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SEPARATE DISTRIBUTION OF DEUTOCEREBRAL PROJECTION NEURONS IN THE MUSHROOM BODIES OF THE CRICKET BRAIN*

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Deutocerebral projection neurones in the brain of the cricket (*Gryllus bimaculatus*) have been investigated by experimental dextran staining, viewed by light and electron microscopy. These neurones of two separate somata clusters innervate two separate primary glomerular neuropils of the deutocerebral segment, either the antennal lobe receiving only antennal nerve sensory input, or the glomerular lobe, receiving input from sensory neurones of lower segmental origin, including chemosensory fibres from mouth parts. Projection neurones of the antennal lobe only invade the anterior calyx of the mushroom body neuropil via the inner antenno glomerular tract, while glomerular relay neurones of the glomerular lobe innervate only the posterior calyx via the tritocerebral tract. All types of projection neurones give rise to presynaptic boutons, forming the central core of microglomeruli with patterned distribution. These projection neurons are cholinergic. The results are discussed in view of maintained segregated modal information, first processed in the separated primary deutocerebral neuropiles and further on in the second order input neuropils of the mushroom bodies. The large posterior calyces are proposed as a compartment for gustatory information.

Keywords: Mushroom bodies – deutocerebral projection neurones – dextran staining – synapses – *Gryllus bimaculatus*

INTRODUCTION

The mushroom bodies (MBs) in the brain of insects are considered higher centres for integration of sensory information and memory storage. There is accumulating evidence from morphological studies that the columnar neuropil of the mushroom bodies is organized in subcompartmental areas, dividing their main gross parts, the calyx, stalk and lobes into delicate smaller parts in a species-specific manner [10]. Prominent examples are the MBs of the bee and cockroach [12, 13]. An important aim is to reveal the obvious compartmental organization from its constituting neurons, and to relate the structure to functional specializations. Interestingly, in the ant

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brain projection neurons (PNs) from visual and other lower neuropil centres are distributed in MB neuropils, so that their geometric order is maintained [6] and converted to a topographic map of sensory information in the MBs. The meaning of the delicate compartmentalization in insect neuropils is poorly understood, though recent studies using Ca-imaging have cast some light on sites of olfactory information processing [4, 5].

The MBs receive chemosensory and other modality information from preprocessing primary deutocerebral neuropils via massive tracts of PNs [1]. In crickets two clearly separated glomerular deutocerebral neuropils can be distinguished: the antennal lobe (AL) and the glomerular lobe (LG). While the first receives mainly olfactory information from the ipsilateral antenna the latter is not connected with antennal nerve sensory fibres [11]. It receives mixed input from the maxillary palpes [2, 3, 7]. Moreover, the LG is subdivided into two glomerular neuropils, a medial (mLG) and a ventral part, the vLG [7]. Thus within the deutocerebrum different sensory information is processed in spatially distinct areas before being transported to the MBs via massive tracts.

We show for the cricket mushroom bodies by use of tracing experiments that the two deutocerebral sensory glomerular neuropils of the AL and LG are connected by different PN types invading separate regions of the ipsilateral bipartite calyx. The PNs of a tract either project completely into the anterior calyx (iACT-PNS) or into the posterior calyx (TT-PNs). Within the calyces PNs form either spheroidal or indented boutons in separate subcompartments. Electron microscopy proves the presynaptic nature of dextran-filled boutons, synaptically coupled to abundant dendritic spines of Kenyon cells, forming patterns of microglomeruli in both calyx parts. Furthermore, immunocytochemistry shows that these different PN types are mainly if not exclusively cholinergic neurones.

MATERIAL AND METHODS

Approximately 90 adult crickets *(Gryllus bimaculatus)* of both sexes up to 7 days after their imaginal moult, from the institute's breeding colony (28 °C, 12h:12h light and dark cycle) were used for experimental dextran tracing. Fluorescent dextrans topically administered into the MB calyces or deutocerebral neuropils were allowed to diffuse for 1–3 hours in isolated brains kept in insect Ringer; thereafter brains were fixed overnight with 4% paraformaldehyde and cut into serial vibratome sections (diameter 50 μm). For double staining we used dextran tetramethylrhodamin, dextran fluorescein and dextran biotin, all 3000 MW (Molecular Probes, USA), the latter being processed in a second step with Cy2-conjugated streptavidin (Rockland, USA). For immunolabeling of vibratome sections an antibody against acetylcholine (Molecular Probes, USA, Code GE-1023) was employed. Electron microscopy of PNs labelled with dextran tetramethylrhodamin and biotin, was performed using the DAB-ABC method (Vectastain elite kit, Vector Lab., USA).

