Anatomy of the Mushroom Bodies in the Honey Bee Brain: The Neuronal Connections of the Alpha-lobe

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ABSTRACT
Neural connections between the mushroom body (MB) and other protocerebral areas of the honeybee’s brain were studied with the help of cobalt chloride and Golgi staining methods. Focal injections of cobalt ions into the α-lobe neuropil of the MB reveal seven clusters of somata located in the protocerebrum and deutocerebrum of each brain hemisphere. These neurons connect the mushroom body neuropil with protocerebral areas and number approximately 400. They contact the layered organization of the α-lobe at different locations. Some project not only into the α-lobe, but also into the β-lobe and pedunculus neuropils. Fifteen cell types which form intraprotocerebral circuits are morphologically described. They can be divided into three categories: 1) unilateral neurons, with projection fields restricted to the ipsilateral protocerebrum; these neurons connect the α-lobe with areas in the protocerebral lobe and ramify with densely layered arborisations arranged perpendicularly to the longitudinal axis of the α-lobe; 2) recurrent neurons, which interconnect subcompartments of the MB, forming loops at different levels of the neuropil; their arborisations are mainly restricted to the α-lobe, β-lobe, pedunculus, and calyces of the ipsilateral MB; they also ramify sparsely around the neuropil of the α-lobe; and 3) bilateral neurons, which either interconnect both α-lobes or connect the ipsilateral α-lobe and protocerebral lobe with the dorsolateral protocerebral lobe of the contralateral hemisphere. The connections of different compartments of the MB with other parts of the protocerebrum as revealed in this study are discussed in the context of hypotheses about the functional role of MBs in the honeybee brain. © 1993 Wiley-Liss, Inc.

Key words: Apis mellifera, insect nervous system, protocerebrum, cobalt chloride staining

Among the most striking structures of the insect brain are two bilaterally symmetric neuropils in the dorsal protocerebrum (Fig. 1), the so-called mushroom bodies (MBs). Since they were first discovered, their fascinating shape, extremely dense packing, and parallel arrangement of the intrinsic neuron axons have attracted speculation concerning their function (Dujardin, 1850; Kenyon, 1896; Jonescu, '09). Hymenopteran insects have particularly large MBs. Thus the question has been raised whether the MB could be a center for complex brain functions underlying social behavior or learning and memory. Indeed, a positive correlation exists between the volume of the MB (relative to brain size) and sophistication of social behavior (von Alten, '10; Howse, '74; Jaffe and Perez, '89). Since olfactory communication is a major component in the social organisation, enlarged MBs were also interpreted to indicate refined olfactory coding and processing (Howse, '74). These speculations were further substantiated by the discovery that the establishment of long-lasting olfactory memory in honeybees is related to normal functioning of the MBs during the consolidation period following olfactory associative learning (Menzel et al., '74; Erber et al., '80). Although the importance of the MB for memory formation in bees is well documented (Menzel et al., '88; for review see Menzel, '90), an understanding of the specific contributions of neuronal elements and compartments which make up the MB requires a detailed knowledge of its intrinsic organisation and connections to other neuropils of the central nervous system.

The structural organization of the MBs of insects has been the subject of numerous anatomical investigations (Kenyon, 1896; Jonescu, '09; Vowles, '55; Jawolsky, '58; Goli, '67; Pearson, '71; Schürmann, '74; Weiss, '74; '81; Mobbs, '82, '84). They consist of densely packed intrinsic neurons, the Kenyon cells (K-cells), which in the worker bee number some 170,000 for each MB (Withtho, '67). The dendritic arborisations of the K-cells form the cup-shaped

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neuropils of the median and lateral calyces. Each calyx is subdivided into three compartments: the lip, collar, and basal ring neuropils. From their cell bodies, located mainly in the center of the calyces, the K-cell axons project into the bipartite pedunculus. At the base of the pedunculus each fibre bifurcates, sending one branch each into the α-lobe and the β-lobe (Kenyon, 1896).

Electron microscopic studies indicate that the calyces are the main input regions and the α- and β-lobes are both output and input regions (Steiger, ’67; Mancini and Fron- tali, ’67; Schürmann, ’74; for review see Schürmann, ’87). Within the MB, intrinsic K-cell axons project ventrally in a highly ordered fashion into the pedunculus, where they form three major horseshoe-shaped bands, each deriving from a different calyceal compartment. At the junction of the pedunculus, the α-lobe and β-lobe, the K-cell bundles subdivide into several strata, which continue into the α- and β-lobe. In essence, the arrangement of the K-cells represents a transformation of the polar organization within the calyces into a Cartesian map within the lobes. Thus the three major compartments in the calyces can be allocated to discrete bands in the pedunculus and layers in the two lobes (Mobbs, ’82). A schematic summary of the MBs intrinsic topography is given in Figure 2c.

In contrast to K-cells, which are restricted to the MBs (Kenyon, 1896), the so-called extrinsic neurons, as defined in different insect species, project to or from the MB, connecting it with other brain areas (e.g., Mobbs, ’82; Schäffer, ’83; Schürmann, ’74).

In the honeybee, sensory fibre tracts originating in the optic and antennal lobes project into substructures of the calyces. The antennal projections terminate in the lip, whereas the visual fibres project to the collar neuropil. The basal ring receives input from different modalities (Satz- inger, ’80; Mobbs, ’82; Arnold et al., ’85). Extrinsic neurons to the pedunculus and the two lobes overlay the arrangement of the intrinsic elements resulting in a complex bundling pattern. In the α- and β-lobe several layers, oriented horizontally and vertically respectively, can be visualised with conventional histological methods (e.g., ethyl-gallate: Fig. 2b,c).

The objective of our study is to investigate the detailed structure of the extrinsic neurons invading the α-lobe and their connections with other regions of the brain. This paper presents a detailed account of the origin, number, and projection patterns predominantly of those neurons which could be traced after focal injection of cobalt chloride into the α-lobe neuropil. A nomenclature is introduced based on the origin, individual distribution, and branching patterns of these neurons in the protocerebrum. For tracts or internal MB compartments that have already been described, the nomenclature of Mobbs (’82) is used.

