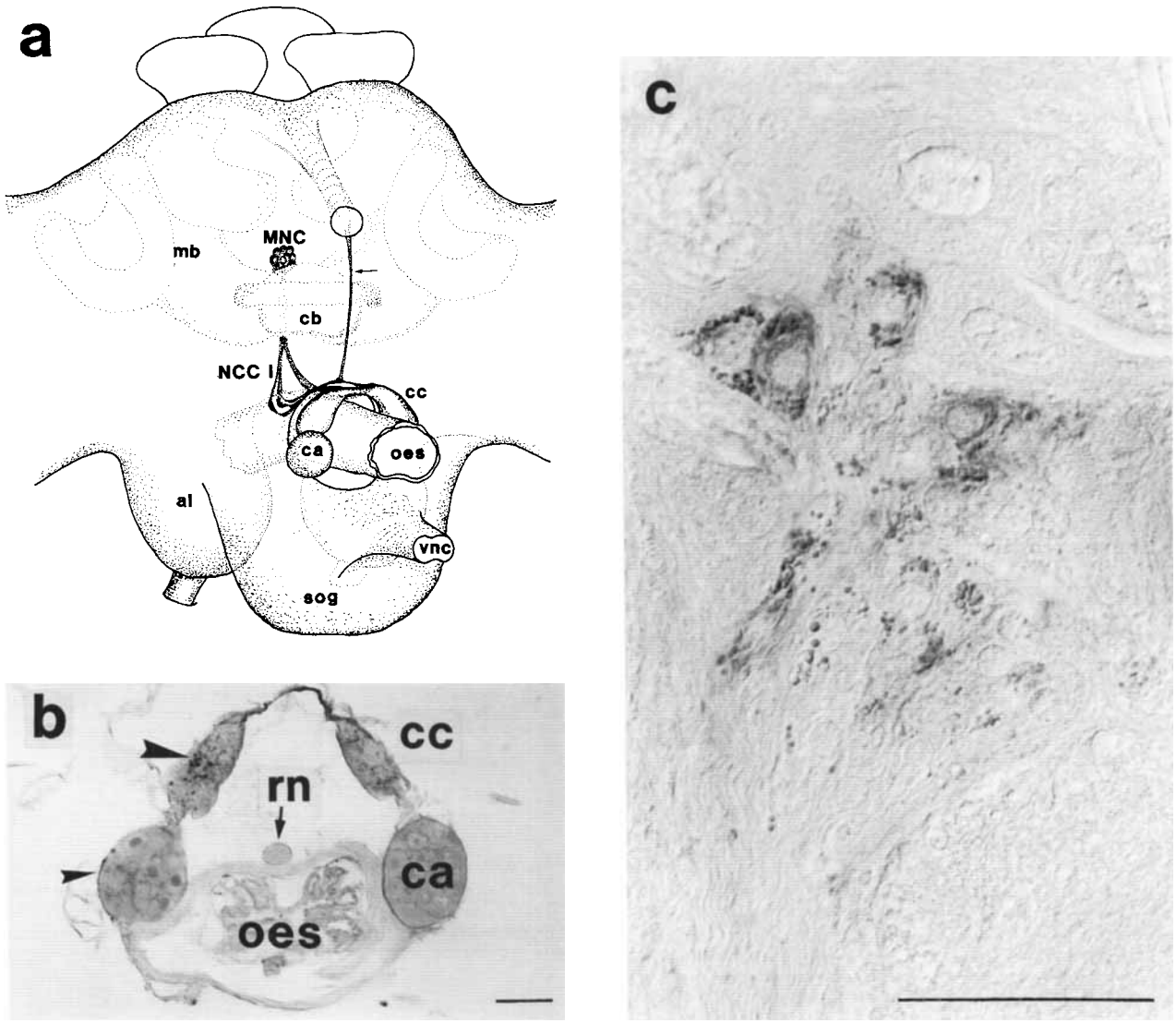


Fig. 3. **a:** Posterior view of the brain showing OA-IR in neurohemal structures. Fibers originating from median neurosecretory cells in the pars intercerebralis project into the corpora cardiaca through the median NCC I. A labelled blood vessel ascends dorsally from the fusion point of NCC with the corpora cardiaca up to the median trachea (arrow). **b:** OA-IR was detected in varicose structures of the corpora cardiaca (large arrowhead) and in very fine fibers surrounding the large diameter neurosecretory cells of corpora allata (small arrowhead). Both neurosecretory structures and the esophagus enclose the dorsal aorta. **c:** Median sagittal section through the pars intercerebralis shows immunoreactive grana in medium sized somata of the median neurosecretory cells. Scale bars = 50 μm.

cluster (approximately 45 cells) is positioned in the midline of the dorsal anterior part of the protocerebrum within the group of median neurosecretory cell bodies (Eichmüller et al., 1991; Fig. 3a,c). Another group of eight to ten somata (cluster 2) was found mediodorsal of the antennal lobes scattered on both sides of the anterioventral midline of the protocerebrum, whereas cluster 3 was found as a compact group of approximately six to seven cells medial to each antennal lobe (Fig. 8b,c). One soma was found on either side of the most posterior part of the central body upper division (group 4). Positioned between the lateral protocerebral lobes and the dorsal lobes of the deutocerebrum a group of approximately four OA-IR was detected in each hemisphere of the brain (group 5).

The somata give rise to numerous varicose OA-IR fibers that invade all neuropils of the cerebral ganglion (Fig. 2). OA-IR in the neuropils will be described in the following paragraphs.

Neurosecretory cells

Octopamine-IR was only detectable in a few cases in neurosecretory cells of the brain and the neurosecretory complex that is generally considered to be the main release site into the hemolymph. In three independent experiments, animals were captured on the evening before experimentation and kept without feeding at 12°C until dissection on the following day. Immunoreactivity in neurosecretory cells and organs could be detected only in these starved animals.

