Tritocerebral tract input to the insect mushroom bodies

Sarah M. Farris*
Department of Biology, West Virginia University, 3139 Life Sciences Building, 53 Campus Drive, Morgantown, WV 26506, USA

ARTICLE INFO

Article history:
Received 19 February 2008
Accepted 21 May 2008

Keywords:
Antennocerebral tract
Calyx
Gustatory
Lobus glomerulatus
Mechanosensory
Pulp

ABSTRACT

Insect mushroom bodies, best known for their role in olfactory processing, also receive sensory input from other modalities. In crickets and grasshoppers, a tritocerebral tract containing afferents from palp mechanosensory and gustatory centers innervates the accessory calyx. The accessory calyx is uniquely composed of Class III Kenyon cells, and was shown by immunohistochemistry to be present sporadically across several insect orders. Neuronal tracers applied to the source of tritocerebral tract axons in several species of insects demonstrated that tritocerebral tract innervation of the mushroom bodies targeted the accessory calyx when present, the primary calyces when an accessory calyx was not present, or both. These results suggest that tritocerebral tract input to the mushroom bodies is likely ubiquitous, reflecting the importance of gustation for insect behavior. The scattered phylogenetic distribution of Class III Kenyon cells is also proposed to represent an example of generative homology, in which the developmental program for forming a structure is retained in all members of a lineage, but the program is not ‘run’ in all branches.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The insect mushroom bodies are higher brain centers best studied for their roles in sensory integration and in certain types of learning and memory (cricket, Schildberger, 1984; cockroach, Li and Strausfeld, 1997, 1999; honey bee, Menzel, 2001; fruit fly, Roman and Davis, 2001; Heisenberg, 2003). Both types of studies have identified the mushroom bodies as centers of particular importance for olfactory processing and discrimination, a finding that has been well-supported by physiological studies (locust, Perez-Orive et al., 2002; honey bee, Szyszka et al., 2005). Dedicated sensory input neuropil of the mushroom bodies, called calyces, receive massive input from primary olfactory centers called the antennal lobes via one or more antennocerebral tracts (ACTs) (house fly, Strausfeld, 1976; cockroach, Strausfeld and Li, 1999a; honey bee, Kirschner et al., 2006). A strong case can also be made for the adaptive significance of this neural pathway, as olfactory cues guide key aspects of insect behavior such as the location of food, mates and oviposition sites (reviewed in Christensen and Hildebrand, 1987; van der Goes van Naters and Carlson, 2006). Higher processing functions provided by the mushroom bodies, for example learning the content of particular cues or associating an array of cues in time and space, may be an important component of these olfactory-guided behaviors (Liu et al., 1999; Liu and Davis, 2006).

While olfactory inputs to the calyces are widespread among the insects and are therefore likely to have been present in a common ancestor, the influence of species-specific behavioral ecologies on calyx innervation becomes apparent when comparisons are made among insects with unique evolutionary specializations. For example, diminutive antennae and the reduction or loss of the entire antennal lobe – inner ACT – mushroom body calyx pathway is observed in notonectid Heteroptera, which have adopted a fully aquatic lifestyle devoid of airborne olfactory cues (Strausfeld et al., 1998). Another example occurs in the Hymenoptera, particularly in bees and wasps, in which the calyces receive visual input from the medulla and lobula of the optic lobes (Groenenberg, 2001; Groenenberg and Hölldobler, 1999). This is likely related to the important role that vision plays in learning the location of food sources and nest sites and even in identifying conspecifics in these insects (Menzel et al., 2005; Tibbetts, 2002). The association between mushroom body afferents and behavior is further supported by the finding that ant species that rely less on vision for the above tasks have a consequent reduction in visual input to the calyces (Groenenberg and Hölldobler, 1999).

Another important sensory modality for insects is contact chemoreception or gustation, which is used to determine the suitability of food sources and oviposition sites on contact (Justus and Mitchell, 1996; Chapman, 2003; Romani et al., 2005; van der Goes van Naters and Carlson, 2006). Mechanosensation is also of importance as it allows insects to gather information about texture that may be used to discriminate and learn about different foods (Kevan and Lane, 1985; Goyret and Raguso, 2006). Gustatory receptors are distributed in species-specific patterns across the
antennae and mouthparts and usually occupy sensilla that also contain mechanosensory receptors (Mitchell et al., 1999). These gustatory sensilla characteristically possess a single tip pore and a moveable base (Staudacher et al., 2005). In holometabolous insects, gustatory sensilla are typically found on parts of the maxillae and their palps, or on modified structures of the labium (such as the long tongue-like glossa in bees and the sponge-like labellum of flies) (Mitchell et al., 1999), and on the antennae (Haupt, 2004; Jorgensen et al., 2006). However gustatory sensilla are arranged peripherally, the receptor neurons of holometabolous species project to the subesophageal ganglion (SEG) and in some cases the tritocerebrum (TC), both of which appear to function as primary gustatory and mechanosensory centers in the Holometabola (Igeln and Hansson, 2005; Jorgensen et al., 2006; Mitchell et al., 1999).