Fluorescent sections were viewed with a conventional fluorescent microscope (Axioskop, Zeiss). Images were taken with a CCD-camera (SPOT, Diagnostic Instruments Inc.) and edited with the corresponding and UTHSCSA Image Tool software. Additionally a laser scanning microscope (LSM 510 Zeiss) was employed. Electron microscopic images were captured by electron microscope Zeiss CEM902. Schematic drawings were produced using Corel Draw 8.0.

RESULTS

Fluorescent dextran staining revealed two main morphological types of deutocerebral PNs with different projection areas in the bipartite calyx, the only input region of these PNs in the mushroom bodies (Figs 1, 2 and 4). Separated somata clusters of both PN types are located in the dorso-anterior deutocerebrum (Figs 1, 6 and 7). From there PN axons either run towards the antennal (Fig. 6) or the glomerular lobe (Fig. 7) where their dendrites contact chemosensory neurones. PNs of the AL form

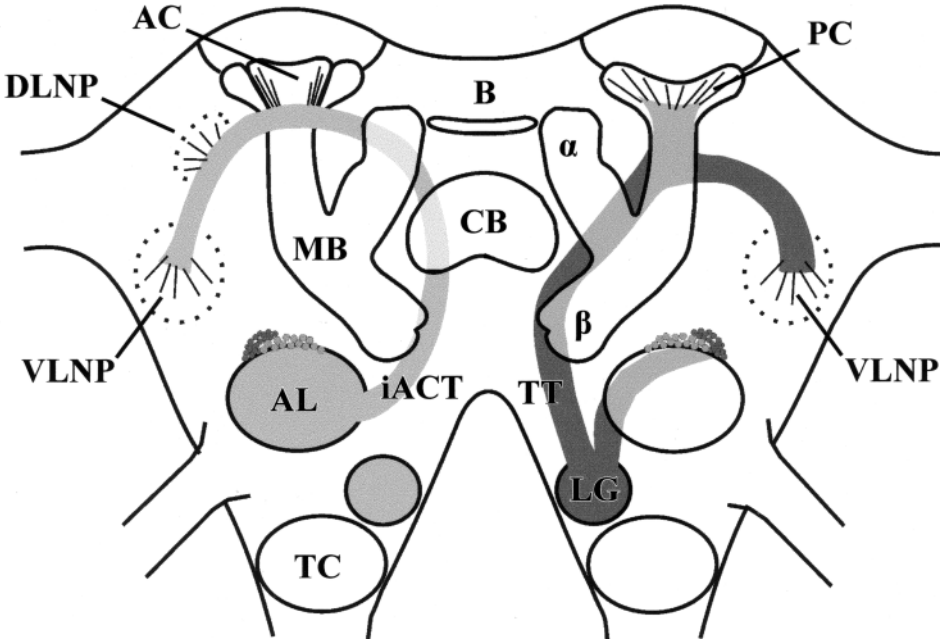
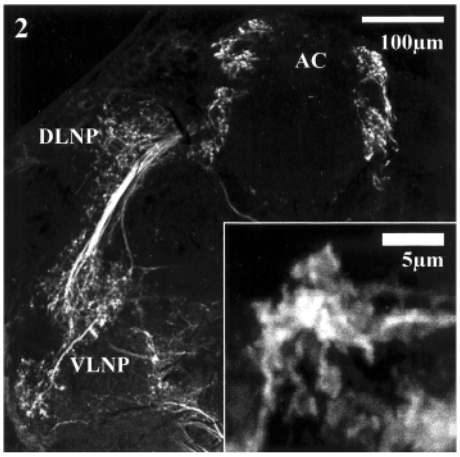
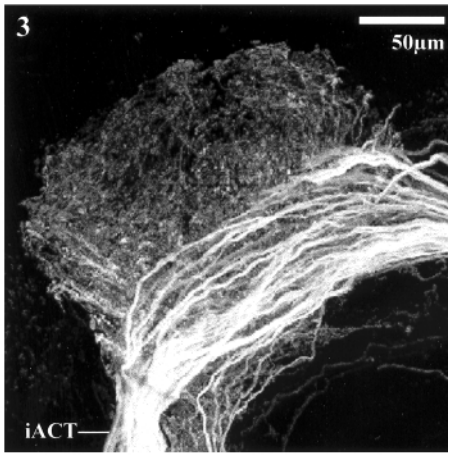
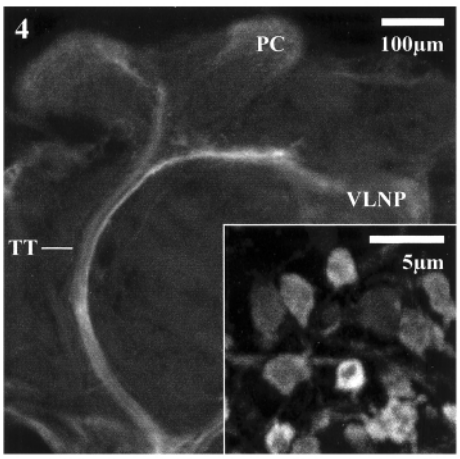
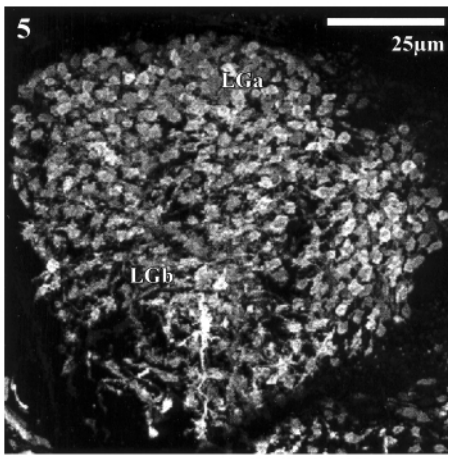