Immunoreactive grana were localized in approximately 45 somata of the median neurosecretory cells in the pars

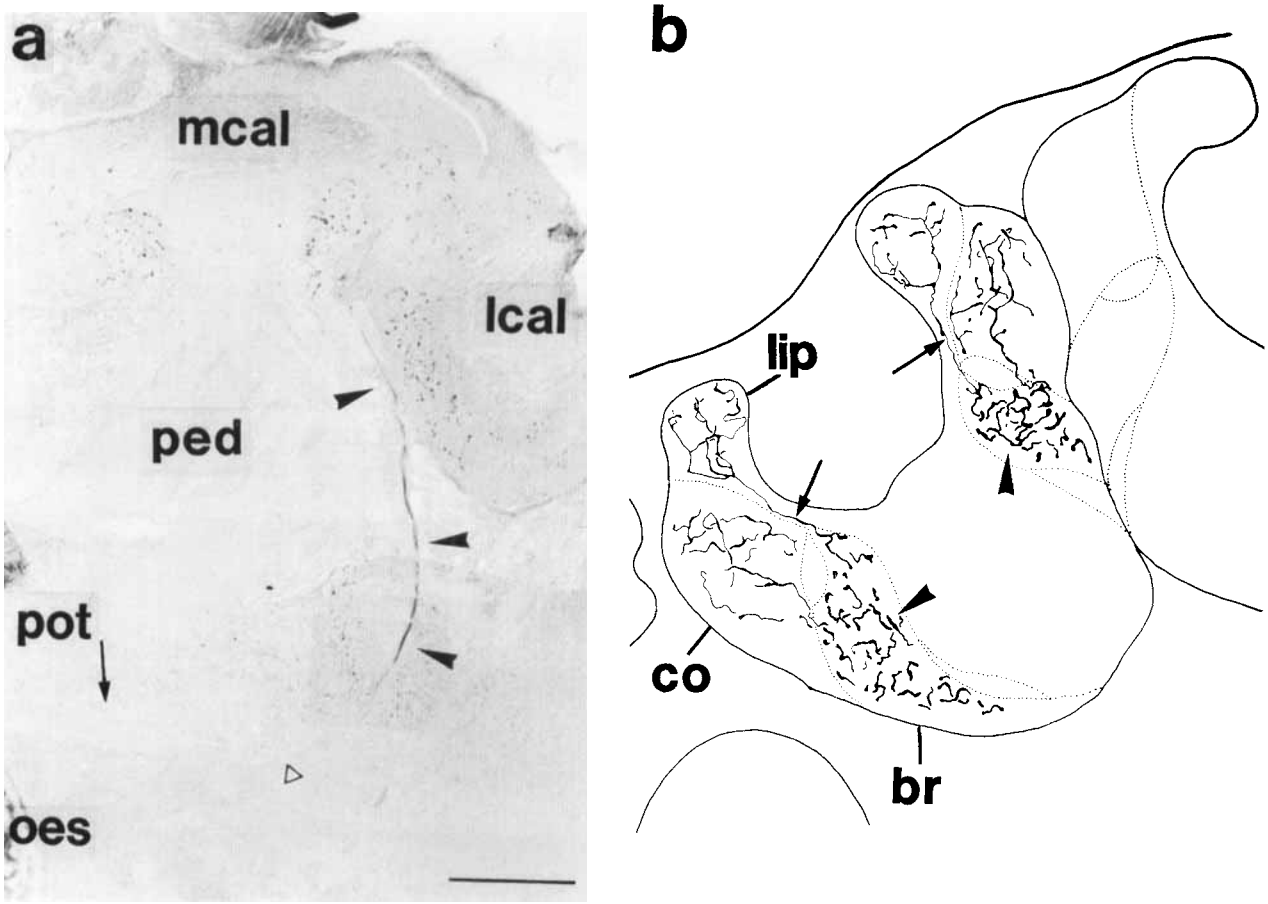


Fig. 4. **a:** Compound photomicrograph of frontal sections. OA-IR profiles enter the calyx of the mushroom body through the lateral antennoglomerular tract (arrowheads). Labelling in the mushroom bodies is largely confined to the calycal neuropil. Numerous OA-IR fine processes are visible in lip, collar, and basal ring of the calyx. Open arrowhead indicates unlabelled part of the lateral antennoglomerular tract. **b:** Reconstruction of several serial frontal sections through one calyx shows higher density of labelling within the basal ring than the other subcompartments (arrowheads). Stained profiles within the lip connect to basal ring processes (arrows) but are separate from neurites within the collar. Scale bar = 100 μm.

intercerebralis. It appears that these cells project through the NCC I, which fuses in the ventral protocerebrum with the fibers running in median and lateral NCC II to form the NCC of the honeybee (Eichmüller et al., 1991) into the corpora cardiaca (Fig. 3a,b). A blood vessel that ascends dorsal from the fusion point of NCC with the corpora cardiaca up to the median trachea shows intense OA-IR (Fig. 3a, arrow). The storage tissue of the corpora cardiaca contained densely packed varicose immunoreactive structures. In the corpora allata, very fine OA-IR fibers surrounding the large diameter neurosecretory cells could be detected (Fig. 3c). Both neurosecretory structures form the dorsal aorta in the honey bee (Freudenstein, 1928).

Mushroom bodies

The mushroom body neuropil consists of two cuplike calyces that are thought to be the predominant input areas, the pedunculus and the α- and β-lobes (Mobbs, 1982). The shape and internal structure of the mushroom body neuropil is largely reflected in the branching pattern of the intrinsic Kenyon cells. Fibers of the Kenyon cells project from their calycal input areas in a parallel arrangement through the pedunculus and finally branch into the α-and β-lobes. The calycal neuropil is subdivided into lip, collar, and basal ring regions.

Our investigations clearly demonstrate the absence of OA-IR in the mushroom body intrinsic Kenyon cells (Fig. 4a). The calyces, however, are innervated by extrinsic OA-IR fibers. We could trace two fibers entering the lateral antennoglomerular tract dorsal to the inferior optic commissure (Mobbs, 1985; Maronde, 1991) joining its fibers up to the calyces of the mushroom bodies (Fig. 4a).

The calycal subcompartments expressed considerable differences in the density of immunoreactive innervation. The basal ring contains a higher density of immunoreactivity than collar and lip regions (Fig. 4a,b). Reconstructions of serial sections revealed a subset of fine fibers that, after leaving the basal ring, pass the collar without detectable ramifications, finally invading the lip region of the calyces by regularly distributed labelled projections (Fig. 4b).

Compared to the presence of numerous fine varicose labelled fibers in the surrounding protocerebral neuropil, OA-IR is completely absent in the pedunculus, β-lobe, and large parts of the α-lobe of the mushroom body (Fig. 5a,b). Only a few tenuous mushroom body extrinsic OA-IR fibers