Ascending pathways from centers receiving input from gustatory sensilla in the SEG and TC to the mushroom bodies have been described in just one holometabolous insect, the honey bee (Schroeter and Menzel, 2003). Neurons comprising the subesophageal-calycal tract (SCT) have cell bodies residing along the lateral edge of the SEG-TC junction, and dendritic fields that overlap with the termini of sensory receptor axons originating from the mouthparts (Rehder, 1989). The SCT runs first along the medial deutocerebrum and protocerebrum, where it appears nearly indistinguishable from the inner ACT (iACT) carrying olfactory projection neuron axons from the antennal lobes. At approximately the level of the mushroom body medial lobe, however, the SCT cuts away from the iACT, passes ventral to the calyces, and provides collaterals to mushroom body intrinsic neurons comprising distinct calycal subcompartments before terminating in the lateral protocerebrum.

As Schroeter and Menzel (2003) point out, a similar ascending pathway is well known from another group of insects, the hemimetabolous Orthoptera (crickets and grasshoppers). Comparative studies by Jawlowski (1954) and Weiss (1981) identified what was termed the tritocerebral tract (TT) emerging from a glomerular neuropil at the tritocerebral-deutocerebral border (the lobus glomerulatus or LG). The TT follows the exact trajectory of the SCT through the protocerebrum, but unlike in the honey bee where SCT collaterals target subcompartments of the primary calyces (that also receive visual and olfactory input), TT axons in the Orthoptera provide collaterals to a physically separate mushroom body neuropil termed the accessory calyx. More recent studies have verified this finding using neuronal tracing and immunohistochemistry techniques (Frambach and Schirrmann, 2004; Homborg et al., 2004).

Of greater interest to the present account are the cell populations in the mushroom bodies that are targeted by TT/SCT axons. Variation is already evident in the above comparison between orthopterans and bees, as the TT projects to a separate accessory calyx in the former, but to subcompartments of the primary calyces in the latter. Developmental analyses of accessory calyces in two species, the cricket Acheta domestica and the cockroach Periplaneta americana, reveal that the component intrinsic neurons (termed Class III Kenyon cells by Farris and Strausfeld (2003) are the first-born during embryonic development (Malaterre et al., 2002; Farris and Strausfeld, 2003). Aside from forming an accessory calyx, Class III Kenyon cells characteristically produce physically separate, delay line-like tracts that run alongside or wrap around the pedunculus and lobes (Malaterre et al., 2002; Farris and Strausfeld, 2003; Sjoholm et al., 2005).

In the Orthoptera where they are best studied, Class III Kenyon cells receive massive input from the LG via the TT (Frambach and Schirrmann, 2004). In the honey bee, in which Class III Kenyon cells have not been observed in the adult (Farris et al., 2004), TT axons instead provide collaterals to a subcompartment of the primary calyx, which is composed of Class II and Class I Kenyon cells (Schroeter and Menzel, 2003; Strausfeld, 2002). This suggests that when present, Class III Kenyon cells serve a specific function in gustatory processing, but that other cell populations in the mushroom bodies can receive this input modality as well.

The goal of the current study is to compare the distribution of TT/SCT inputs to the mushroom bodies across representatives of several insect orders. Using immunostaining and neuronal tract tracing, TT/SCT inputs are found to be ubiquitous but diverse, while accessory calyces and their constituent Class III Kenyon cells have a less uniform distribution.

2. Materials and methods

2.1. Insects

Thermobia domestica (Lepismatidae: Zygentoma) were reared in a laboratory incubator at 35 °C on a 24 h dark cycle. They were fed Meow Mix® cat food ad libitum and moisture was provided by pans of tap water placed at the bottom of the incubator. Periplaneta americana (Blattidae: Dictyoptera) and Acheta domestica (Gryllidae: Orthoptera) were reared in an incubator at 28 °C on a 12:12 light-dark cycle and fed cat food. Water was provided by soaking paper towels contained within plastic cups, that were replaced every week. Oncopeltus fasciatus (Lygaeidae: Hemiptera) were reared in an incubator at 28 °C on a 12:12 light-dark cycle and provided with raw, shelled sunflower seeds for food. Water was provided in a closed cup with a central wick. Tetrix sp. (Tettigidae: Orthoptera), Ceuthophilus sp. (Raphidophoridae: Orthoptera), Forficula auricularia (Forficulidae: Dermaptera), Onthophagus hecate and Maladera castanea (Scarabaeidae: Coleoptera) were captured live in the Morgantown, WV area and prepared within 24 h for neuronal filling. Tachycines asynamorhous (Raphidophoridae: Orthoptera) were collected in Chincoteague, VA and prepared within 48 h for immunostaining.

The following sample sizes were used for this study: Cason’s staining Thermobia first instar n = 8; Cason’s staining Thermobia adult n = 6; DC0/phalloidin staining Oncopeltus n = 6; DC0 staining/deutocerebrum fills Forficula n = 2; DC0/phalloidin staining Acheta n = 2; DC0/phalloidin staining Tachycines n = 2; DC0/phalloidin staining Periplaneta n = 2; antenial nerve fills Thermobia n = 10; deutocerebrum fills Thermobia n = 5; deutocerebrum fills Periplaneta n = 5; deutocerebral fills Tetrix n = 2; deutocerebral fills Ceuthophilus n = 2; deutocerebral fills Onthophagus n = 2; deutocerebral fills Maladera n = 5.

