Chapter 5

Concluding Remarks

The purpose of this study was to elucidate the olfactory structures of the locust, *Schistocerca americana*. Three broad classes of cells in three successive relay stations of the olfactory system were presented in anatomical detail and related to brain structures: projection neurons of the antennal lobe, Kenyon cells receiving input in the mushroom bodies, and extrinsic cells receiving the output of, and providing feedback to, the mushroom bodies.

5.1 New Methods of Analysis and Data Presentation

The processing and analysis of the majority of the data presented in this thesis was aided greatly by the development of MATLAB scripts. This process reduced the time required to isolate filled fluorescent neurons from noisy background structures within image stacks that were often comprised of 300 or more images, enabling the analysis of over 150 individual neurons in detail.

The isolated cell images were then combined with manually traced brain structures to produce three dimensional reconstructions in order to better present the anatomical data visually. This is important because brain structures are complicated and hard to decipher, even in 2-dimensional schematics by individuals not accustomed to them. This way of showing the data makes it more accessible to others without the requirement of years of experience with anatomical data from insects. Hopefully, this

will enable easier functional studies of identified cells and structures.

5.2 Summary of Results

Three sets of cells are described in this thesis with relation to brain structures as well as to each other. The projection neurons are shown with respect to the antennal lobe, as well as the calyx of the mushroom body and the lateral horn. Specifically, PNs are shown to have overlapping glomeruli, providing specific evidence that each of the 1000 glomeruli are the site of multiple PN innervation (figure 5.1). In 65 fills, including individual cells and cells filled concurrently — each PN's processes are shown to populate the AL as a pinwheel structure. These pinwheels lie in parallel planes throughout, with varying spoke radii within and across planes.

In the calyx, PNs are shown to innervate one specific division out of four total calycal divisions. This innervation of the outer-calyx is seen in 15 total calycal fills. Each PN's axonal arbor is seen to effectively cover the entire OC in all three dimensions. The PN axon terminals in the lateral horn are shown to be restricted to a physically separable area: the lateral horn lobe.

The calyx of the MB is shown here to have four zones, rather than the two typically described in the literature, the primary and accessory calyces. Specifically, the primary calyx is shown to be divided into three regions based on physical separability and differential innervation by specific neurons.

This thesis defines KCs into three different classes based on the zones of the calyx they innervate. Cells are defined as innervating the outer-calyx only (OC-exclusive), both the outer- and mid-calyces (OC/MC), or both the outer- and inner-calyces (OC/IC). Depending on the inputs to those regions, these KC morphological types may comprise functional types as well (figure 5.2). KC axons are also shown to fall into two different morphological types (spiny and smooth) which segregate to different parts within the β lobe. This β lobe segregation itself may be important because a class of extrinsic cells are found that innervate the spiny division preferentially (figure 5.3).