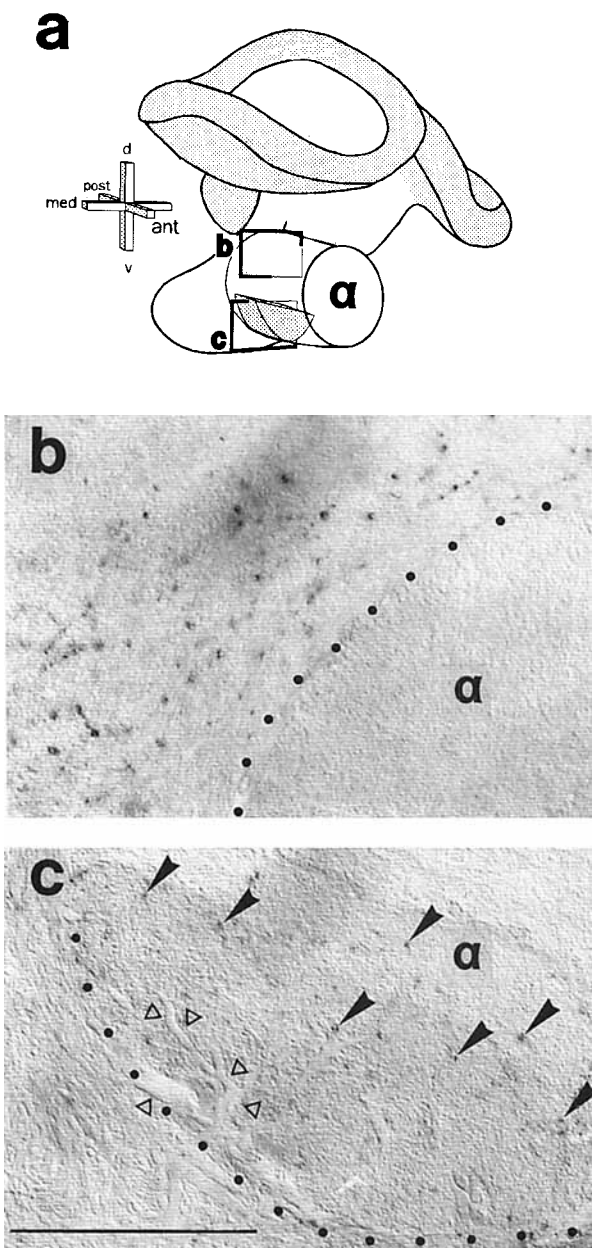


Fig. 5. **a:** Schematic drawing of the mushroom body illustrating positions of photomicrographs b and c. Innervation with OA-IR fibers is indicated as gray shading. **b:** Frontal section through the most anterior part of the protocerebrum at high resolution. The photomicrograph shows the absence of OA-IR in the α-lobe of the mushroom body as compared to the presence of numerous fine, varicose, labelled fibers in the surrounding neuropil. **c:** Fine OA-IR fibers (examples are indicated by arrowheads) were detected in a small segment in close vicinity of unlabelled large diameter axons entering the α-lobe (open triangles). The boundary between α-lobe and surrounding protocerebral neuropil is indicated by a dotted line. Scale bar = 50 μm.

innervate a small sector of the α-lobe orthogonal to the parallel projecting Kenyon cell axons. The labelled fibers within this sector are in close vicinity of large diameter axons, entering the α-lobe ventrally near the β-exit point (Fig. 5a,c).

Central complex

The strongest labelling in the protocerebrum was found in the central complex, which is a prominent arrangement of neuropils in the protocerebrum. It comprises the central body, two noduli, and the protocerebral bridge. The central body displays a much higher density of OA-IR fibers in its lower than in its upper division (Fig. 6a) that are connected by a regular array of labeled large diameter fibers (Fig. 6b,c,f). The arrangement of fibers is reconstructed in Figure 6e. A sagittal section (Fig. 6f) reveals the relationship between the density of the staining in the central body at various depths and the surrounding neuropil. OA-IR was also consistently detected in the remaining parts of the central complex, the ventral noduli, and the protocerebral bridge (Fig. 6b,d,f).

Optic lobes

The optic lobes contain three neuropilar masses with columnar organization: The lamina, medulla, and lobula. All three neuropils are innervated by immunoreactive fibers, although no OA-IR somata could be detected within the optic lobe area. Reconstructions indicated that a subpopulation of very few cells within soma group 5 innervates the optic lobes. Figure 7a shows OA-IR fibers in the external plexiform layer C of the lamina, which is a narrow stratum nearest to the outer chiasma. In thick vibratome section (40 μm), we could discern a network of immunoreactive neurites that form an alternating array of predominantly tangentially or radially orientated fibers (Fig. 7b). There was, however, no apparent relationship between the immunoreactive fibers and the columnar organization of the optic ganglia.

Antennal lobes

The deutocerebral antennal lobes are the first relay station of the chemosensory receptors of the antennae. Sites of synaptic contacts between sensory neurons, local interneurons, and projection neurons are arranged as spherical glomeruli surrounding the central neuropil.

All glomeruli contain OA-IR processes deriving from the very few profiles which enter the antennal lobes from the ventroposterior and deuto- and tritocerebral areas (Fig. 8a). The varicose OA-IR profiles within the cortical regions of the glomeruli appear to be of considerable large diameter compared to the very thin fibers in the glomerular central neuropil.

The output relay neurons of the antennal lobe project through the antennoglomerular tracts into the mushroom bodies and the lateral protocerebral neuropil area (Mobbs, 1985). The relay neurons were not stained, yet both projection areas are invaded by OA-IR fibers originating from other regions of brain and subesophageal ganglion. For example, as described above, two OA-IR fibers join the lateral antennoglomerular tract en route to the calyces of the mushroom bodies (Fig. 4a).

The most strongly stained soma group was found in the vicinity of the antennal lobe (cluster 3, Figs. 1, 8b,c). These cells are most likely not connected to the neuropil of the antennal lobe because they do not send their primary neurites toward the glomeruli (Fig. 8c).

Subesophageal ganglion and tritocerebrum

The subesophageal ganglion is contiguous with the deuto- and tritocerebral parts of the brain and arises from the