2.2. Fluorescent dye tracing of neuronal trajectories

Prior to TT/SCT filling with fluorescent dextran, insects were anesthetized on ice until movement ceased. Heads were then removed and the brain rapidly dissected under O’Shea–Adams saline (O’Shea and Adams, 1981). All fills were performed with Texas Red conjugated 3000 MW dextran (Molecular Probes, Inc. (Invitrogen), Eugene, OR). Two methods were used to introduce the dye into the brain. In the first, highly concentrated liquid dye (≥5% in distilled water) was loaded into a broken-ended glass electrode, of which the blunt end was attached to a tuberculin syringe via a piece of rubber tubing. The point of the electrode was placed in the desired fill site and the syringe plunger depressed, pressure-injecting the dye into the brain. The second method used a similarly broken electrode, but rather than loading with liquid dye, semi-hardened dye in the brain. In the first, highly concentrated liquid dye (≥5% in distilled water) was loaded into a broken-ended glass electrode, of which the blunt end was attached to a tuberculin syringe via a piece of rubber tubing. The point of the electrode was placed in the desired fill site and the syringe plunger depressed, pressure-injecting the dye into the brain. The second method used a similarly broken electrode, but rather than loading with liquid dye, semi-hardened dye into the brain. The second method used a similarly broken electrode, but rather than loading with liquid dye, semi-hardened
the TT/SCT. After the fluorescent dextran was placed in the desired location, the brain was incubated in vigorously swirling physiological saline on an orbital shaker for 2–4 h. The brain was then transferred to 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) and fixed at 4 °C at least overnight.

Backfills of the antennae were also performed in Thermodinia to identify the location of the putative antennal lobe. Antennae were trimmed approximately 0.5 mm from their base on Thermobia heads that had been fixed for at least 24 h at 4 °C in 4% paraformaldehyde. Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) or DiO (3,3'-dioctadecyloxycarbocyanine perchlorate) crystals (Molecular Probes (Invitrogen) Eugene, OR) were placed in the cut ends of the antennae and the heads returned to fixative for 1 week prior to dissection (McConnell et al., 1994).

2.3. Preparation and imaging of tracer-labeled tissue

Following fixation, dye-filled tissue was washed 3 × 10 min in PBS and then embedded in 7–8% agarose prior to vibratome sectioning at 70 μm, the only exception being that the small Thermobia brains were transferred directly to the next step as whole mounts, due to the ability of the confocal to penetrate through the tissue without sectioning. In the other species, sections were washed for several hours after sectioning in PBST (PBS containing 0.1% Triton X-100) and cleared for 1 h in 60% glycerol and 1 h in 80% glycerol, both diluted in PBS. Sections were mounted in 80% glycerol and coverslipped prior to viewing on an Olympus Fluoview 1000 confocal microscope. Brains that were filled with Dil or DiO were prepared for imaging in a similar manner, with the exception that washes in PBST were omitted to prevent leaching of the lipophilic dyes from the sections. Imaging of the tissue employed one or two lasers: the green HeNe laser to capture the Texas Red fluorophore localizing the dextran fill, and the multi-Argon laser to capture autofluorescence and allow localization of structural landmarks if they were not sufficiently visible using the green HeNe laser. Confocal stacks of whole vibratome sections were first collected using 2 μm (20× objective) and 1 μm (40× objective) optical sections. TIFF images were generated by merging one to three optical sections, and then processed for brightness, contrast and color balance using Adobe® Photoshop® 7.0 for Macintosh (Adobe Systems Incorporated, USA).

2.4. Fluorescent immunohistochemistry and Cason’s histology

The overall structure of the mushroom bodies in several species was illuminated using an anti-DC0 polyclonal antibody (a generous gift of Dr. Daniel Kalderon) that is directed against the catalytic subunit of Drosophila melanogaster protein kinase A. This protein is highly enriched in the fly mushroom bodies (Skoulakis et al., 1993), and anti-DC0 reliably labels all Kenyon cell subpopulations in the developing and adult mushroom bodies of insects (Farris, 2005a; Farris et al., 2004; Farris and Strausfeld, 2003). The anti-DC0 primary antiserum was used at a 1:1000 concentration and visualized using a Texas Red conjugated goat anti-rabbit secondary antiserum (Molecular Probes, Eugene, OR). Alexa 488 conjugated phalloidin was applied at a 1:500 concentration (Molecular Probes, Eugene, OR) to label filamentous actin, which is enriched in the microglomeruli formed by the spines of Kenyon cell dendrites in the calyces, and in the processes of newborn Kenyon cells during development (Farris et al., 2004; Frambah et al., 2004; Kurusu et al., 2002). Visualization of fluorescence double staining was performed as described above for dextran fills.