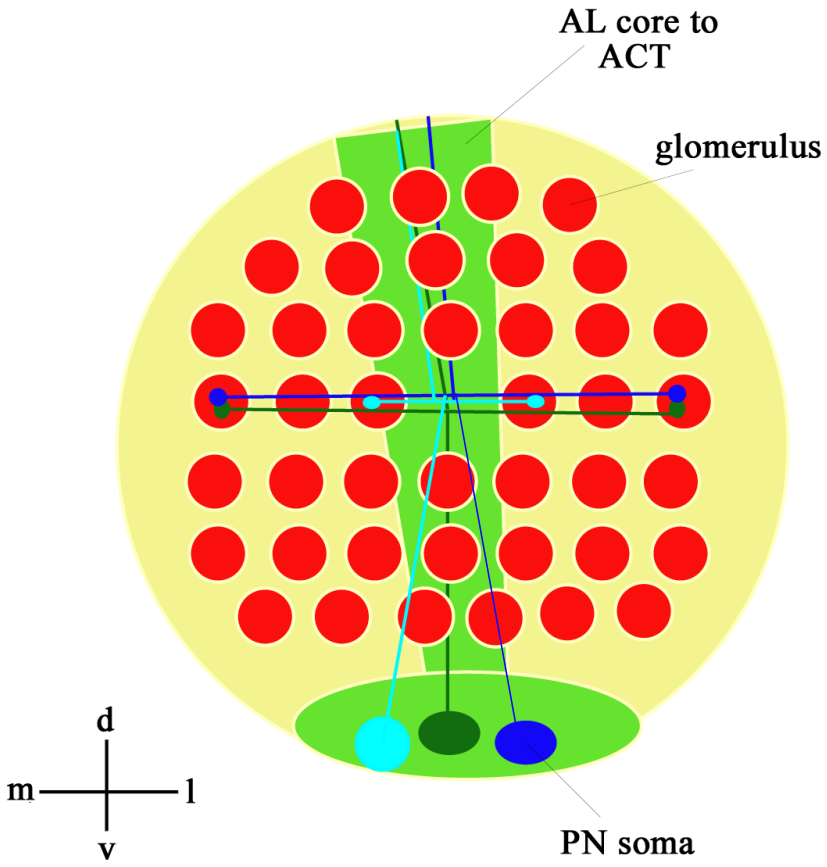


Figure 5.1: Schematic of the locust antennal lobe showing innervation pattern in one plane. Dark blue and green cells innervate the same glomeruli and lie within the same plane. Light blue cell lies in the plane with the other two cells, but innervates glomeruli at a different radius.

In all, two classes of mushroom body extrinsic cells are described in detail. Class I cells are shown to specifically feed into the LH lobe where PN axons terminate. CI cell co-localization with NADPH diaphorase immunoreactive zones is also shown in the peduncular heel, suggesting a preference of this cell for KCs expressing this neuromodulator. It is postulated that CI cells may be comparing unprocessed PN olfactory information from the LH, and processed olfactory information from the KCs in the β lobe, and then modulating KC output via connections in the α lobe and pedunculus.

Class II extrinsic cells are determined to be a group of 9 to 12 cells with somata

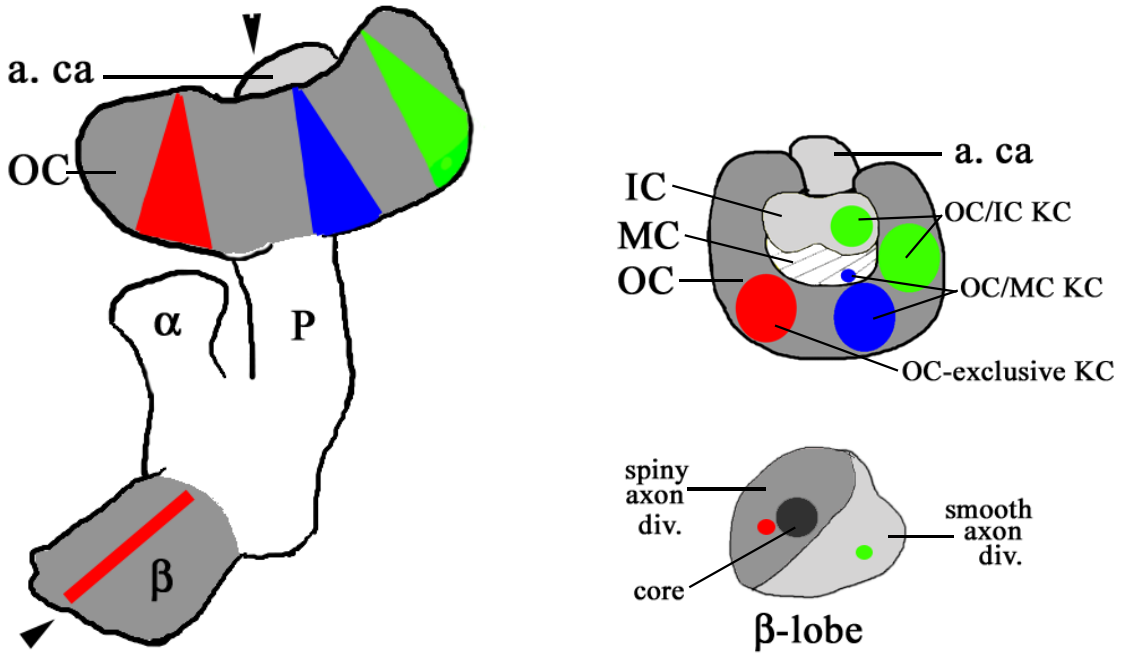
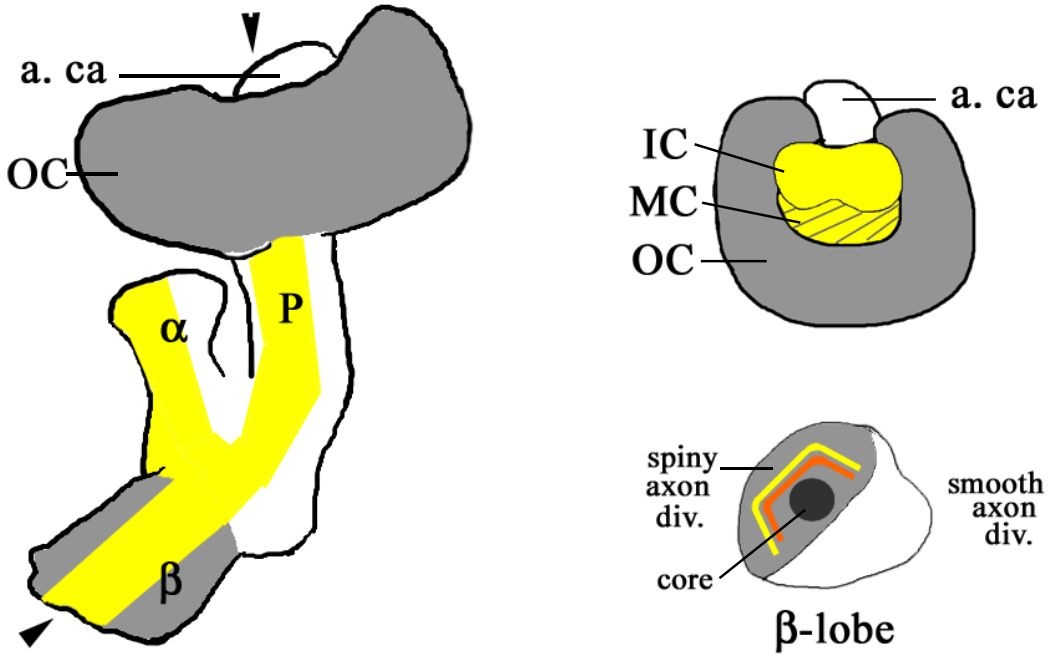