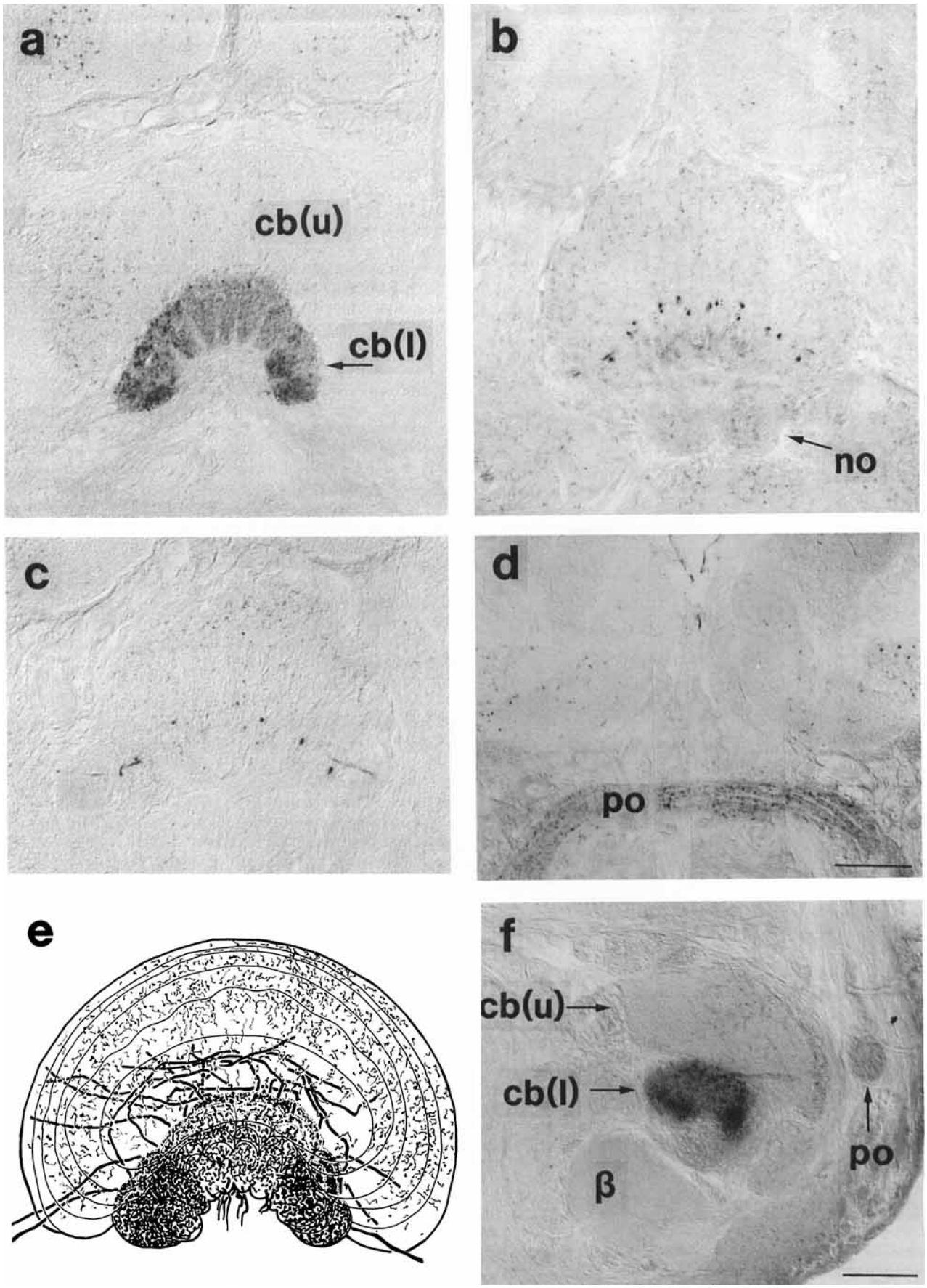


Figure 6

embryonic fusion of three neuromeres: the mandibular, maxillary, and labial neuromeres. The subesophageal ganglion innervates the muscles of the neck and the proboscis (Rehder, 1988) and serves as a relay station for the information flow between the brain and the ventral nerve cord.

The tritocerebrum and parts of the subesophageal ganglion are innervated by primary neurites of the OA-IR soma group 5. Their cell bodies are positioned between the lateral protocerebral lobes, the dorsal lobes of the lateral deutocerebrum, and proximal ventral optic lobes (Figs. 1, 9c). In the posterior part of the subesophageal ganglion, their fibers join the dorsal rim of the median ventral tract (Fig. 9c). Four axons descend through the cervical connective. At least two of these axons originate from group 5. As mentioned above, cluster 5 also contains a few cell bodies that send projections toward the optic lobes.

The subesophageal ganglion contains one labelled cell body in the lateral soma layer (group 6) and three groups of cell bodies containing six to ten somata each (group 7), which are clustered in the ventral median parts of the mandibular, maxillary, and labial neuromeres (Figs. 1, 9a,c). In the labial neuromere, the ventral median somata were found in a rather posterior location, close to the cervical connective. However, in several preparations, up to three of these cells could also be found in a dorsal soma position (Fig. 9a).

Most of the ventral median cells send their neurites dorsally through the midline tracts and show a characteristic symmetric bifurcation (Fig. 9b), whereas the neurites of two OA-IR cells in each neuromere follow the ventral cell body neurite tracts (Rehder, 1988) before entering the neuropil of the subesophageal ganglion. One cell body, positioned in the most anterior part of the lateral soma rind of the mandibular neuromere, sends ascending projections into the trito- and deutocerebrum, which run parallel along the esophagus (Fig. 9c). No OA-IR could be detected in the peripheral nerves of the subesophageal ganglion containing the axons of motoneurons that serve the feeding apparatus.

DISCUSSION

Variability of staining intensity

Biochemical assays have provided evidence that octopamine levels in a variety of arthropod species can be influenced by the behavioral state (Davenport and Evans, 1984; Evans, 1985; Kravitz, 1988; Hirashima and Eto, 1993a,b). Using HPLC with electrochemical detection, Harris and Woodring (1992) reported handling stress-induced elevations of octopamine levels in the brains of honeybees and also measured seasonal differences in the titers of the three amines, octopamine, serotonin, and dopamine. The majority of our immunocytochemical experiments, which were