MATERIALS AND METHODS

Worker honeybees (Apis mellifera carnica) were caught at the hive entrance, immobilised at 4°C, and fixed in metal tubes with sticky tape. To gain access to the brain’s surface, the head capsule was opened ventrally by means of an incision between the antennae, compound eye, and ocelli. Glands and tracheal sheaths were removed. The dense round neuropil of the α-lobe was easily distinguishable from the surrounding protocerebrum. The tips of glass capillaries were filled with an aqueous 5% solution of either cobalt-(2)-chloride or cobalt-hexamminecobalt chloride (Sigma). The capillary was positioned above the α-lobe, and lowered anteroposteriorly into the α-lobe neuropil, between 20 and 200 μm below the brain surface. The intraneuropil pressure method (Mobbs, ’84) was applied to inject volumes of 1–5 nl cobalt chloride solution.

To reveal trajectories and neuronal shapes of single neurons in more detail, smaller amounts of cobalt were injected iontophoretically. Electrodes used for extracellular filling were placed in different regions of the α-lobe. The resistances in the tissue ranged between 5 and 30 MΩ. For intracellular staining of single neurons, electrode tips (80– 120 MΩ) were filled with hexaminecobalt chloride and backed up with 0.1 M LiCl solution. The electrodes were inserted at the ventral margin of the α-lobe, a region characterized by large diameter fibres penetrating the MB (Figs. 4, 8). Depolarizing DC (2–10 nA) or depolarizing current pulses of 1 Hz and 0.2 second duration were applied with a Grass SD-9 Stimulator for 10–15 minutes. Silver wire inserted into the thorax served as an indifferent electrode.

Brains were excised and placed in a buffered MOPS-Ringer’s solution (Coles and Orkland, ’83). The cobalt ions were allowed to diffuse for a period of 0.5 to 2 hours. Following precipitation of cobalt with an H2S saturated Ringer’s solution, the brains were fixed in Carnoy’s solution for 30 minutes and then block-intensified (Bacon and Altman, ’77). After dehydration, specimens were transferred into methyl salicylate and viewed either as whorl mounts or sectioned (20–50 μm) after embedding in Durcupan (Flucal).

For Golgi preparations (Colonnier, ’64) brains were fixed for 4 hours in 2.5% glutaraldehyde in cacodylate buffer. After washing in buffer, brains were chromatized for 2–3 days in an unbuffered mixture of 1 part 25% glutaraldehyde and 4 parts potassium dichromate supplemented with surose to a final concentration of 0.1 M. Following a wash in potassium-dichromate, the tissue was rinsed in 0.1%
silver nitrate in distilled water until the solution remained clear. For the subsequently performed silver impregnation, the tissue was incubated in 0.75% silver nitrate for 12 hours. Thereafter, brains were washed in distilled water and dehydrated in ethanol and propylene oxide prior to embedding in Durcupan.

The osmium-ethyl gallate procedure (Wigglesworth, '57) was performed with the same fixative and buffer as described for the Golgi procedure. Following several washes in buffer, brains were osmicated in 2% OsO₄ in cacodylate buffer for 1 hour in the dark. Tissue was then transferred to 0.5% ethyl gallate (Merck) in distilled water for 1 to 4 hours. The solution was changed until the blue-grey color disappeared. Following thorough washing in distilled water, specimens were dehydrated and embedded in Durcupan. Both Golgi and ethyl gallate preparations were sectioned at 10–25 μm.

Results were documented by camera lucida drawings and black and white photography (Kodak Panatomic-X) with a Reichard-Jung Polyvar microscope. Depth measurements were carried out from the frontal brain surface and are indicated in the figures by numbers standing for depths in microns. They were not corrected with respect to an initial shrinkage factor (13.8%: Strausfeld, '76). For comparison with other insect species, it should be borne in mind that anatomical features of neurons were given with reference to the body axis (Fig. 2a).

RESULTS

Location of cell bodies and number of α-lobe extrinsic neurons

Our analysis unambiguously identified the detailed morphology of 15 cell types which exemplify the arborisations of about 400 cell bodies supplying the α-lobe. They are located in seven somata clusters (A1–A7) in the protocerebral and deutocerebral rind of each brain hemisphere (Figs. 3, 4). The number of somata in each cluster ranges from 30 to 70, with the exception of only four in the case of the A5 neurons (Table 1). The primary neurites traverse in different fascicles through the protocerebral lobe to regions around the outer margins of the α-lobe. Here they branch.

Fig. 2. (At right.) The central brain of the honey bee in three different planes. a: Sagittal section showing the internal topographic organization of the intrinsic Kenyon cells in the mushroom body (after Mobs, '82). The calyces (MC and LC; LC out of plane) are subdivided into the lip (β), collar (α), and basal ring (βr) neuropil. Kenyon cells axons (small arrow) run ventrally through the pedunculus (Pe) and form layers in the alpha- and beta-lobe (α-L, β-L). Shaded areas indicate corresponding zones of Kenyon cell arborizations in the calyces and the two lobes. The large arrows b and c refer to the body axis, where the frontal plane (b) runs parallel to the dorsoventral axis and the horizontal plane (c) lies in an anterior-posterior orientation. b,c: Frontal and horizontal section in ethyl-gallate preparations. Note the striations in the α- and β-lobes. Scale bar = 100 μm.
bands are produced by fine, smooth endings, presumably input sites (stratified dendritic fields, e.g., Figs. 7–9). Within the areas to which these neurons project (the calyx, pedunculus, and α-lobe, as well as the protocerebral lobe), α-lobe neurons exhibit swelled varicosities (blebs, e.g., Figs. 9a,b, 11c) which are presumed to be indicative of presynaptic structures. Therefore, arborisations in the α-lobe are described as dendritic and those in other projection areas as axonal or terminals.

The seven somata clusters (A1–A7) comprise neurons of three major morphological groups distinguishable according to their projection patterns and cell body location in the proto- and deutocerebrum (summarized in Fig. 5): 1) unilateral neurons (Figs. 6–9): clusters A1, A2, A4, and A5; 2) recurrent neurons (Fig. 11): clusters A3d and A4; and 3) bilateral neurons (Figs. 12–14): clusters A6 and A7.

**Unilateral neurons**

The projection fields of these neurons (clusters A1, A2, A4, and A5) are restricted to one protocerebral hemisphere. They invade the α-lobe ventrally to form dense horizontal bands in the ventral (A4, A5), ventral and medial (A5), and medial and dorsal (A1 and A2) α-lobe, thus connecting these regions with the neuropil around the α-lobe and the LPL.