Cason’s stained brains of Thermodinia were prepared from Carnoy’s fixed, paraffin embedded tissue exactly as described in (Farris, 2005a), and images were collected using a light microscope.

3. Results

3.1. Distribution of Class III Kenyon cells across the insects

Brains of insects representing several taxa were stained using the anti-DC0 antibody and counterstained with fluorophore-tagged phalloidin to reveal Kenyon cell bodies and the calyx, pedunculus and lobe subcompartments (Fig. 1). Using the criteria for identification described by Farris 2005b-first-born neurons with peripherally located cell bodies, separate calyx neuropil, axons forming a tract external to the pedunculus and lobes-, Class III Kenyon cells were observed in the milkweed bug (Hemiptera, Fig. 1B, arrow) and an earwig (Dermaptera, Fig. 1C, arrow), and confirmed in a cricket, camel cricket (Orthoptera, Fig. 1D,E, arrows) and blattid cockroach (Fig. 1F, arrows). Cason’s stained brains of the apterygote firebrat Thermodinia (particularly in adult animals >10 mm body length) revealed dense, ovoid glomeruli in the ventral calyx (Fig. 1A, arrows). The structure and location of these neuropils was somewhat reminiscent of the small accessory calyces of the camel cricket and blattid cockroach (Fig. 1E,F; arrows). Developmental and anatomical studies of Thermodinia mushroom bodies, however, have suggested that they are composed of a single population of Kenyon cells that innervate all parts of the structure (Farris, 2005a). Based on this data it is unlikely that these ventral calyx glomeruli represent the separate dendritic arborizations of a distinct population of intrinsic neurons, as do Class III Kenyon cells in the accessory calyces of other insects. This is supported by histological preparations of mushroom bodies in newly hatched (Fig. 1 inset A1) and mid-development (Fig. 1 inset A2, 5–6 mm body length) Thermodinia, which failed to reveal disproportionately large ventral glomeruli that would be expected if the component cells, like Class III Kenyon cells, were born first in development followed by Kenyon cells that make up the remainder of the calyx.

Adding the immunostaining and histological data of the present study to a review of the mushroom body anatomy literature (Farris, 2005b) the occurrence of Class III Kenyon cells can be mapped onto a phylogenetic tree of the insect orders (Fig. 2). The distribution of this trait is scattered across the tree, and implies that Class III Kenyon cells have been lost and/or reacquired multiple times in the insects. Furthermore, innervation of the mushroom bodies by the SCT/TT is not always accompanied by the presence of Class III Kenyon cells.

3.2. Tritocerebral and antennocerebral tract inputs to the mushroom body calyces of a basal insect, the firebrat Thermodinia domestica

Fluorescent dextran placed into the medial deutocerebrum of adult Thermodinia labeled outputs from a glomerular AL and a glomerular LG to the mushroom bodies and lateral protocerebrum (Fig. 3A,B). AL outputs formed a recognizable iACT that passed over the calyx surface producing synaptic terminals en passant (Fig. 3B, arrowheads). Axons of the iACT continued laterally past the mushroom bodies and ended in a profusion of punctate terminals in the lateral protocerebrum (data not shown). Similarly, outputs from the LG formed a TT that cut a sharper angle to the calyx, passing ventrally beneath it and providing ramifications only to two ventral glomeruli of this neuropil (n = 2; Fig. 3B, arrows). TT axons also terminated in the lateral protocerebrum.

Histological preparations of Thermodinia brains showed the tritpartite organization of the deutocerebrum, composed of a dorsal glomerular AL, a striate ventral antennal mechanosensory and motor center (AMMC) reminiscent of the ventral flagellar area described in crickets (Staudacher and Schildberger, 1999/2000) and a small medial LG (Fig. 3C). Dil crystals placed in the cut end of the antennae reliably demonstrated that axons from this appendage...
innervated both the AL and the AMMC, but not the LG \((n = 10; \text{Fig. 3D})\). It is presumed that the LG is targeted by receptor afferents from the maxillary and/or labial palps, but repeated attempts at DiI and dextran application were unable to fill the nerves innervating these exceedingly slender and delicate appendages.

Additional fills of the *Thermobia* medial deutocerebrum confirmed the presence of non-overlapping inputs of the iACT and TT to the calyx. Axons of the iACT defasciculated at the medial margin of the calyx, wrapped the entire neuropil in fibers decorated with large, flocculent boutons \(\text{Fig. 3E, arrowhead}\), and converged at the lateral margin of the calyx where they continued into the lateral protocerebrum. A similar type of iACT projection neuron with large en passant synapses was observed in a pterygote insect, the earwig *Forficula auricularia*, in which the morphology of iACT projection neurons and their terminals was nearly identical to that of *Thermobia* \(\text{Fig. 3F, arrowheads}\).