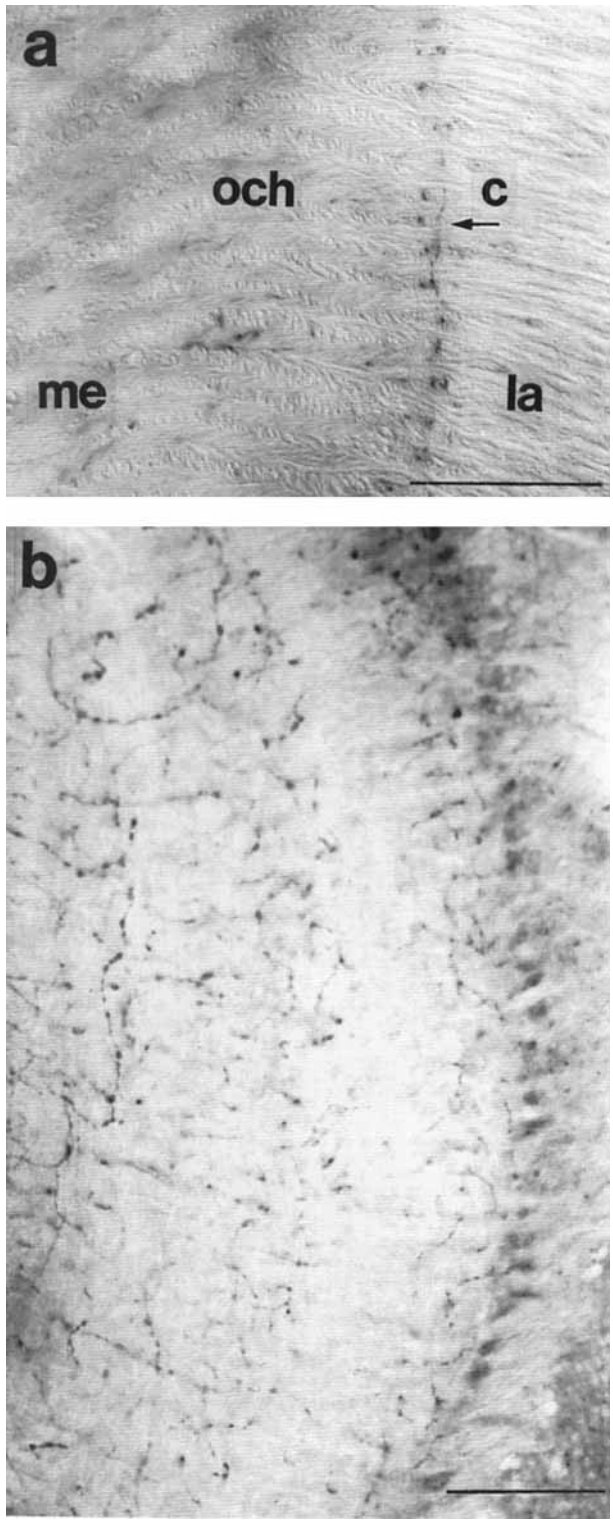
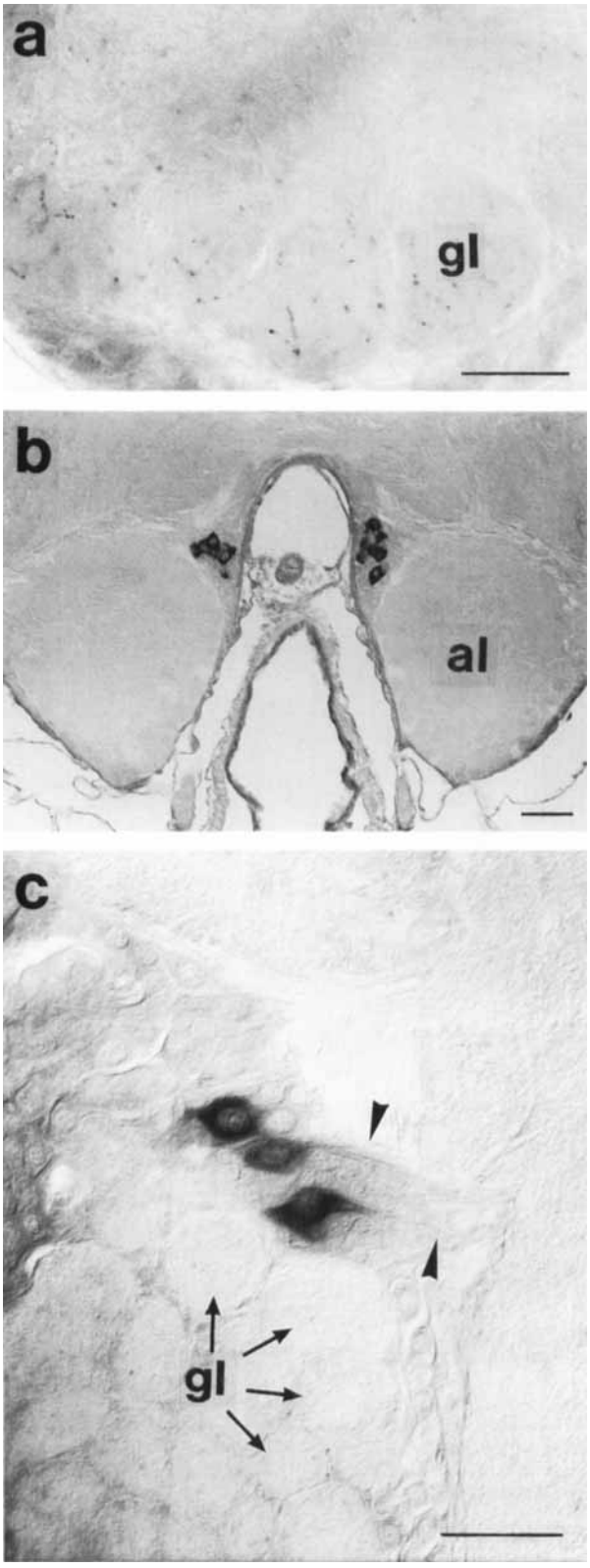


Fig. 7. **a:** Frontal section of the first optic ganglion shows OA-IR fibers in the external plexiform layer C of the lamina. **b:** Vibratome section (40 μm) cut in a frontal plane. A net of labelled fibers invades the medulla, which is predominantly tangentially or radially oriented. Scale bars = 50 μm.

Fig. 6. Series of frontal sections through the central complex from anterior to posterior. **a:** A much higher density of OA-IR fibers is observed in the central body lower division than in its upper division. **b,c:** A regular array of large diameter fibers connects the lower division of the central body with its less densely labelled upper division. **d:** OA-IR in the protocerebral bridge. **e:** Reconstruction of serial sections through the central body. **f:** Sagittal section through the central complex showing OA-IR in the central body divisions with connecting large diameter axons and labelling of fibers in the cross section of the protocerebral bridge. Note the complete absence of labelling in the β-lobe of the mushroom body. Scale bars = 50 μm.

performed on bees caught at their hive prior to foraging flights, indeed showed considerable variability in the intensity of the labeling in some neurons. The basic pattern of stained somata and processes, however, was always constant despite the variability of the immunocytochemical staining intensity.



Neurosecretory cell of the pars intercerebralis expressed only immunoreactivity in starved and quiescent animals. We could show OA-IR in the median neurosecretory cells, the NCC I, the corpora cardiaca, and fine branches in the corpora allata in the honeybee, but no immunoreactive cells bodies in the region of lateral neurosecretory cells or in the median or lateral NCC II (Eichmüller et al., 1991). Our immunocytochemical experiments suggest that the pars intercerebralis contains efferent neurons capable of releasing OA into glands and the hemolymph.