**Cluster A1 and A2.** These neurons form small bands in the median and dorsal α-lobe and connect the α-lobe with the anterior ipsilateral protocerebral lobe. Some small masses of these neurons exist, specific morphological types can not be distinguished.

The somata are located anterior dorsomedially (A1) and dorsally (A2) to the antennal lobe at depths of about 80 and 50 μm, respectively (Figs. 3 and 6a). The bundle of neurites from each cluster extends to the ventromedial margin of the α-lobe, and sends off thick fibers anteriorly and posteriorly through the protocerebral lobe. Branches, which course anteriorly, wrap around the α-lobe and give off collaterals which arborise in large fields in the anterior protocerebral lobe around the α-lobe. They form fine endings, unlike the swollen, bleb-like varicosities which are found for most other α-lobe unilateral neurons in the protocerebral lobe. Collaterals of both neuron groups run posteriorly to enter the α-lobe medially (A1) or ventromedially (A2) at a depth of 115–125 μm (arrows in Fig. 6a). Within the α-lobe the axons turn anterodorsally (Fig. 6a). Small iontophoretic injections of cobalt at the ventromedial margin of the α-lobe resulted in the labelling of a small subset of five A1 neurons (Fig. 6b). Axons of these cells extend to several areas in the median and dorsal α-lobe, where they arborise and exhibit fine endings. A characteristic feature of some of these neurons is that they form several bands within the α-lobe (multistratified neurons: Fig. 6b). One of these A1 neurons also sends collaterals into a thin layer in the anterior β-lobe.

**Cluster A4.** The somata of the A4 neurons are located in the median rim of the antennal lobe. They occupy a depth of between 150 and 200 μm (Fig. 4b). Their primary neurites extend dorsally into the protocerebral lobe and bifurcate ventrally to the α-lobe (Fig. 4a). Two distinct morphological types (A4-1 and A4-2) can be distinguished according to their projection pattern in the ventral α-lobe and in the surrounding protocerebral lobe (Fig. 7a–d).

In the protocerebral lobe the bundle of primary neurites of the A4-1 cells turns laterally and runs through the ventral protocerebral lobe. A characteristic feature of these neurons is a loop formed at the ventral side of the α-lobe. From here large axons enter the α-lobe at a depth of 70 to 90
Upon entrance the axon diameter of these neurons increases markedly, forming tree-like arborisations that ramify throughout the ventral part of the α-lobe (Fig. 7a). These neurons continue as very fine branches, creating a dense, stratified banding pattern oriented perpendicularly to the length of the intrinsic K-cell axons. Golgi preparations of these bands reveal fine smooth fibers, without exhibiting typical spines, as shown in Figure 11f for the A3 cells. The dorsal margin of the A4 band establishes a sharp border to the dorsal parts of the α-lobe (Fig. 4a). An intracellular stained A4-1 cell reveals the typical banding pattern which, in this case, is restricted to about 20 μm in an anteroposterior direction. In the ventral protocerebral lobe, branches of A4-1 neurites turn anteriorly and form swollen en passant varicosities in the ventral protocerebral lobe. A bundle of A4-1 fibers, the α-lobe to lateral protocerebral lobe tract (α-LPL.t.), courses from the ventral protocerebral lobe laterally. Fine processes emanate from this bundle, wrap around the α-lobe, and arborise in the tangle neuropil around the α-lobe. The α-LPL.t. itself continues posteriorly into the LPL and bifurcates twice at depths of about 130 and 150 μm (Fig. 7a). These arborisations extend from about 50 to 300 μm in an anteroposterior direction, and form flat shields which traverse the protocerebral lobe vertically. Detailed morphology of the axonal arborisations of A4-1 neurons in the LPL reveals varicosities which are mostly bleb-like and sometimes spiny in appearance (Fig. 9a).

Iontophoretic application of cobalt ions into the medial part of the ventral α-lobe reveals small subsets of both A4-1 and K-cells. The K-cell somata, located beneath the neuropil of the medial calyx, give rise to neurites which project with bunch-like specializations into the most anterior parts of the lip and collar neuropil of the median calyx (type K-15; Mobbs, '82; Fig. 7d). Their axons pass through the pedunculus and bifurcate, the branches running into the ventromedial α-lobe and anteromedial β-lobe. In the α-lobe, the axons of the K-cells cross the A4 band ventrolaterally in an area of

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**Fig. 4.** Frontal view of a preparation in two section planes in which cobalt chloride was pressure-injected into the α-L neuropil of the left mushroom body. a: α-L and protocerebral lobe at the level of the α-exit (100 μm); α-L extrinsic somata groups (A1, A2, A3) are located in the rind of the protocerebrum. Prominent inputs to the α-L neuropil are located at its lateral (open arrow) and ventral (A4 neurons) margin. A5 axons penetrate the α-L medially (single arrowheads). b: At the β-exit level (170 μm), a large dendrite (the Pe-1 neuron) penetrates the α-L medioventrally (arrow) flanked by two fibre bundles (open arrows). A main projection area of these neurons is the dorsal and lateral protocerebral lobe (single arrowheads). A large fibre penetrates the antennal lobe at its dorsolateral margin (small arrow). d: dorsal; l: lateral. Scale bars = 100 μm.
about 10–20 μm diameter, and end anterior to the A4 band. The fine structure of a Golgi stained K-(5) cell in the lip region of the median calyx is shown in Figure 10.

The ramifications of the A4-2 neurons in the α-lobe and in the protocerebral lobe are both located more posteriorly than arborisations of the A4-1 neurons (Fig. 7c). The primary neurites of A4-2 neurons bifurcate at the ventrolateral margin of the α-lobe, where large diameter collaterals are sent into the α-lobe neuropil. Figure 7c shows the projection pattern of a single A4-2 collateral in the α-lobe: a main branch crosses the lobe, ramifies into finer branches, and forms a band in the α-lobe, the A4-2 band. This band lies posteriorly to the A4-1 band, and reaches the base of the α-lobe near the pedunculus. In the protocerebral lobe, the projection areas of A4-2 cells are restricted to the LPL: a fascicle of axons runs laterally within the α-LPL1t and branches off close to the axonal areas of A4-1 neurons. Here the terminals have a bleb-like appearance (Fig. 9b). A4-2 neurons do not ramify around the circumference of the α-lobe and in the ventral protocerebral lobe.