The *Forficula* mushroom bodies displayed another feature in common with those of *Thermobia* in possessing innervation of a calyceal subcompartment by axons of the TT. In *Forficula*, however, TT axons targeted a distinct accessory calyx that lay entirely separate from the tripartite primary calyx \(\text{Fig. 1C, arrow}\). Dextran fills of both the iACT and TT in *Thermobia* revealed that TT axons projected to two glomeruli in the ventral portion of the same neuropil innervated by the iACT \(\text{Fig. 3B,E, arrows}\). These ventral glomeruli very likely correspond to the structures that stained densely with Cason's stain in the adult insect \(\text{Fig. 1A, arrows}\).

### 3.3. Tritocerebral tract inputs to accessory calyces of orthopterans and the dictyopteran *Periplaneta americana*

While not obvious from histological preparations, a recent study combining anti-DC0 immunostaining with Golgi impregnated...
material revealed that the mushroom bodies of the cockroach *Periplaneta americana* possess a small number of Class III Kenyon cells. In the Dictyoptera, Class III Kenyon cells are found only in the basal cockroaches and in the termites: they appear to have been lost in crown cockroaches (indicated by hatched circle). Some accounts report TT-like inputs to the accessory calyces (dotted circle; see text). Insect phylogenetic tree is adapted from Grimaldi and Engel, 2005.

3.4. Tritocerebral tract inputs to the primary calyces of scarab beetles

In holometabolous insects, Class III Kenyon cells have been observed in the Lepidoptera (Pieridae and Sphingidae) and the Neuroptera (Chrysopidae) (Ali, 1974; Farris, 2005b; Pearson, 1971; Sjöholm et al., 2005), but input to the mushroom bodies via the TT has been demonstrated only in the honey bee (Schröter and Menzel, 2003). Honey bees, however, do not possess Class III Kenyon cells as adults (Farris et al., 2004) and the TT (or SCT as it is called in this species) projects directly into subcompartments of the primary calyces (Schröter and Menzel, 2003). Scarab beetles also lack an accessory calyx or any other indication of Class III Kenyon cells (Larson et al., 2004; Farris and Roberts, 2005). Dextran fills applied to the medial deuto cerebrum of both *Onthophagus hecate* (Scarabaeidae; Fig. 5A,B) and the *Scarabaeinae* in the lateral protocerebrum after passing the mushroom bodies.
Fig. 3. Innervation of the calyx in the firebrat Thermobia by projection neurons of the inner antennocerebral tract and the tritocerebral tract. A. Fluorescent dextran applied to the medial deutocerebrum labels projection neurons in the inner antennocerebral tract (iACT) from the antennal lobe (AL) and in the tritocerebral tract (TT) from the lobus glomerulatus (LG). Axons in both tracts provide collaterals to the mushroom body calyx (Ca) and to the lateral protocerebrum (LPc; data not shown for iACT). Frontal section. B. Higher magnification view of TT axon collaterals in the calyx. Only two glomeruli in the ventral calyx received TT input (arrows), while axons from the iACT form en passant synapses over the remainder of the calyx (arrowheads). C, D. Frontal view of Cason’s staining (C) and DiI fills of the antenna reveal compartments in the deutocerebrum. A dorsal, glomerular antennal lobe (AL, glomeruli indicated by arrowheads) and a ventral, striate antennal mechanosensory and motor center (AMMC) are innervated by axons projecting from the antennal nerve (AN). The small lobus glomerulatus (LG) at the medial margin of the deutocerebrum does not receive antennal input. E. iACT and TT innervation of the calyx revealed by fluorescent dextran filling of the medial deutocerebrum. iACT axons pass along the dorsal and lateral surface of the calyx and innervate the neuropil with flocculent boutons (arrowhead). In contrast, TT terminals innervate just two compact glomeruli in the ventral calyx (arrows). Based on shape and location, it is likely that the two glomeruli innervated by TT axons correspond to the two large ventral glomeruli that are densely labeled by Cason’s staining (Fig. 1A). F. Frontal view of dextran-filled axons of the iACT in the earwig Forficula auricularia are strikingly similar to those observed in Thermobia, including the generation of large, flocculent boutons (arrowheads) by projection neurons as they pass over the calyx surface. Scale bars in A–C, E, F = 20 μm; D = 50 μm.
observed surrounding the TT in the protocerebral neuropil ventral to the calyces. Anti-DC0 immunostaining of generalist scarabs has not revealed a diffuse accessory calyx like that of *Periplaneta*, so it is unlikely that these latter terminals represent inputs to the mushroom bodies of *Maladera*. As in hemimetabolous species, the TT of both scarab species continues past the mushroom bodies to terminate in the lateral protocerebrum.

4. Discussion

The mushroom bodies of orthopteran species – crickets, grasshoppers and their kin – at first appear unusual among the insects due to the structure and innervation of their calyces. In these insects a primary calyx receives olfactory input via an inner antennocerebral tract (iACT) and an accessory calyx receives gustatory input via the tritocerebral tract (TT). Recent studies, including the present account, demonstrate that neither possession of an accessory calyx and its component neurons (Class III Kenyon cells), nor innervation by the TT is unique to the mushroom bodies of Orthoptera (*Farris and Strausfeld, 2003; Schröter and Menzel, 2003*). In addition to the Orthoptera, TT inputs to the mushroom bodies are now documented in three holometabolous species (the honey bee and two scarab beetles), two hemimetabolous species (a cockroach and an earwig) and one apterygote (the firebrat). In most insects TT axons provide collaterals to an accessory calyx when present, and to the primary calyx/pedunculus when an accessory calyx is lacking. Given the broad phylogenetic distribution of taxa surveyed, it is now plausible to conclude that input carried via the TT is a universal feature of insect mushroom bodies.