Neuropilar compartments and soma clusters

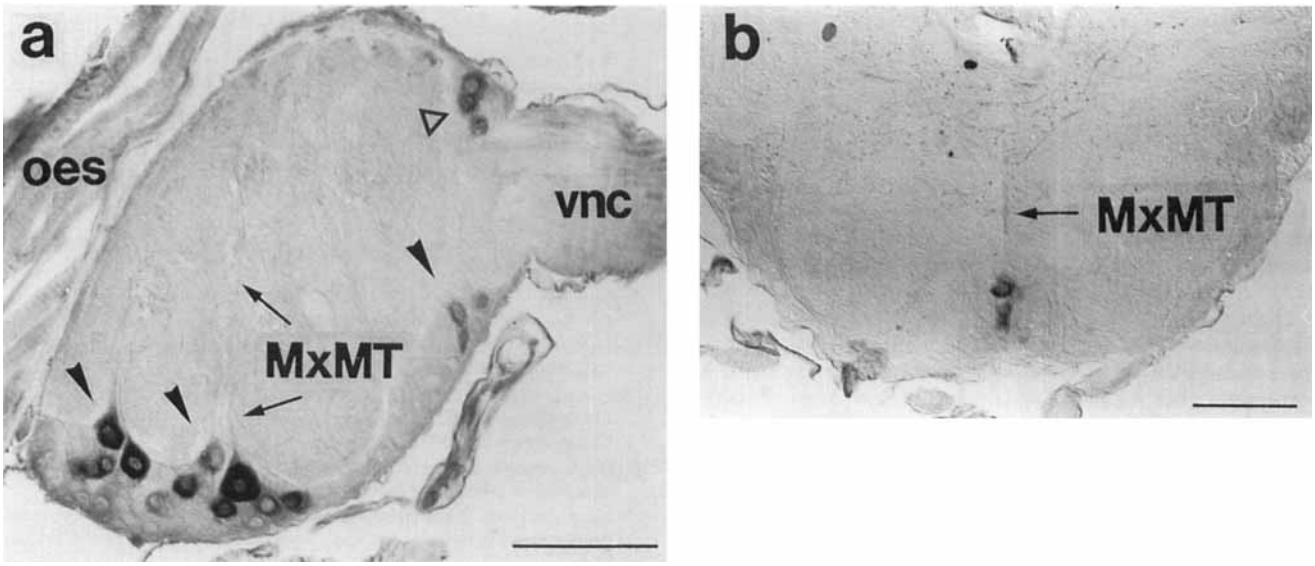
A hallmark of OA immunoreactivity in the nervous system of the bee is the rather homogeneous distribution of labelled fiber profiles without apparent layering or compartmentalization in the major neuropil areas. With the exception of the pedunculi of the mushroom bodies and large parts of the α- and β-lobes, varicose immunoreactive fibers invaded all parts of the brain and the subesophageal ganglion. Even though all three neuropils of the optic lobes are innervated by immunoreactive fibers, there was hardly any indication of a discrete lamination as has, for example, been described for serotonin-IR (Schürmann and Klemm, 1984; Schäfer and Bicker, 1986), GABA-IR (Schäfer and Bicker, 1986), and acetylcholinesterase-histochemistry (Kreissl and Bicker, 1989).

Two neuropilar areas of the central brain are clearly distinct with respect to a compartmentalized pattern of OA-IR. The central body displays a much higher density of OA-IR fibers in its lower than in its upper division (Fig. 6a). The most striking compartmentalization is found in the mushroom bodies. The calyces are innervated by extrinsic varicose OA-IR fibers, whereas large parts of the α- and β-lobes are devoid of OA-IR fibers (Figs. 2, 4). Mushroom body extrinsic fibers, however, innervate a small sector of neuropil orthogonal to the parallel projecting Kenyon cell axons in the α-lobe (Fig. 5). Comparison of this rather complex pattern of immunoreactivity with autoradiographic binding studies of radiolabelled octopamine reveals a further complication: a high density of octopamine binding sites in the pedunculi and α-lobes (Erber et al., 1991, 1993) contrasts rather sharply with the absence of OA-IR. The functional significance of this apparent mismatch of receptors and transmitter is not presently known.

Our investigations clearly demonstrate the absence of OA-IR in the mushroom-body-intrinsic Kenyon cells. As in locusts (Konings et al., 1988), Kenyon cells of the honeybee are therefore not octopaminergic, contrary to previous suggestion (Mercer et al., 1983).

An identified ventral unpaired median neuron (VUMmx1) of the subesophageal ganglion with unique morphology has recently been described by intracellular recording and staining (Hammer, 1993). The soma position of VUMmx1 is among the median ventral cell bodies in the maxillary neuromere, and its other anatomical characteristics are

Fig. 8. **a:** Frontal section through the antennal lobe at high magnification. All glomeruli of the antennal lobes contain OA-IR processes. **b:** Frontal section through the ventral protocerebrum and antennal lobes. In the deutocerebrum, one soma cluster (group 3) was found medial to each antennal lobe. **c:** Sagittal section through the deuto- and tritocerebral area shows OA-IR in somata and primary neurites (arrowheads) of soma group 3 (posterior to the right). Scale bars = 50 μm.