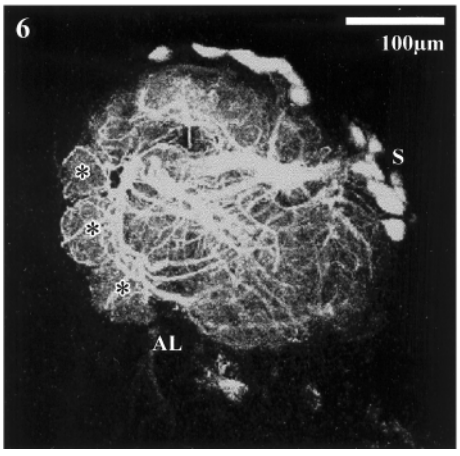
Fig. 1. Scheme of the cricket central brain
Abbreviations: AC = anterior calyx; AL = antennal lobe; α = alpha lobe; B = bridge; β = beta lobe; CB = central body; DLNP = dorso-lateral neuropil; iACT = inner antenno-cerebral tract; MB = mushroom bodies; LG = Lobus glomerulatus; PC = posterior calyx; TC = tritocerebrum; TT = tritocerebral tract; VLNP = ventro-lateral neuropil


2
100µm
AC
DLNP
5µm
VLNP


3
50µm
iACT


4
PC
100µm
VLNP
TT
5µm


5
25µm
LGa
LGb


6
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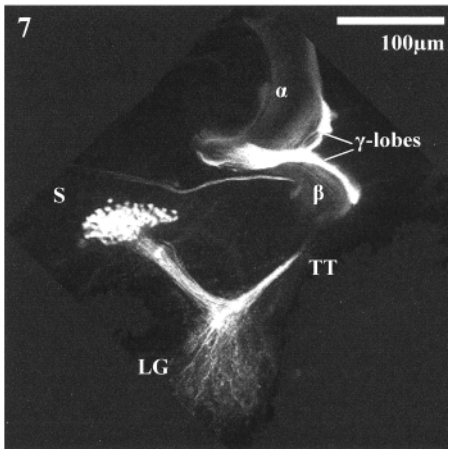
I
S
AL