**Cluster A5.** A small group of at least four somata, the A5 cluster, is positioned 10–20 μm below the anterior brain surface, close to the midline. It is located contralateral to the cobalt-injected mushroom body (MB). The somata are of strikingly large diameter (up to 30 μm). From the A5 cluster the primary neurites project medially and cross the midline at a depth of 30 μm towards the medioventral margin of the α-lobe, where they bifurcate. A5 neurons are a diverse group of neurons exhibiting several unique forms with a complex projection pattern within the α-lobe.

The A5-1 neuron is one of the largest α-lobe extrinsic cells with respect to the size of the soma and the area of innervation in the α-lobe (Fig. 8a). Its primary neurite arises from the contralateral soma and loops ipsilaterally. One axon invades the α-lobe medially at a depth of 70 μm, and branches off into processes which occupy a narrow horizontal layer in the anterior α-lobe, forming large varicosities. Along the length of this area collaterals pass down posteriorly and connect to a posterior layer in which the processes contribute to a very dense banding pattern (Fig. 9c,d). Other collaterals project into this posterior band emanating from the main branch entering the α-lobe medioventrally and ventrally at a depth of between 100 and 130 μm (Fig. 8a). Figure 9d shows the fine structure of A5-1 endings in the posterior layer at a higher resolution. A5 collaterals that wrap around the α-lobe project into the ventral and median protocerebral lobe and have mainly blebby processes. One axon surrounds the α-lobe laterally and forms a prominent loop from which several branches project into the dorsal and lateral protocerebral lobe.

The A5-2 cell gives rise to a neurite that bifurcates medioventrally to the α-lobe (Fig. 8b). A large diameter dendrite penetrates the α-lobe ventrally and ramifies in the ventral α-lobe. In the ventral protocerebral lobe, an axon is wrapped around the ventrolateral edge of the α-lobe and sends two main branches anteriorly into the optic tubercle (OT). Here they form two oval axonal fields in which the processes exhibit bleb-like varicosities. Other side branches form a ring around the α-lobe. The main axon continues to the dorsolateral protocerebral lobe, curves posteriorly, and divides into side branches which project to the protocerebral neuropil adjacent to the pedunculus. All the processes of the A5-2 neuron within the protocerebral lobe form blebby varicosities (Fig. 8b).

The huge soma (30 μm) of the A5-3 neuron is located in the anteromedial contralateral protocerebral lobe. Ipsilaterally a fine primary neurite bifurcates medioventrally to the α-lobe. Collaterals wrap around the α-lobe, and three large axons enter the α-lobe ventrolaterally. Here they ramify into several branches which end in distinct dendritic fields (diameter of 40 μm) in the ventral α-lobe. Posteriorly these dendritic fields fuse to form a horizontal band at the base of the α-lobe.

Another A5 neuron (A5-4) was incompletely stained and its projections obscured by parallel staining of other neurons which terminate in this region. Nevertheless, this cell clearly ramifies in the ventral protocerebral lobe neuropil adjacent to the α-lobe, penetrates the ventral α-lobe, and projects contralaterally to the ventral protocerebral lobe around the β-lobe.

**Recurrent neurons.** Neurons that connect the α- and/or β-lobes with the calyces have been documented in several insect species (Goll, '67; Pearson, '71; Weiss, '78). In the honeybee, electrophysiological (Homborg and Erber, ’79; Groenenberg, ’87) and anatomical (Mobbs, ’82; Bicker et al., ’85) studies show that feedback neurons of the protocerebrocalycal tract (p.c.t.) arborise in the α- and β-lobes and pedunculus and connect these neuropils with the calyces.

**Clusters A3-d and -v.** The present study reveals subpopulations of this type of MB extrinsic neurons originating in two large clusters of somata: A3-v and A3-d (Figs. 3, 4a), located in the anterior lateroventral protocerebrum. They form different loops connecting various compartments of the MB. The primary neurites traverse the protocerebral lobe in two separate bundles which cross each other halfway to the α-lobe. The A3-d fascicle runs anteriorly to the A3-v bundle and enters the α-lobe at its lateral margin; the A3-v fibers course posteriorly and enter the α-lobe 20–30 μm beneath the A3-d fibers (Fig. 11a).

The A3-v fascicle is identical to the anterior lateral protocerebral tract (a.l.p.t.) described by Mobbs (’82). Neurons running in this bundle bifurcate in the protocerebral lobe dorsolaterally to the α-lobe. One branch joins the p.c.t., and the other loops ventrally to enter the α-lobe at the α-exit. From there A3-v neurons spread out horizontally throughout the dorsal and medial layers of the α-lobe and arborise in local areas in different layers and depths. Some of them send branches posteriorly which run parallel to the K-cell axons, pass the base of the α-lobe, and project into the β-lobe and pedunculus (Fig. 11a). A Golgi stained preparation revealed the spiny appearance of single A3-v fibers in the dorsal α-lobe (Fig. 11f).

A3-v collaterals in the protocerebral lobe run within the p.c.t. posteriorly, branch, and enter the ipsilateral median and lateral calyx via the inner ring tracts (i.r.t., Mobbs, ’82; Fig. 11a). They terminate everywhere in the calyces, but
ALPHA-LOBE NEURONS

Fig. 5  Schematic drawings of the projection patterns of α-lobe extrinsic neurons (shaded areas) in the MB and surrounding protocerebral lobe. a: A1 and A2 neurons @ connect small regions of the dorsal and median α-lobe with the ipsilateral PL. A4 ⃝ and A5 ⃝ neurons terminate in the ventral α-L and connect these regions to the ipsilateral PL and also with the optic tubercle. b: A3 neurons connect various subcompartments of the MB; they terminate in the dorsal and median α-L and project through the PL into both calyces A3-v. ⃝ A subset of A3-d neurons © connect the α-L with the β-L. c: A6 neurons © and A7 neurons © connect the ipsilateral MB and PL with the opposite brain hemisphere.
predominantly in the lip and basal ring regions. Cobalt iontophoresis into the dorsal α-lobe reveals an A3-v subpopulation with arborisations restricted to the basal ring of both calyces. A conspicuous oval region which extends throughout the calyces between basal ring and collar neuropil is more densely innervated by A3-v fibers than the basal ring neuropil. This area, obvious even in ethyl gallate preparations, is named here the dorsal basal ring region (d-br in Fig. 11a,b). From the i.r.t. side branches of the A3-v neurons innervate the basal ring and send processes into the central layer of the dorsal parts of the pedunculi (Fig. 11c). Thus, the same fibers innervating the basal ring terminate in a layer of the pedunculus which represents the basal ring neuropil (see opening paragraphs and Fig. 2). In the pedunculus the varicosities of A3-v terminals exhibit slightly different, more claw-like specialisations in comparison to the round bleb-like structures of the same fibers in the basal ring neuropil (Fig. 11c). No differences were found between the innervation pattern of A3-v cells in the lateral and the median calyx neuropil. In Figure 11a the dendritic branching of a simultaneously stained basal ring K-cell (type 3: Mobbs, '82) is shown. In other preparations A3-v cells were stained together with large groups of K-cells which both project into the dorsal α-lobe. K-cell somata were estimated to number more than 1,000 cells positioned in the center of the medial calyx. The dendritic branches of these cells arborise within the basal ring of the medial calyx. The K-cell axon bundle runs within the inner layer of the pedunculus, bifurcates at the base of the α-lobe, and extends into the posteriormedial β-lobe and the dorsomedial α-lobe.