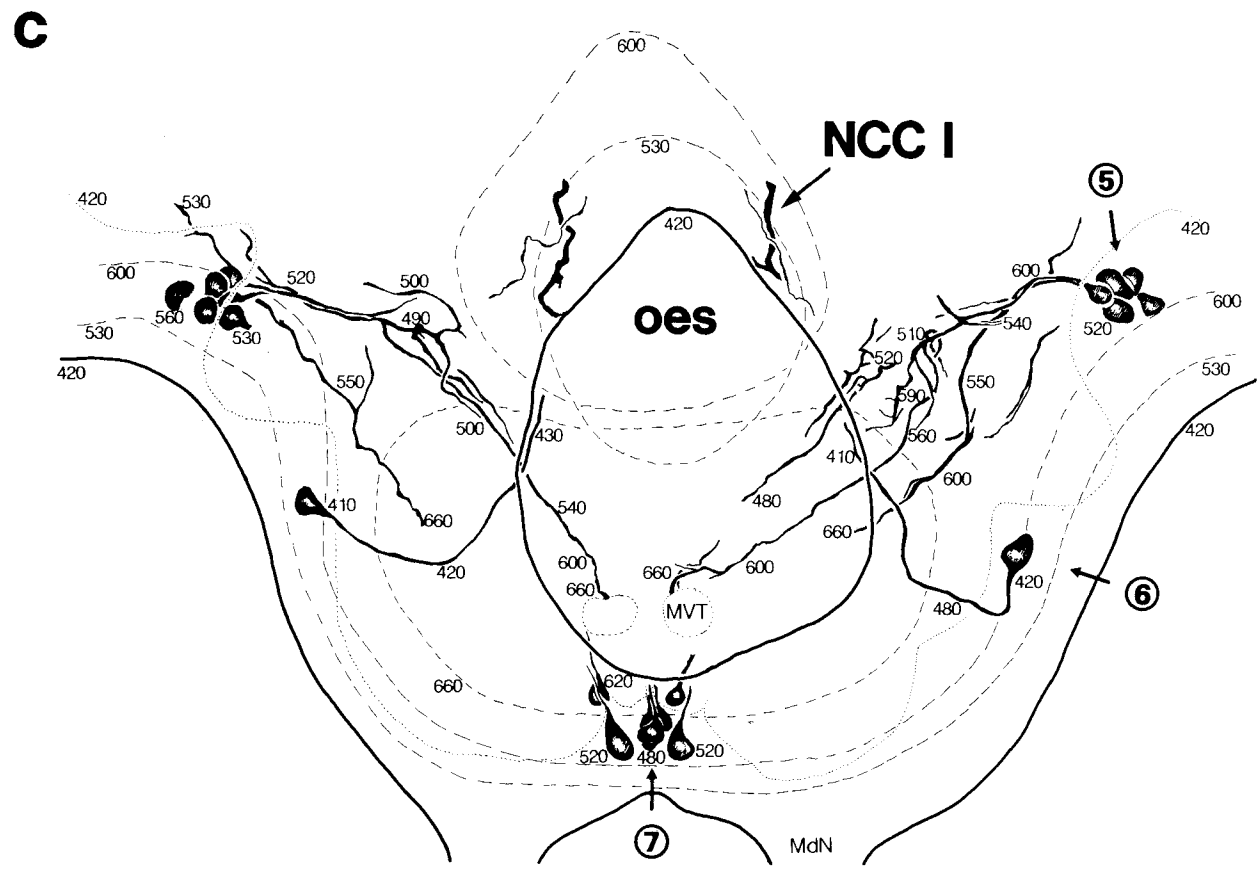


Fig. 9. **a:** Sagittal section through the midline of the subesophageal ganglion shows three clusters of ventral median OA-IR cells, one in each of the three neuromeres (arrowheads). Median cells of the labial neuromere in this preparation are in ventral (arrowhead) and dorsal (open triangle) positions, close to the exit of the ventral nerve cord. **b:** The primary neurite of a median ventral cell of the maxillary neuromere projects through the midline tract and bifurcates. **c:** Subset of OA-IR cells in the subesophageal ganglion as obtained by a reconstruction of serial frontal sections from one preparation. Primary neurites of soma group 5 descend toward the ventral nerve cord. The two lateral somata (group 6, cp. Fig. 1b) of the mandibular neuromere project anteriorly in parallel to the esophagus. Two paired somata with projections in the ventral cell body neurite tracts, and four somata with projections in the maxillary midline tract are shown as examples of ventral median cells. Numbers indicate the distance (μm) from the frontal surface of the brain. The anteriormost section is shown with a solid outline, subsequent sections are stippled. Scale bars = 100 μm.

symmetric bifurcation in the subesophageal ganglion, bilateral projections into the antennal lobes, the lateral protocerebra, and via the lateral antennoglomerular tracts into the calyces (Hammer, 1991). The facts that 1) the soma position is among the ventral OA-IR somata in the maxillary neuromere (Fig. 9a,b), 2) all projection neuropils of this neuron show fine branches of immunoreactive fibers (Figs. 4, 9a), 3) OA-IR neurites enter the lateral antennoglomerular tract at the same position than VUMmx1, and 4) OA-IR neurites innervate collar and lip of the calyces separately (Fig. 4) suggest that VUMmx1 is part of a group of octopaminergic cells. Intersegmentally projecting neurons of median soma position and similar morphology have also been described in other insect species. For example, the subesophageal ganglion of the locust has been shown to contain OA-IR dorsal unpaired median (DUM) neurons with a similar innervation pattern of the principal neuropils of the central brain (Bräunig, 1991). Despite the difference in soma position with respect to the surface of the subesophageal ganglion, the unique morphological characteristics and the common transmitter immunoreactivity (Bräunig, 1991) would suggest a homologous relationship between VUM and DUM cells of bees and locusts. In a study of OA-IR in the ventral nerve cord of the locusts, Stevenson et al. (1992) noted variations in cell body locations across several preparations causing somata of identified DUM neurons to shift into a ventral median position in abdominal neuromeres.

Octopamine-IR neurons and behavioral plasticity

The widespread branching patterns of OA-IR fibers in the visual system seems to indicate that octopamine release by single neurons will influence integrative processes across large neuropilar areas simultaneously. The OA-IR neurons in the visual ganglia may contribute to the modulation of the visual antennal reflex (Erber et al., 1991, 1993).

The chief neuropils involved in associative proboscis extension learning (Kuwabara, 1957; Menzel et al., 1974; Bitterman et al., 1983) are the antennal lobes, the mushroom bodies, lateral parts of the protocerebrum, and the subesophageal ganglion, all of which contain OA-IR processes. Depolarizing intracellular stimulation of an identified ventral unpaired median neuron (VUMmx1) of the subesophageal ganglion was sufficient to substitute for the unconditioned stimulus in the proboscis extension learning (Hammer, 1993). This immunocytochemical study presents evidence that VUMmx1 is part of an octopaminergic cell group in the subesophageal ganglion. The involvement of octopaminergic cells in the neural pathway of the unconditioned stimulus would also explain the specific effects of octopamine injections onto associative memory (Menzel et al., 1988, 1991; Bicker and Menzel, 1989; Hammer et al., 1993). Systemic pharmacology provided evidence that octopaminergic neurotransmission is not only involved in associative learning but also in nonassociative plasticity of the honeybee's proboscis extension reflex (Mercer and Menzel, 1982; Menzel et al., 1988, 1991; Bicker and Menzel, 1989; Braun and Bicker, 1992; Hammer et al., 1993). It remains a challenging task to elucidate the relationship between the functional anatomy of OA-IR neurons innervating the neuropils containing pathways of the proboscis extension reflex and the different forms of its associative and nonassociative neuronal plasticity.

ACKNOWLEDGMENTS

We thank Sybille Schaare and Astrid Klawitter for photographic assistance and Drs. P. Stevenson and M. Hammer for helpful comments on an earlier version of this paper. This work was supported by grants of the Deutsche Forschungs Gemeinschaft (Pf 128/6-3).

LITERATURE CITED

Agricola, H., W. Hertel, and H. Penzlin (1988) Octopamin—Neurotransmitter, Neuromodulator, Neurohormon. Zool. Jb. Physiol. *92:*1–45.

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

Bitterman, M.E., R. Menzel, A. Fietz, and S. Schäfer (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J. Comp. Psychol. *97:*107–119.

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

Bräunig, P. (1991) Suboesophageal DUM neurons innervate the principal neuropils of the locust brain. Phil. Trans. R. Soc. London Biol. *332:*221–240.

Davenport, A.P., and P.D. Evans (1984) Stress-induced changes in the octopamine levels of insect haemolymph. Insect Biochem. *14:*135–143.

David, J.C., and J.F. Coulon (1985) Octopamine in invertebrates and vertebrates, a review. Progr. Neurobiol. *24:*141–185.

Eckert, M., J. Rapus, A. Nürnberger, and H. Penzlin (1992) A new specific antibody reveals octopamine-like immunoreactivity in cockroach ventral nerve cord. J. Comp. Neurol. *322:*1–15.