7
100µm
α
γ-lobes
S
β
TT
LG

the inner antenno cerebral tract (iACT) to finally terminate in the lateral protocerebrum (Fig. 2). On their way axons send off collaterals to the periphery of the anterior calyx cup, to contact dendrites of Kenyon cells (KCs) (Fig. 3). PNs of the other so-called tritocerebral tract [7] either bend laterally to end in the lateral protocerebrum or run straight towards the mushroom body calyx where they contact KCs only in the posterior calyx (Figs 4 and 5). PNs of the LG joining the TT are of two types: type LGa forming spheroidal boutons (Fig. 4 inset) and type LGb forming irrregularly shaped, indented boutons within the posterior calyx. No separate clusters of LG PNs could be discriminated. The posterior calyx is clearly subdivided into a marginal outer and an inner neuropile region [9, 15]. The PN LGa exhibit a regular pattern of boutons in the marginal parts of the posterior calyx, while boutons of the PN LGb are restricted to its deeper inner parts (Fig. 5). The form of PN LGb boutons resembles the large PN iACT boutons (Fig. 2 inset) in the anterior calyx very much, the latter only to be stained from the AL or with its somata and dendrites from the anterior calyx. In dextran stainings of the calyces, boutons were consistently identified by electron microscopy as divergent presynaptic elements (Figs 8–11). They appear as central elements of microglomeruli surrounded by and synaptically coupled to abundant Kenyon cell dendritic spines, representing the majority or even the total of postsynaptic elements (Figs 9–11). There is evidence that in the marginal posterior calyx the ovoid PN LGa boutons may exclusively represent the central core of the microglomeruli, examined by electron microscopy of dextran labeled neurones (Fig. 11). ACH-immunostaining (Figs 12–13) reveals a pattern of boutons that matches the bouton pattern of PN dextran stainings within the anterior and posterior calyx. Thus both types of PNs appear cholinergic and coupled to KC dendrites within the calyces.

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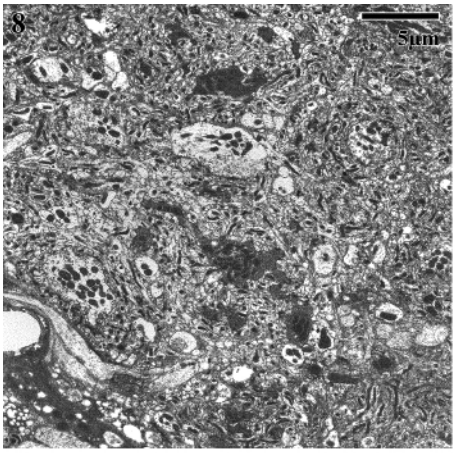
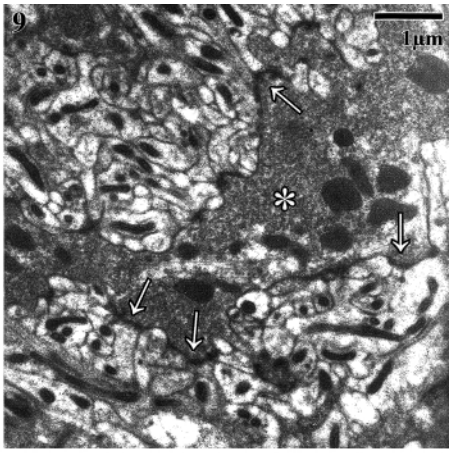
Figs 2, 3, 5–7. Laserscan images of projection neurons; *Fig. 4* conventional epi-fluorescent image of projection neurones: S somata, Sa somata of Kenyon cells of the anterior calyx, Sp somata of Kenyon cells of posterior calyx; other abbreviations see Fig. 1. – Fig. 2. iACT PNs project into outer rim of the cup shaped anterior calyx and into the dorsal and ventral part of the lateral protocerebrum. Inset: Irregularly shaped boutons of iACT PNs within the anterior calyx. – Fig. 3. IACT PNs send off collaterals on their way to the lateral protocerebrum to contact Kenyon cell dendrites of the anterior calyx. – Fig. 4. PNs of the glomerular lobe either contact the posterior calyx or run towards the lateral protocerebrum. Inset: ovoid LGa boutons. – Fig. 5. A regular pattern of LG PN boutons within the posterior calyx. The posterior calyx is divided into a marginal part with ovoid LGa boutons and in an interior part with indented LGb boutons. – Fig. 6. Staining of the antennal lobe reflecting its glomerular texture (asterisks) via dextran application into the anterior calyx. Somata of iACT PNs are clustered around the lobe. – Fig. 7. Dextran application onto the posterior calyx. Staining of the glomerular neuropil and somata of glomerular lobe PNs. Note selective heavy staining of KCs in the γ-lobe shaping the posterior calyx while the KCs of the α- and β-lobe remain unstained