A neurite bundle originating from a subset of about 30 neurons with somata located in the A3-d cluster (Fig. 11a,d,e) runs directly to the lateral α-lobe anterior to the A3-v bundle. The fibres spread into the anterodorsal α-lobe. Before the A3-d fascicle enters the α-lobe, it gives rise to a small branch which sends fine processes into the α-lobe (Fig. 11d,e). Outside the α-lobe the branches cross the main tract anteriorly, loop ventrally, and run posteriorly to the β-lobe (α-lobe-to-β-lobe tract: α-β.t). The α-β.t enters the β-lobe at its lateral margin at a depth of 160 μm, and fine ramifications spread throughout the β-lobe (Fig. 11d,e). Here, the stratified banding pattern, which is typical for the α-lobe, is lacking.

Mass fills of cobalt have shown that cells originating in the A3-d cluster and invading the α-L also run in the p.c.t and project into the calyces.

**Bilateral neurons**

Most α-lobe extrinsic neurons project within the ipsilateral protocerebrum. A smaller number of neurons originating in the A6 and A7 cluster projecting to the α-lobe (and partly into the β-lobe) also project to the contralateral side of the brain and connect the ipsilateral lobes with the contralateral protocerebral lobe and anterior α-lobe.

**Cluster A6.** This cluster of somata in the dorsolateral protocerebral lobe (A6) is located ventrally to the lateral calyx, about 180 μm below the frontal brain surface (Fig. 3). A heterogeneous group of neurons originates in this cluster, but they are similar in that their arborisations in the α-lobes are restricted to its anterior parts. Two types, A6-1 and A6-2, could be identified.

Neurites from type A6-1 pass through the dorsal protocerebral lobe, parallel for part of its length the p.c.t, and extend anteriorly (Fig. 12). Within this bundle axons run to the dorsolateral edge of the α-lobe. One branch approaches the α-lobe laterally and enters at its ventrolateral margin (Fig. 13a). It arborises within the most anterior parts. A

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**Fig. 7.** A4 neuron types in the protocerebrum. a: Four A4-1 somata located in the antennal lobe give rise to loop-forming axons (arrow) in the protocerebral lobe. In the α-lobe they arborise in its ventral region. Target regions in the PL are the neuropil surrounding the α-L and, via the α-LPL.t. (triangle), the dorsal and lateral PL. b: An intracellularly stained A4-1 neuron. Note the typical banding pattern in the ventral α-L (arrowhead). c: A4-2 neurons connect an area in the posterior α-L with the LPL (triangle: α-LPL.t). d: Simultaneous labelling of A4-1 neurons and Kenyon cells (type K-'5'), within the proto- and deutocerebrum (the arrow indicates the injection site of cobalt). The K-'5' cells with their somata positioned beneath the median calyx have small dendritic fields in the lip and collar region of the MC. In the α-L, their axons cross the A4-1 band (arrow). d, dorsal; m, median. Scale bars = 100 μm.
Figure 7
Fig. 9. Fine structure of A4 and A5 neurons. a: A4-1 terminals in the lateral protocerebral lobe. Blebs (small arrow), often arranged in complexes up to 4-5 μm, and spiny processes (arrowhead) appear on the same fiber. b: A4-2 endings in the same area exhibit bleb-like varicosities. c,d: The A5-1 neuron in whole-mounts. c: A large branch (long arrow) enters the α-L and terminates in an extensive area of the ventral α-L (posterior band). Note the bunch-like arborisations in the ventral protocerebral lobe (short arrow). d: Fine arborisations in the posterior band at higher resolution. Scale bars = 10 μm for a,b; 20 μm for c,d.

side branch runs posteriorly and ramifies within the lateral protocerebral lobe (not shown Fig. 13a). A collateral of this cell passes through the dorsal protocerebral lobe and crosses the midline of the brain via the anterior median protocerebral commissure (a.m.p.c.; Fig. 12). It continues into the contralateral protocerebral lobe where the fibers terminate, forming bleb-like structures in the dorsolateral neuropil around the α-lobe (Fig. 13a).

A6-2 neurons have their somata positioned ventrally to the lateral calyx in the A6 cluster. The primary neurites form a loop directed posteriorly and then turn dorsally to run part of their length parallel to other A6 neurites. Dorsal to the α-lobe they course medially, approach the dorsal margin of the α-lobe, and surround its medial side. During this course the neurites give off several collaterals that

Fig. 8. Camera lucida drawings of A5 neuron types. Their somata are located contralaterally in the anterior protocerebral lobe. a: The bistratified A5-1 neuron enters the α-lobe at different locations, arborizes in two areas (arrow), and connects these zones with protocerebral areas around the α-L, and the dorsolateral PL. b: The A5-2 arborises in the ventral α-L (reconstructed from a preparation provided by J. Mauelshagen). In the PL it sends branches anterior into the optic tubercle. For position of A5 neurons in the protocerebrum, see Figure 5a; d, dorsal; l, lateral. Scale bars = 100 μm.

Fig. 10. A Kenyon cell from type 6 innervates the lip region of the median calyx. The soma lies outside the calyx (arrow). d, dorsal; l, lateral. Scale bar = 20 μm.
details on the ipsilateral side are obscured. Another cell (type 2; Fig. 13c) enters the contralateral α-lobe and spreads out as described above but exhibits a denser stratification in the medial and dorsal part of the α-lobe.