In the zygentoman *Thermobia*, TT projection neurons terminate in two ventrally positioned calyceal glomeruli that superficially resemble the small accessory calyces of *Periplaneta* and some orthopterans like *Ceuthophilus* and *Tachycines*. Unlike these latter species, however, *Thermobia* possesses just one Kenyon cell subpopulation (*Farris, 2005a*), which precludes the existence of a separate accessory calyx composed of Class III Kenyon cells. It is therefore likely that in *Thermobia* TT axons are innervating a dendritic subcompartment, restricted to the two dense ventral gomerali of the calyx, of the single morphological population of Kenyon cells that make up the mushroom bodies of this species. Similar patterns of sensory input are observed in the primary calyces of the cockroach *Periplaneta americana*, where afferent
Interestingly, expression of the maxillary palps of Drosophila has been lost in holometabolous species, perhaps having been fused with the antennal lobe in these insects (Anton and Homberg, 1999). The lobus glomerulatus (LG) therefore appears to have become an olfactory rather than a gustatory processing center in the fly (de Bruyne and Warr, 2005; Olsen et al., 2005; Schachtner et al., 2005). An LG is perhaps also present in the milkweed bug *Leucophaea maderae* and silverfish *Lepisma saccharina* (Rottman, 1957; Jorgensen et al., 2006). This latter study also filled and recorded from gustatory sensilla in the antennae and found the targets of their receptor axons to be in the TC and SEG as well. In orthopterans gustatory receptor neurons have been identified physiologically on the maxillary palp (Chapman and Ascoli-Christensen, 1999), and mass backfills of the palp nerve label axons targeting the SEG and/or LG (Johansson and Li, 1999b). Even in social Hymenoptera, such as the honey bee, different sources of visual input project to different levels of the dendritic tree in the same populations of Kenyon cells (Ehmer and Gronenberg, 2002). It is not known what the exact function of these input configurations may be, although it is likely that they provide a basis for integration of sensory inputs at the level of Kenyon cell dendrites.

The source of TT projection neurons in the Orthoptera is the lobus glomerulatus (LG), which receives first order gustatory and mechanosensory inputs from the palps (Ignell et al., 2000; Nishino et al., 2005). Gustatory sensilla of hemimetabolous insects are located on the mouthparts and on the antennae, and besides the LG target brain regions including the SEG and tritocerebrum, and the deutocerebral dorsal lobe and the protocerebrum (Ignell et al., 2000; Nishino et al., 2005). The LG has also been identified in the cockroaches *Diploptera punctata* and *Leucophaea maderae* (Blaberida: Dictyoptera; Chiang et al., 2001; Hofer et al., 2005) and in the silverfish *Lepisma saccharina* (Lepismatidae: Zygentoma; Hofer et al., 2005; Schachtner et al., 2005). An LG is perhaps also present in the milkweed bug *Oncopeltus fasciatus* according to Johansson (1957) and Pflugfelder (1936), who designated it part of the mechanosensory deutocerebrum but noted that it projects to an accessory calyx of the mushroom bodies. The LG appears to have been lost in holometabolous species, perhaps having been fused with the antennal lobe in these insects (Anton and Homberg, 1999). This hypothesis is supported by the fact that sensory neurons from the maxillary palps of *Drosophila* target a subset of glomeruli in the antennal lobes (Olsen et al., 2007). Interestingly, expression patterns of olfactory receptor genes and physiological responses of palp sensory neurons demonstrate that the maxillary palp functions in olfaction rather than gustation in the fly (de Bruyne and Warr, 2005; Olsen et al., 2007). The holometabolan LG therefore appears to not only have become merged with the antennal lobe, but to have become an olfactory rather than a gustatory processing neuropil. A schematic summarizing known ascending olfactory, gustatory and mechanosensory pathways to the mushroom bodies and surrounding protocerebrum, with emphasis on the hemimetabolan organization including an LG, is shown in Fig. 6.

Physiological recordings from TT projection neurons are not currently available, so it is only possible to infer their sensory modality by identifying the sensitivities of the receptor neurons that supply them. Gustatory receptor neurons are located within bimodal sensilla that also contain mechanosensory receptors (Staudacher et al., 2005). These sensilla can be differentiated from unimodal mechanosensory sensilla by the presence of a pore at the distal tip. Backfills of single gustatory sensilla or the appendages upon which they are typically found, the maxillae and labium and their palps, reveal receptor terminals in the tritocerebrum and subesophageal ganglion (SEG), the antennal lobe (AL), and in the hemimetabolous insects, the lobus glomerulatus (LG). Backfills of the labial nerve in the honey bee, which carries the axons of gustatory receptor neurons from the glossa, reveal a field of innervation overlapping the dendrites of TT/SCX neurons in the SEG (Schröter and Menzel, 2003). Similarly, gustatory receptor neurons displaying physiological responses to sucrose and with axons projecting to the TC and SEG were reported on the proboscis of the moth *Heliothis virescens* (Jorgensen et al., 2006). This latter study also filled and recorded from gustatory sensilla in the antennae and found the targets of their receptor axons to be in the TC and SEG as well.