Figure 5.2: Schematics of the proposed structure of the MB and its KC innervation. (**a**) Frontal view of the calyx, showing the pattern of innervation of an OC-exclusive cell (red), an OC/MC cell (blue) and an OC/IC cell (green). It is possible that OC-exclusive cells innervate the spiny axon division of the β lobe (shown in gray). (**b**) Two more views of the MB; top schematic is the calyx viewed from above (topmost arrow in part [a]), showing representative innervation of the three types of KCs. Bottom schematic is a view of the β lobe (bottom arrow in part[a]) showing the spiny axon and smooth axon divisions within the lobe. It is possible that OC/IC cells innervate the smooth axon division of the β lobe. Note also the core of the spiny axon division, as described in chapter 3.

deep within the lateral protocerebrum. The somata are shown to be co-localized with a group of cells that show positive GABA immunoreactivity. CII cells innervate the spiny division of the β lobe, suggesting that morphologically different KCs may be functionally different as well. CII cells are most likely modulatory cells, receiving input from the KCs in the lobes and modulating KCs with connections to them in the pedunculus and calyx.



(**a**) (**b**)

Figure 5.3: Schematics of the proposed structure of the MB and its class II extrinsic cell innervation. (**a**) Frontal view of the MB, with the areas of CII innervation shown in yellow. (**b**) Two more views of the MB; top schematic is the calyx viewed from above (topmost arrow in part [a]), showing representative innervation of the CII extrinsic cells. Bottom schematic is a view of the β lobe (bottom arrow in part[a]), showing the spiny axon and smooth axon divisions within the lobe. CII extrinsic cells innervate the lobe in a characteristic 'C' shape around the spiny axon division's core, in slightly offset areas. Representative of one cell is shown in yellow while another is shown in orange. There are postulated to be 9 to 12 CII cells covering the spiny axon division.