Cluster A7. A7 neurons originate in a soma cluster located in the ventral protocerebrum at a depth of about 90 μm. The cluster is positioned posteriorly, close to the A2 cluster (Fig. 3). Its primary neurites run posteriorly over the β-lobe, wrap around it, turn anteriorly, and enter the MB through the β-exit (Figs. 4 and 14; Mobbs, '82). Based on their innervation pattern within the MB, two types of A7 neurons can be distinguished: A7-1 neurons arborize within the transition between α-lobe and pedunculus (Fig. 14a: arrows) where they form finger-like, vertically orientated dendritic fields which are restricted to the area occupied by the K-cell collaterals projecting into the α-lobe. A7-2 neurons have the same entry as the A7-1 cells but from the β-exit they turn anteriorly within the α-lobe. Their ramifications can be traced to the median and dorsal α-lobe up to 90 μm below the brain surface (Fig. 14b).

In the medial protocerebral lobe, A7 profiles turn contralaterally and project anteriorly, running in the anterior dorsal protocerebral commissure (a.d.p.c.(i): Mobbs, '82). In the contralateral medial protocerebral lobe they form small fields. Some of these fibers continue to wrap around the dorsal α-lobe and form branches with blobby varicosities which terminate in the dorsal and lateral protocerebral lobe. A large bundle, the a.d.p.c., turns back ipsilaterally and surrounds the α-lobe dorsally (Fig. 14). Here the a.d.p.c. divides into several strands (a.d.p.c.(ii)) which project to different areas in the ipsilateral lateral protocerebral lobe where they extend to between 30 μm and 300 μm in an anteroposterior direction.

Other alpha-lobe extrinsic neurons

In addition to the neurons presented above, other neurons were found that do not fit the types described so far. A3-v-I. A single neuron with its soma located in the A3-v cluster sends its primary neurite dorsally through the protocerebral lobe (Fig. 11a,d). It then turns medially and splits into several collaterals which arborise in the protocerebral lobe lateral and dorsal to the α-lobe. Here they form blob-like varicosities. One branch approaches the lateroventral margin of the α-lobe, enters ventrally, and forms extensive arborisations in the ventral α-lobe which extends mediodorsal and anterior.

Pe-I. The Pe-1 cell is one of the most prominent identified single neurons extrinsic to the MB (Mauelschagen, '93). It exhibits structural polarity, with fine, smooth arborisations in the pedunculus and blobby projections in the protocerebral lobe. The soma of this neuron has a diameter of 30 μm, and lies close to the midline 20 μm below the anterior surface of the brain (Fig. 15). The primary neurite courses contralaterally and passes posteriorly through the protocerebral lobe. Along the way it gives off very fine, short branches. At a depth of 210 μm it loops back ipsilaterally, and enters the posterior α-lobe at a depth of about 170 μm, close to the bundle of A7 fibers. Here it forms a large dendrite with a diameter up to 15 μm. The Pe-1 neuron bifurcates and forms tree-like arborisations which run posteriorly. They turn into vertically oriented bands with fine branches that extend finger-like structures from the dorsal base of the α-lobe deep into the pedunculus. Outside the α-lobe, one branch runs anteriorly through the ventral protocerebral lobe, where it arborises. The main
fibre courses posteriorly into the lateral protocerebral lobe. There the axonal projections terminate at a depth of 150–300 μm in an anteroposterior direction.

A6-1. Cobalt injections into the ventral α-lobe also stained a fibre which enters the antennal lobe at its mediolateral edge (Figs. 4h, 16) at a depth of 80 μm. In the antennal lobe, the axon courses anteriorly and arborises in numerous glomeruli. It forms varicosed endings in the periphery of the glomeruli. In the protocerebral lobe the fibre courses anteriorly and approaches the α-lobe on its medial side, from where it could be traced no further. A second fibre connects the pedunculus with the neuropil of the antennal lobe. It loops more posteriorly (200 μm) from the antennal lobe into the protocerebral lobe and runs parallel to the lateral margin of the β-lobe. In the dorsal protocerebral lobe it branches into several collaterals. One axon turns laterally into the LP, and the other approaches the lateral pedunculus where it enters and arborises at the junction of the pedunculus to the β-lobe.

**DISCUSSION**

**Technical considerations**

Intraneuronal and intracellular injections of cobalt ions into central brain areas have frequently been used to resolve the neuronal connections and to mark individual cells in the insect brain (Pitman et al., '72; Strausfeld and Hausen, '77; Bacon and Strausfeld, '80; Mobbs, '84). We employed this technique to label specific subpopulations of neurons projecting into or out of the MB by injecting cobalt ions into the α-lobe neuropil. The focal injection of cobalt solution into this area is facilitated by the fact that the outlines of the α-lobe can be easily recognized. Moreover, even relatively large volumes of injected solution (1–2 nl) remain within the α-lobe, probably because of the compact packing of intrinsic and extrinsic fibers, and because of the presence of glial sheaths within and surrounding the α-lobe. Glial barriers as a constraint of dye-diffusion are also reported in the lamina ganglionaris of dipteran optic lobes (Jarvilehto et al., '85). However, since not all the injected cobalt is taken up by the severed neurons, a lingering cobalt pool often obscures the arborizations of neurons at the injection site.

Nevertheless, preparations with bulk injections provide a valuable overview and were taken to estimate the total number of α-lobe extrinsic neurons (see Table 1). Since the injection of cobalt was carried out in different depths throughout the α-lobe, we assume that the estimated number of cells, about 400 (Table 1), represents the total neurons extrinsic to the α-lobe. It shows clearly that many K-cells (170,000 for each MB; Wittthöft, '67) converge onto a small number of α-lobe extrinsic neurons.

![Fig. 13. A6 neurons in the anterior protocerebrum. a: An A6-1 neuron gives rise to a neurite which bifurcates in the dorsal protocerebral lobe and projects ipsilaterally into the anterior parts of the α-L. Centrally, the neuron projects into the dorsolateral PL. b: An A6-2 neuron surrounds the α-L dorsally and arborises within the ventral layers of both α-L (intracerebral stained and reconstructed by J. Maulschagen). c-d: α-Lobe connecting neurons type 2 (c) and type 1 (d). The projection pattern within the contralateral α-L is extended throughout the horizontal plane. Arrows indicate the injection site of cobalt. d, dorsal; l, lateral. Scale bars = 100 μm.](image)

**Morphological polarity of alpha-lobe extrinsic neurons**

The structural polarity of neurons is often used to correlate structural specializations with dendritic or axonal function, thus supporting the construction of hypothetical functional neuronal circuits. Ultrastructural studies provide evidence that fine branching (smooth endings) or spiny processes are typical for postsynaptic dendrites, whereas varicosities such as blebs on axonal arborizations are indicative of presynaptic sites (e.g., Schürmann, '72, '73; Strausfeld and Bassemm, '85).