Eichmüller, S., M. Hammer, and S. Schäfer (1991) Neurosecretory cells in the honeybee brain and suboesophageal ganglion show FMRFamide-like immunoreactivity. J. Comp. Neurol. *312:*164–174.

Erber, J., P. Kloppenburg, and A. Scheidler (1991) Neuromodulation in the honeybee. Autoradiography, behaviour and electrophysiology. In L.J. Goodman and R.C. Fisher (eds): The Behaviour and Physiology of Bees. Wallingford, UK: CAB International, pp. 273–287.

Erber, J., P. Kloppenburg, and A. Scheidler, (1993) Neuromodulation by serotonin and octopamine in the honeybee: Behaviour, neuroanatomy and electrophysiology. Experientia *49:*1073–1083.

Evans, P.D. (1985) Octopamine. In G.A. Kerkut and L.I. Gilbert (eds): Comprehensive Insect Physiology Biochemistry and Pharmacology Vol. 11. New York: Pergamon Press, pp. 499–529.

Evans, P.D. (1993) Molecular studies on insect octopamine receptors. In Y. Pichon (ed): Comparative Molecular Neurobiology. Basel: Birkhäuser, pp. 286–296.

Evans, P.D., and M. O'Shea (1978) The identification of an octopaminergic neuron and the modulation of a myogenic rhythm in the locust. J. Exp. Biol. *73:*235–260.

Freudenstein, K. (1928) Das Herz und das Cirkulationssystem der Honigbiene. Z. Wiss. Zool. *123:*404–475

Hammer, M. (1991) Analyse der funktionellen Rolle des Neurons VUMmx1 bei der klassischen Konditionierung des Rüsselreflexes der Biene. Thesis, Freie Universität, Berlin.

Hammer, M. (1993) An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature *366:*59–63.

Hammer, M., R. Menzel, and U. Schneider (1993) Octopamine local injections into the mushroom body calyces substitute for the unconditioned stimulus in honeybee olfactory conditioning. In N. Elsner and M. Heisenberg (eds): Gene-Brain-Behaviour. Proceedings of the 21st Göttingen Neurobiology Conference. Stuttgart: Thieme Verlag, p. 848.

Harris, J.W., and J. Woodring (1992) Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. J. Insect Physiol. *38:*29–35.

Hirashima, A., and M. Eto (1993a) Biogenic amines in *Periplaneta americana* L.: Accumulation of octopamine, synephrine, and tyramine by stress. Biosci. Biotechnol. Biochem. *57:*172–173.

Hirashima, A., and M. Eto (1993b) Effect of stress on levels of octopamine, dopamine and serotonin in the American cockroach (*Periplaneta americana* L.). Comp. Biochem. Physiol. *105C:*279–284.

Konings, P.N.M., H.G.B. Vullings, M. Geffard, R.M. Buijs, J.H.B. Diederen, and W.F. Jansen (1988) Immunocytochemical demonstration of octopa-

mine-immunoreactive cells in the nervous system of *Locusta migratoria* and *Schistocerca gregaria*. Cell Tissue Res. *251:*371–379.

Kravitz, E.A. (1988) Hormonal control of behavior: Amines and the biasing of behavioral output in lobsters. Science *241:*1775–1781.

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.

Kuwabara, M. (1957) Bildung des bedingten Reflexes von Pavlovs Typus bei der Honigbiene, *Apis mellifera*. J. Fac. Sci. Hokkaido Univ. Ser. VI Zool. *13:*458–464.

Maronde, U. (1991) Common projection areas of antennal and visual pathways in the honeybee brain. J. Comp. Neurol. *309:*328–340.

Menzel, R., J. Erber, and T. Masuhr (1974) Learning and memory in the honeybee. In L. Barton-Browne (ed): Experimental Analysis of Insect Behaviour. Berlin: Springer, pp. 195–217.

Menzel, R., D.B. Michelsen, P. Rüffer, and M. Sugawa (1988) Neuropharmacology of learning and memory in honeybees. In G. Herting and H.C. Spatz (eds): Synaptic Transmission and Plasticity in Nervous Systems. Berlin: Springer, pp. 335–350.

Menzel, R., M. Hammer, G. Braun, J. Mauelshagen, and M. Sugawa (1991) Neurobiology of learning and memory in honeybees. In L.J. Goodman and R.C. Fisher (eds): The Behaviour and Physiology of Bees. Wallingford, U.K.: CAB International, pp. 323–353.

Mercer, A., and J. Erber (1983) The effects of amines on evoked potentials recorded in the mushroom bodies of the bee brain. J. Comp. Physiol. *151:*469–476.

Mercer, A.R., and R. Menzel (1982) The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee, *Apis mellifera*. J. Comp. Physiol. *145:*363–368.

Mercer, A.R., P.G. Mobbs, P.D. Evans, and A. Davenport (1983) Biogenic amines in the brain of the honeybee, *Apis mellifera*. Cell Tissue Res. *234:*665–677.

Mobbs, P.G. (1982) The brain of the honeybee *Apis mellifera*. I. The connections and spatial organization of the mushroom bodies. Phil. Trans. Soc. London Biol. *298:*309–354.

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.

Rehder, V. (1988) A neuroanatomical map of the suboesophageal and prothoracic ganglia of the honey bee (*Apis mellifera*). Proc. R. Soc. London [Biol.] *235:*179–202.

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.

Schäfer, S., and G. Bicker (1986) Common projection areas of 5-HT-and GABA-like immunoreactive fibers in the visual system of the honeybee. Brain Res. *380:*368–370.

Schäfer, S., and V. Rehder (1989) Dopamine-like immunoreactivity in the brain and suboesophageal ganglion of the honeybee. J. Comp. Neurol. *280:*43–58.

Schneider, H., B.A. Trimmer, J. Rapus, M. Eckert, D.E. Valentine, and E.A. Kravitz (1993) Mapping of octopamine-immunoreactive neurons in the central nervous system of the lobster. J. Comp. Neurol. *329:*129–142.

Schürmann, F.W., and N. Klemm (1984) Serotonin-immunoreactive neurones in the brain of the honeybee. J. Comp. Neurol. *225:*570–580.

Spörhase-Eichmann, U., H.G.B. Vullings, R.M. Buijs, M. Hörner, and F.W. Schürmann (1992) Octopamine-immunoreactive neurons in the central nervous system of the cricket, *Gryllus bimaculatus*. Cell Tissue Res. *268:*287–304.

Stevenson, P.A., H.-J. Pflüger, M. Eckert, and J. Rapus (1992) Octopamine immunoreactive cell populations in the locust thoracic-abdominal nervous system. J. Comp. Neurol. *315:*382–397.