8
5µm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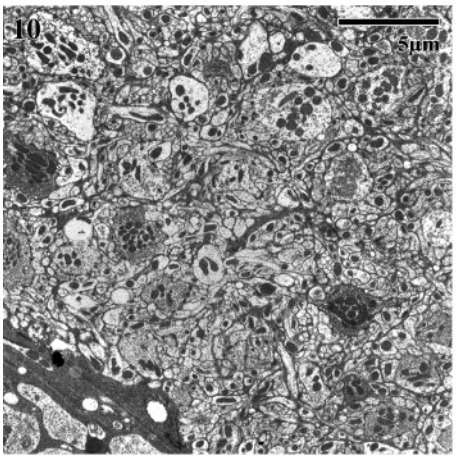
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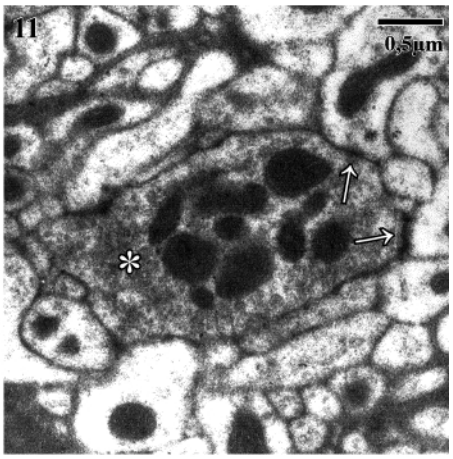
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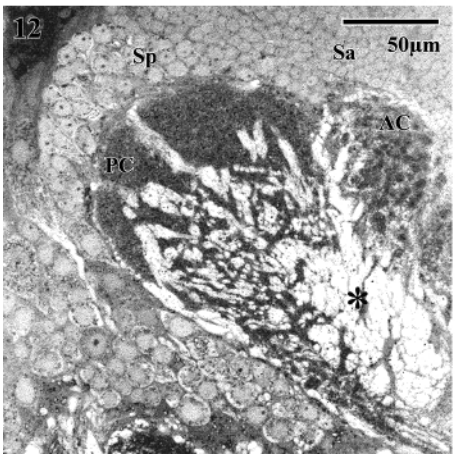
12
Sp
Sa
50µm
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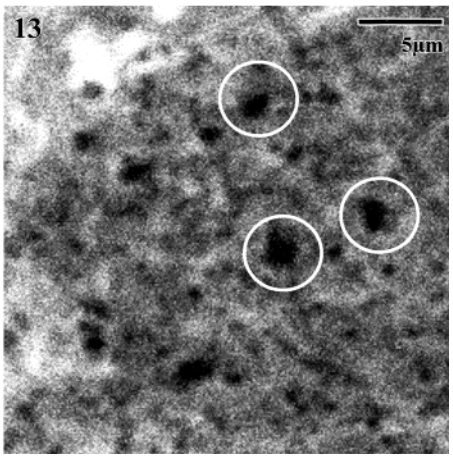
PC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DISCUSSION

The present morphological study presents evidence for a maintainance of separate sensory information conveyed from two primary sensory deutocerebral brain neuropils, the AL and LG, to the protocerebral second order neuropil of the MBs, into either the anterior or posterior part of the bipartite calyx in the cricket, via PNs in separate tracts, the iACT and TT. Morphological diversity of sensory glomerular deutocerebral neuropils and functional specialization has been shown for many groups of insects [1, 5]. This is in particular known for olfactory information processing in the spheroid glomeruli and the macroglomeruli of the antennal lobes.