In electron microscopic studies presynaptic terminals are described as a characteristic feature of the glomerular calyx neuropil (Steiger, '67; Schürmann, '74). These bouton-like structures, also typical for honey bee calyx, may represent the blebs of the terminal arborisations on the antennal-glomerular tract (AGT) (Mobbs, '82) and p.c.t. fibers (Gronenberg, '87; Fig. 11c). The smooth endings of the majority of α-lobe extrinsic neurons within the α-lobe and their blebby endings within protocerebral areas indicate that these neurons receive input from K-cell axons and convey information to other protocerebral regions, namely, the ring neuropil around the α-lobe and the lateral protocerebral lobe.

However, it should be kept in mind that input and output synapses are often located close together on the same axon, may occur in one varicosity, or may even lack a synaptic specialization (e.g., Watson and Burrows, '83; Watson et al., '85). Therefore, light microscopical structures are only indicative and not proof of functional polarity.

**Allocation to physiologically described MB neurons**

In recent studies, neurons of two large tracts, the a.d.p.c. and p.c.t. (this study: A7 and A3 neurons, respectively), have been described (Mobbs, '82; Homberg, '84; Gronenberg, '87). These studies showed that the MB is woven into a complicated net of looping neurons interconnecting different parts of the MB with each other. Additionally, Mobbs ('82) described connections between all parts of both MBs via the a.d.p.c. This cannot be confirmed for the A7 fibers found in our study. Their projections to the contralateral side are restricted to the median and dorsolateral protocerebral lobe (Fig. 14).

Recurrent neurons which interconnect the different compartments of the same MB were described as a conspicuous feature of this neuropil in many insects (ants: Goll, '67; crickets: Schürmann, '73. Orthoptera: Weiss, '81). In this study two large somata clusters in the anterior ventrolateral protocerebrum, the A3-v and A3-d, are identified as the origins of a set of fibres which have been described as feedback interneurons in anatomical (Mobbs, '82) and electrophysiological (Homberg and Erber, '79; Gronenberg, '87) studies. Immunohistochemistry reveals that this cell type is immunoreactive to γ-aminobutyric acid (GABA) (Bicker et al., '85). A3 fibers enter the α-lobe via the α-exit and connect the α-lobe with the ipsilateral calyx neuropil via the p.c.t. Their fine structure, varicosed endings in the calyx of a putatively presynaptic nature (Schürmann and Elekou, '87), and spiny appearance in local bands of the α-lobe (Fig. 11f) indicate an information flow from the α-lobe to the calyces and support their possible role as a feedback pathway of the MB.
Fig. 14. A7 neurons in frontal sections: the primary neurites pass underneath the β-lobe and enter the α-lobe at its medioventral margin. In the medial protocerebral lobe they project dorsally and send processes contralaterally. Ipsilaterally the neurons surround the dorsal α-L (not shown in a). In the α-L A7-1 neurons branch in long, finger-like strips at the base of the α-L (arrows) (a) or project to its dorsal parts (type A7-2) (b). d, dorsal; l, lateral. Scale bar = 100 μm.
Focal cobalt injections into the dorsomedial α-lobe reveal an A3-v subpopulation with restricted projections in the basal ring and a central zone in the pedunculus, indicating specific connections between the dorsal α-lobe and the basal ring area (Fig. 11a–c). Although we could not trace any individual A3 neuron connecting equivalent zones between α-lobe and calyx, intracellular staining of an A3 neuron has demonstrated such a specific connection (Grünewald, personal communication). Thus, K-cell activity evoked in the basal ring and transferred to the dorsal α-lobe may be controlled by the action of topologically specific feedback neurons.

Dopamine-like immunoreactivity has been recently demonstrated for three α-lobe extrinsic neurons groups, A1, A2, and probably A6-2 cells (Figs. 6, 13b). The cell groups called C1, C2, and C3 by Schäfer and Rehder ('89) correspond to the clusters A1, A2, and A6, respectively. Although the arborisations of these neurons in the α-lobe could not be resolved entirely in the immunocytochemical preparations, the correspondence to fibre tracts described in our study can be deduced on the basis of the position of the cluster of somata and the course of the neurite bundles.

**Deutocerebral connections**

In the honey bee the major pathways from the antennal lobe to the MBs are mediated by the AGTs (Satzinger, '80; Mobbs, '82; Arnold et al., '85). Connections were also reported between the α-lobe and antennal lobe (Arnold et al., '85) and neurons with widespread arborisations in the antennal lobe connecting the calyx with the antennal lobe (Mobbs, '84). The latter were interpreted as a possible feedback pathway. Our study reveals an antennal lobe extrinsic fibre (AL-1, Fig. 15) with multiglomerular projections within the antennal lobe. Comparison with the preparation from Satzinger ('80) implies that AL-1 is identical to projections of a large neuron that innervates a specific band in the α-lobe. The varicose endings in the AL are consistent with the interpretation of a feedback pathway from the MB to the deutocerebrum (Vowles, '64).

**General organisation and connectivity of the MB**

The internal, topographic organisation and connectivity of the MB in the honey bee has been studied extensively (Koenen, 1896; Jones and Vowles, '69; Schürmann, '74; Mobbs, '82, '84). Visual fibre tracts terminate in the collar, whereas antennal relay neurons project via the m- and l-AGT into the lip neuropil of the MB calyces (Satzinger, '80; Mobbs, '84). Another second-order olfactory neuropil, the ventral lateral protocerebral lobe (V.p.n.a.; Mobbs, '84), receives input from m-AGT and l-AGT collaterals. A third important pathway originating in the antennal lobe is the m-AGT which projects into the dorsal and lateral parts of the LPL without sending collaterals into the calyces of the MB (Satzinger, '80; Fig. 17a).