5.3 Discussion

In the locust, the primary calyx is divided into three regions based on physical separability and differential innervation by specific cells. Weiss [101] described orthopteran primary calyces as having three zones, but defined two of those zones (the *zona interna* and the central zone) as areas devoid of KC dendrites. Specifically, he described the *zona interna* as a region of KC primary neurites on their way to branch in the calyces, and the central zone as the region of KC axons on their way to the pedunculus. Here, I've described a new division of the primary calyx — the inner-calyx

(IC) — which can been seen as a physically separable zone of the primary calyx and which does house KC dendritic branches (figure 5.2). I've also re-described Weiss' central zone as the mid-calyx, containing not only passer KC axons but also areas of synaptic specializations. This was possibly missed by Weiss due to the small and stubby nature of the dendritic specializations of the OC/MC cells. In this account, the area defined by Weiss as the *zona interna* has been included as part of the soma layer.

The calycal divisions and KC types described here have possible correlates in the cricket, another orthopteran order member. Schurmann [78] has described three types of KCs based on dendritic form, size, and position of axons. One particular class of cells — the KII cells — are reminiscent of locust OC/IC cells described here. KII cells are shown to occupy a similar region within the calyx interior (surrounding the anterior end of the pedunculus), and their axons descent down the core of the pedunculus similar to OC/IC cells (figure 5.2). Schurmann's KIII cells closely resemble some portion of OC-exclusive cells shown here, in their axonal innervation of the pedunculus and lobes. In cricket, these KCs have spiny axons that course through the periphery of the pedunculus, and end in the medial portion of the β lobe. In the locust, some OC-exclusive cells are seen to occupy the peripheral peduncular fibers and the medial portion of the β lobe - the spiny-axon division - with their highly branching axons. One large difference, however, seems to be that, in cricket, KIII cells are KCs of the accessory calyx, while in the locust, accessory calyx KCs probably innervate the smooth division of the β lobe.

In other well-studied insects, especially the honeybee and cockroach, calyces are shown to have physically separable divisions that show differential innervation by the input neurons, KCs, and extrinsic neurons. The locust too has clearly separable calycal regions: by afferent input (PNs innervate the OC and not the MC or IC), by KC innervation (IC-specialized and MC-specialized branches on some KCs), and extrinsic cell innervation (CII cells innervate the IC and MC). The locust OC is most likely a purely olfactory neuropil and reminiscent of the lip region in honeybee, which has been shown to be purely olfactory [96] [36]. In cockroach, four zones exist in

the calyx. Zone II is a relatively thin central zone and receives visual information [93] [64] [81]. In locust, the mid-calyx has physical resemblance to this zone. Further anatomical investigation should explore whether there are indeed visual inputs to this or other locust calycal zones.

In honeybee, cockroach and fly, stratification in the pedunculi have been demonstrated. Though separate fibers are seen in the locust pedunculus, it is unclear how different types of KCs translate into those fibers. OC-exclusive and OC/MC cells project through all peduncular fibers and only the OC/IC cells show a preference for a particular fiber (the core fiber). However, there is probably functional significance to peduncular arrangement in locust. If viewed as a tube, the peripheral KC axons are spiny and the core axons are smooth (figure 5.4). CII extrinsic cells innervate the pedunculus heavily, but seem to be confined to the periphery, suggesting that the spiny axons in the peduncular periphery are modified to specifically contact these and perhaps other peripheral extrinsic cells. Furthermore, the β lobe itself is divided into two regions: one receiving spiny axons and one receiving smooth axons. It is likely that spiny axons within the peduncular periphery travel to the spiny axon division of the β lobe, while the pedunculus central/core fibers travel to the smooth axon division. However, this probably does not exclude "switching" of the fibers from spiny to smooth or vice versa, as they transition from pedunculus to β lobe.

The β lobe divisions shown here in the locust also have specific correlates in the other insects, especially within *Drosophila*, where two major types of KC axons exist — the unbranched and the highly branched. These axons are segregated in *Drosophila* into the β and β' lobes (highly branched axons) and the β'' lobe (unbranched axons) [89]. These lobes are analogous to the spiny axon and smooth axon divisions described in the locust β lobe, respectively. It may well be the case that the locust spiny axon β lobe division is itself further divided into two regions based on KC axons, as a 'hollow' core is seen to exist that physically divides this portion of the lobe. Immunocytochemical studies, as well as fills of KCs down to the lobes will need to determine if in fact there are distinguishable KCs within the spiny beta lobe division.