Extensive structural separation of deutocerebral neuropils has especially been reported for orthopteran insects in both ensiferen and caeliferen groups [3, 7]. The AL receives primary olfactory input solely from the antennal nerve sensory neurons, whereas the LG obtains input from the maxillary palp sensory neurons, among them contact chemosensory receptors [2, 7]. These sensory neurons ascend from the SOG via the circumesophageal connectives to form uni-, bi- and multiglomerular terminals in the LG. The dendritic innervation patterns of the AL and LG by its PNs, selectively stained from the separate large MB calyx parts, fits to the size of the glomeruli. Moreover, we found small and large dendritic fields of PNs in the LG, but from methodological limitations we were not able to associate the dendrites to subtypes of the LG PNs. This finding of two types of PN dendrites is suggestive for supplying the LG subcompartments [7] receiving segregated sensory input from lower segments.

The bipartite organization of the orthopteran MB calyces has been shown previously [9, 15]. The anterior calyx contains large indented boutons, whereas the posterior calyx houses spheroid boutons exclusively in an outer marginal zone and indented boutons restricted to an inner neuropil zone, shown before by Golgi impregnations [9]. The topical dextran mass staining of PNs via the AL and/or LG confirms these previous findings and clearly proves the deutocerebral localization of two somata clusters of the PNs. Tritocerebral somata of ascending PNs were so far not found by dextran infusion into the MB posterior calyx. The contribution of tritocerebral and lower order segment neurones to calycal input [7] was not evaluated by our

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Figs. 8–13. Electron microscopy of dextran stained PNs within the calyces. – Fig. 8. Electron dense boutons in the anterior calyx. – Fig. 9. Anterior calyx; a presynaptic bouton (asterisk) contacting many postsynaptic Kenyon cell dendrites. Presynaptic active zone of PN boutons (arrows). – Fig. 10. A regular pattern of dextran stained and unstained glomerular lobe PN boutons within the posterior calyx. – Fig. 11. PN boutons constituting the centre of microglomeruli (asterisk); presynaptic active zones (arrows). – Fig. 12. Anti-ACh IR in the calyces, dark spots obtained by inversion of laser scan images. – Fig. 12. Within the immunoreactive neuropil of the anterior and posterior calyx tracts of KC axons remain unstained (asterisk). Somata of the KCs shaping the anterior calyx (Sa) are smaller than somata of the posterior calyx KCs (Sp). – Fig. 13. The ACh IR pattern (dark spots) resembling the bouton pattern of dextran stainings within the anterior and posterior calyx. Bouton immunoreactive dark spot in centres of microglomeruli are encircled

experiments. The dextran fillings of PNs do, however, show the preponderant and patterned distribution of their bouton types in the different MB calyx neuropils. Furthermore, the study gives additional support for suggesting a modal and submodal topical segregation of sensory input proposed for the primary brain neuropils of different species [6, 7, 14]. This segregation is proposed for the higher secondary MB neuropil, the calyces, the only MB input area for deutocerebral PNs. Modal integration of sensory input to the AL and LG takes place in these neuropils as shown by the response types of PNs [8], in the MBs and probably in the lateral protocerebral neuropil (third and terminal station of PN arborizations). The different KC types [10] can be expected to serve for a first multimodal integration of PN input types, because the latter synaptically converge to KC dendrites. We see the anterior calyx as a MB input station for olfactory information, and the posterior calyx mainly for gustatory information. The latter neuropil is the dendritic area of KCs forming the MB y-lobe [12].

The presynaptic nature of all bouton types has been deduced previously from conventional electron microscopy [10]. The dextran electron microscopy of deutocerebral PNs gives for the first time convincing evidence that their boutons form the prevailing elements of the central core of microglomeruli, consistently being presynaptic and coupled to abundant dendritic tips of KCs, also observed from experimental staining (Frambach and Schürmann, unpublished). Input sites to presynaptic boutons have been rarely encountered. This is consistent with the recent study on the giant cholinergic boutons in the fruit fly MB calyces [16]. For the cricket, the cholinergic nature of calyx boutons is shown by immunocytochemistry, and this pattern fits to the distribution and size of MB calyx bouton types. The minor extent of synaptic contribution of GABA, 5-HT, octopamine and of neuropeptide fibres to the microglomeruli of the cricket MB calyces [10] remains to be demonstrated. The understanding of the delicate neuropil compartmentalization needs further investigation using single cell staining, recording and other approaches. The separation of input to the cricket bipartite calyx offers favourable conditions to test the hypothesis of a segregated gustatory, olfactory and mechanosensory input by Ca-imaging, successfully used for PN boutons in the fruit fly calyces [4].

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