As has previously been suggested for the honey bee, direct input from first order sensory neuropils serving gustation suggests that processing and learning and memory of this modality may be an important role for the insect mushroom bodies (Fahrbach, 2003; Schröter and Menzel, 2003). Mushroom body function has to date
been interpreted primarily with regard to olfaction (Perez-Orive et al., 2002; Syszka et al., 2005; Cassenaer and Laurent, 2007; Krashes et al., 2007; Lin et al., 2007). This study, combined with those demonstrating significant visual inputs to the mushroom bodies of the Hymenoptera (Gronenberg, 2001) and herbivorous scarab beetles (Farris, in press) and multimodal afferents to the pedunculus and lobes (Schildberger, 1984; Li and Strausfeld, 1997, 1999), emphasize the need for caution when interpreting the functions of these complex neuropils with regard to a single sensory modality such as olfaction.

Class III Kenyon cells and their associated structures are broadly distributed across the insects, but in contrast to the apparent ubiquity of TT innervation, this cell population is clearly absent in some species. Yet, where present, Class III Kenyon cells are strikingly similar in developmental origin and in morphology. The cells that supply the accessory calyx are the first-born mushroom body intrinsic neurons, as determined by BrdU incorporation studies or by the position of their cell bodies in the adult (Malaterre et al., 2002; Farris and Strausfeld, 2003). Class III Kenyon cells characteristically produce separate calyx and lobe systems, the latter composed of stout fibers that form delay line-like tracts devoid of synaptic specializations (Farris and Strausfeld, 2003; Sjöholm et al., 2005, 2006). With these features as well as input from the TT in common, it seems unlikely that Class III Kenyon cells arose independently in at least five separate lineages as indicated in Fig. 2. A potential explanation for this phylogenetic distribution that does not invoke multiple independent origins is that of generative homology (Butler and SaidaI, 2000, 2003). Generative homology posits that the underlying developmental program for producing Class III Kenyon cells is present in all insects, having been present in a common ancestor, and has been repeatedly turned on (or turned back off) in different lineages. For example, the presence or absence of Class III Kenyon cells could be determined by the expression of a small number of genes at the top of a regulatory cascade during mushroom body neurogenesis. This concept is reminiscent of the experimental manipulation of Hox genes to activate latent pathways for appendage formation in a novel location (Carroll et al., 2005). In lineages in which Class III Kenyon cells are present, their morphology and innervation would thus be remarkably similar because the first-order regulatory genes that have been switched on act through a conserved framework of downstream effectors for differentiation and maturation.

The hypothesis of generative homology also predicts that when the trait does appear, it will be present in a cluster of related species reflecting the activation of the developmental program in a common ancestor (Butler and SaidaI, 2003). This is exactly the case for Class III Kenyon cells that appear to characterize entire orders such as the Orthoptera and the Lepidoptera. The distribution of Class III cells in the Dictyoptera is particularly supportive of generative homology; they are present in basal cockroaches and in the sister group of cockroaches, the termites, but have been lost again in the crown cockroaches (Farris and Strausfeld, 2003).

4.1. Class III Kenyon cells and tritocerebral tracts in transition in Periplaneta

A curious “intermediate” condition of TT innervation is observed in the cockroach Periplaneta americana, which possesses just a small number of Class III Kenyon cells that produce only a diffuse accessory calyx. TT axons in this species provided collaterals to both the accessory calyx and the lower two-thirds of the primary calyces. This innervation pattern is particularly interesting in light of the fact that Periplaneta belongs to the basal blattid family of cockroaches, and that the Blaberidae and Blattellidae, i.e. crown cockroaches both seem to lack Class III Kenyon cells altogether (Farris and Strausfeld, 2003). Additionally, the termites possess Class III Kenyon cells, and recent phylogenetic reconstructions place this taxon as a sister group of the blattids (Inward et al., 2007). If Periplaneta truly represents an intermediate step in the loss of Class III Kenyon cells and the accessory calyx, it may be expected that all TT inputs would be displaced to the primary calyces in crown cockroaches. Unfortunately, many attempts to test this hypothesis for the present study were unsuccessful, as dextran fills to the deutocerebrum of blatellid and blaberid cockroaches consistently labeled so many descending neurons from the protocerebrum that TT collaterals in and around the mushroom bodies could not be clearly discerned.