In the MB the spatial segregation of sensory inputs into the calyces is maintained in the pedunculus and the lobes (Mobbs, '82; Fig. 2). This observation is confirmed at least for parts of the collar and basal ring region by our finding that K-cell bundles project from these calyceal zones to corresponding areas in the α-lobe (Fig. 11, text).
Fig. 16. Injection of cobalt into the ventral α-lobe stains a fibre projecting into the antennal lobe, where it arborises into numerous glomeruli. Scale bar = 100 μm.

Since many extrinsic neurons form bands narrower than the three layers of the MB, Mobbs ('84) suggested that MB extrinsic neurons create a fine grain map which may reflect the order of inputs with a higher resolution than the gross three-layered map of the MB. This is supported by the structure of the A4 neurons (Figs. 7, 17) described in our study. The A4 cells may receive input from K-cells with dendritic arborisation in the lip, namely K-(1) cells that project to the ventral α-lobe (Mobbs, '82). Therefore, A4 neurons may integrate olfactory information. The A4 fibres project via the α-LPL into the dorsal and lateral protocerebral lobe. In this area they overlap with the projections of the AGT fibres (Fig. 17). Therefore, the LPL may integrate two kinds of olfactory information, one which is processed via the MB, and one which is directly relayed from the antennal lobe. These findings suggest that olfactory information spatially segregated in the MB is transferred to other brain regions (protocerebral lobe) by specific subsets of α-lobe output neurons. The convergence of two kinds of olfactory information has to be tested with both anatomical and physiological experiments.

The small field K-(5) cells (Fig. 10) do not follow the regular projection pattern of the other K-cells. They project from different calycal zones together to spots in the ventral α-lobe (Fig. 7). This region may therefore also integrate sensory information from different modalities. Although no physiological data exist from the A4 cells, intracellular recordings of A3 and A7 neurons provide evidence for multisensory sensitivities in the MB (Erber, '78; Homberg, '84; Gronenberg, '87). These results were explained by different mechanisms, e.g., multimodal inputs to one region of the calyces (via AGT neurons to the lip; Homberg, '84), existence of feedback neurons which connect nonequivalent zones between calyx and α-lobe (Gronenberg, '87), and/or synaptic connections between K-cells (Schürmann, '74). Our findings suggest that multimodal response characteristics of MB output neurons might result from intrinsic network properties of the MB itself which are mediated via the K-(5) cells.

The anatomy of other α-lobe extrinsic neurons indicates that they integrate information across the intrinsic banded subdivisions of the α-lobe. The large A5 cells and the Pe-1 neuron (Mauelshagen, '93; this study: Fig. 15) form extensive dendritic fields in the α-lobe and pedunculus, indicating that information derived from different calycal zones is integrated in these areas.

Regarding the input pattern of neurons to the calyces and arborisations of neurons invading the α-lobe, no evidence was found indicating functional differences between the two calyces (Mobbs, '82; this study). α-Lobe extrinsic neurons (with dendritic bands equally organized across the lateral-medial axis of the α-lobe) receive equally strong input from both calyces: K-cell bundles run separately from the two calyces into the lobes, in such a way that the lateral calyx is represented in the lateral part of the α- and β-lobes, whereas the median calyx is represented in their medial parts.

With respect to the innervation pattern of extrinsic fibres, the α-lobe is roughly divided into three major compartments (Fig. 17a,b). The dorsal and median α-lobe is dominated by recurrent cells which connect different parts of the MB, while the ventral part is invaded by unilateral neurons that relay information to the protocerebral lobe. The morphology of A4 neurons indicates that these cells are part of a loop within the olfactory system of the honey bee (Fig. 17a). The most anterior parts of the α-lobe are invaded by bilateral A6 neurons (Fig. 17b).

Outlook

With respect to the α-lobe our study supports the idea that MB extrinsic neurons are elements of intraprotocerebral circuits, as stated by Boeckh and Ernst ('87). The two main target areas of α-lobe extrinsic neurons, the ring neuropil around the α-lobe and the LPL (Figs. 5, 17), also exist in other insects (e.g., Schübler, '83). The latter is reported as an important area for odor processing (Arnold et al., '85; Christensen and Hildebrand, '87). In this study an initial step was taken to localize different compartments within the protocerebral lobes. Further studies will be concerned with the question of whether α-lobe output neurons contact the antennal lobe projections in the LPL or project directly to descending neurons as reported for diptera (Strausfeld et al., '84).

The MB are involved in learning and memory, as shown by genetic disruption and reversible cooling experiments (Heisenberg et al., '85; Menzel et al., '74; Erber et al., '80). Further evidence for the role of the MB in mnemonic processes comes from single gene mutants in Drosophila (Nighorn et al., '91; Han et al., '92). In the honey bee the MB are an essential neural substrate for the formation of the stable, long-lasting olfactory memory (for review, see Menzel, '90). Recent results of intracellular recordings of neurons connected to the MB support the notion that the
Fig. 17. Diagrams illustrate major circuits involving the α-lobe and related protocerebral areas. **a** Right side: Layers in the ventral α-lobe (unilateral A4 neurons) are connected via the α-L.P.L.t. with the dorsal and lateral protocerebral lobe (dark-shaded areas). In the L.P.L they overlap partly with projections of the antenno-glomerular tracts (m-, mL-, and I-AGT) (arrow). AGT collaterals also reach the lip neuropil of both calyces (lightly shaded areas). Left side: A system of recurrent fibres connects dorsal and median layers in the α-lobe with different calyceal compartments of the MB. **b** Horizontal view: Bilateral A6 neurons connect the ipsilateral α-lobe with the contralateral α-lobe and protocerebral lobe via two tracts, the a.m.p.c. and α-L.t. (arrowhead). (A4: A4 neurons, see also a.) Arrows indicate the possible information flow between the neuropils. a, anterior; d, dorsal; l, lateral; v, ventral.
MB are involved in learning. First, the Pe-1 neuron (Fig. 15), a single identified MB extrinsic neuron, was shown to change its response properties to olfactory stimuli specifically during associative learning (Maeselschagen, '90). These adaptive changes are transient. Second, the VumX1, a large modulatory neuron that was shown to substitute for the sucrose reward in a single learning trial during conditioning of the proboscis extension reflex (PER) invades the calyces, the LPLs, and antennal lobes (Hammer, '92).

The neural connectivity described in this paper will help us to perform further neurophysiological and anatomical studies on pathways. Such studies will help us to elucidate the functional roles of the mushroom bodies in the honeybee brain.

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LITERATURE CITED