A caveat of the interpretation that the TT provides two types of inputs to the Periplaneta mushroom bodies is that the morphology of TT collaterals into the primary calyces is very much like that of some antennal lobe projection neurons (Stausfeld and Li, 1999a). The identity of TT axons in the preparation pictured in Fig. 5B,D could not be determined due to mass dextran filling in the deutocerebrum that obscured the origination of ascending axons. The possibility can, therefore, not be ruled out that the TT collaterals in the primary calyces are produced by antennal projection neurons. This would be extremely unusual, however, as antennal lobe...
In insects lacking Class III Kenyon cells, TT collaterals terminate on Class I and Class II Kenyon cells in the primary calyx. In the honey bee, visual and olfactory afferents appear to target mostly separate populations of Kenyon cells (the collar and lip, respectively (Gronenberg, 2001; Strausfeld, 2002)). TT inputs innervate a tiny region near the junction of the calyx lip and collar (Schröter and Menzel, 2003), that appears to be composed of Kenyon cells that also extend into visual and olfactory input zones (for example, see Figs. 2E, 3K and 4C in Strausfeld (2002)).

If so, then gustatory information from the TT is integrated with other sensory input at the level of Kenyon cell dendrites. In contrast, TT input to Class III Kenyon cells, when present, remains in a separate processing stream at least until it reaches efferent neurons in the lobes. This latter arrangement would, therefore, serve to retain the unique identity of TT sensory cues through an additional layer of processing, although exactly how this would facilitate gustatory processing in insects possessing Class III Kenyon cells is unclear.

Another peculiar feature of Class III Kenyon cells is the separate, meandering tracts formed by their axons prior to inserting into the lobes proper. These tracts are reminiscent of a delay line that might serve to retard the transmission of sensory information carried by Class III cells to lobe efferent neurons, relative to olfactory and other cues carried in the remainder of the mushroom bodies. Delay line circuits are known in the vertebrate auditory system, where they encode interaural time differences to allow sound localization (Carr and Konishi, 1988; Hyson, 2005). In this example, however, the delay line allows comparisons of the same sensory modality so that its source may be determined; in the mushroom bodies, however, a delay line system provided by Class III Kenyon cells would be comparing two different modalities (or perhaps even three, if mechanosensory information is also being delivered by Class III Kenyon cells). A food source may be successfully localized using olfaction alone, however, gustatory cues would be received by mushroom body efferents once the food source was reached. It thus seems that a circuit for encoding the timing of a gustatory cue relative to an olfactory cue for the purpose of localizing the source of both cues would be unwieldy and redundant.

A cueing receiver is an activity indicator that focuses subsequent levels of processing on relevant features of an input (Tsui, 1986). In this device, one input stream prenews the next processing circuit, while a delay line holds a second input stream. When the second stream exits the delay line, it feeds into the circuit that was preneted by the direct input stream. Thus, the delayed input is processed in the context of direct input received at the same time. A cueing receiver-like function for Class I/Class II vs. Class III processing streams is attractive, since the direct stream does not need to be of the same modality as the delayed stream in order to impact the next level of computation. Perhaps Class III Kenyon cells allow gustatory information to be modulated in a novel way by simultaneously received olfactory input. Again, it is not immediately clear why this arrangement would prove particularly adaptive to orthopterans, earwigs, bugs and moths, but dispensable for other insect species.

4.3. Insight into the ur-mushroom body

The apterygote Thermobia domestica, which as a member of the Zygentoma represents the most basal living lineage of insects with mushroom bodies (Hanström, 1940; Strausfeld et al., 1998), was recently shown to be in clear possession of a calyx supplied by the progeny of two small groups of neuroblasts per hemisphere (Farris, 2005a). The current study emphasizes the similarity between the Thermobia calyx and that of more recently derived species, all of which receive input from both the antennal lobes (AL) and the TT. Based on the basal phylogenetic position and relatively un-specialized behavioral ecology of the Zygentoma, it is possible that the olfactory and gustatory processing pathways observed in modern zygentomans closely resemble those of the earliest insects.

Electron microscopical studies of the antennae of zygentomans and of the most basal living insect, the Archaeognatha, reveal the presence of multiporous sensilla, which are characteristic of olfactory sensilla (de Bruyne et al., 1999; Berg and Schmidt, 1997). This supports the findings of this study that zygentomans such as Thermobia can detect and process information about airborne odorants, and strongly suggests that archaeognathans can do so as well, although these insects have been reported to lack mushroom bodies (Hanström, 1940; Strausfeld et al., 1998). Further study is needed to determine the presence or absence of chemosensory pathways in the archaeognathans, but the present results for Thermobia strongly suggest that olfactory and gustatory processing is an ancient functional role that was present in the first insect mushroom bodies.

Acknowledgements

Dr. Erich Staudacher provided comments, corrections and references that greatly improved this manuscript. I would also like to thank Kelly Baldwin B.S., for the immunostained preparation of Oncopeltus fasciatus, Todd Steucke M.S. for collecting Tachycines asynamorous specimens, and Dr. Daniel Kalderon for providing the anti-DC0 antibody.

References


Hanström, B., 1940. Insektorisk Ortegare, Sinnesorganer och Nervsystemet des Kopfes einiger niederer Insektenordnungen. Kungl Svenska Vetenskaps Akademiens Handlingar 18, 1–266.