The β lobe divisions in the locust may have correlates within the α lobe. The

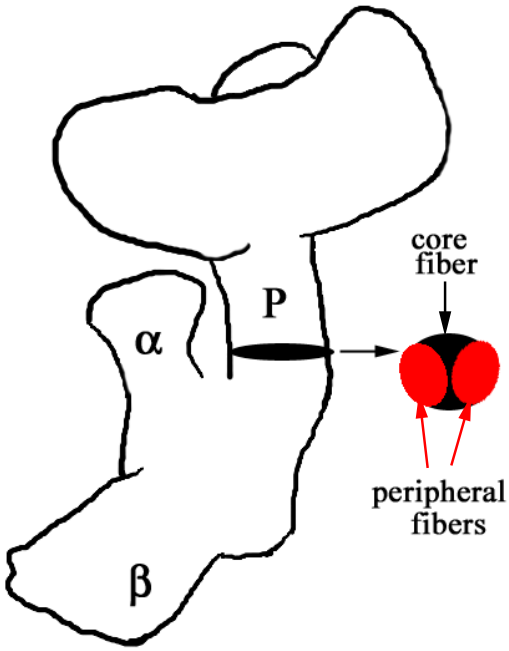


Figure 5.4: Schematics of the MB showing the proposed structure of the peduncular fibers.

medial side of the α lobe seems to contain the axon collaterals of KCs innervating the smooth division of the β lobe. CII extrinsic cells also follow this division. As shown in figure 5.3, these cells only innervate the spiny axon division of both β and α lobes (shown in yellow). The light grey region in figure 5.5 indicates the smooth axon region of the α lobe.

Extrinsic cells of orthopterans (crickets) were described in some detail by Schildberger [77]. In his account, Schildberger describes a number of extrinsic cells that innervate the calyces and MB lobes. However, neither of the extrinsic cells described here were described by him in cricket. This could be due to species-specific differences, or to that fact that the only extrinsic cells studied here were required to be responsive to many odors (see chapter 3). It is unclear if any/all of the extrinsic cells looked at in previous accounts were responsive to odorants.

In mammalian brain, it has been proposed that divergent inputs to different cortical areas from the olfactory bulb allow parallel and differential processing of olfactory receptor inputs [106]. In the locust olfactory system, PNs output to the OC (broadly)

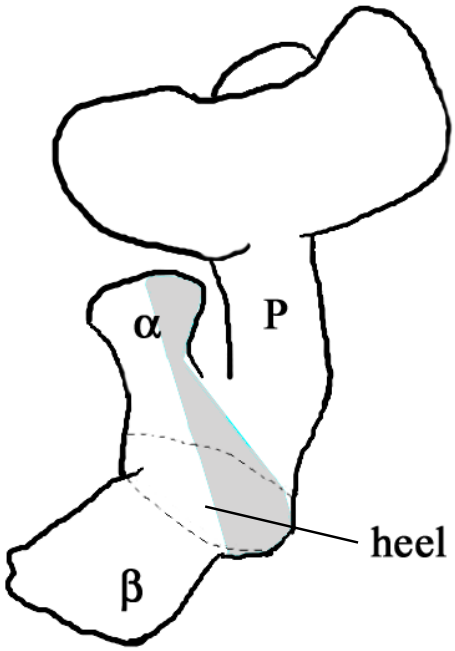


Figure 5.5: Frontal schematic of the MB showing the region (in gray) of the α lobe that contains the axon collaterals of KCs which innervate the smooth axon division of the β lobe.

and to the lateral horn lobe. It has been shown here that CI extrinsic cells, specifically, seem to receive processed olfactory inputs of PNs via KCs in the β lobe and direct PN inputs in the lateral horn lobe. These cells are a prime candidate for differential processing of olfactory information from the AL and the MB calyx. It remains to be seen if their effects are indeed modulatory on KC outputs in the α lobe and pedunculus, as suggested here.

Analysis of most types of cells described — PNs, KCs, and CII extrinsic cells — shows that different classes of cells of the locust olfactory system occur as repeated and morphologically similar units that occupy slightly offset regions of the structures they innervate. From one stereotaxic coordinate to another, the cells display a gradual morphological shift. Two cells of a structure that are relatively far apart may look morphologically dissimilar, but usually an intermediate cell can be found in an intermediate position between the two.

In discussing the origin of lamina within insect MB lobes, Strausfeld states:

> If extrinsic neurons would substantially contribute to one or another lamina [within the MB]...then many identical dendritic trees would be required to provide an isomorphic structure (lamina) extending from the calyces to the distal ends of the lobes. This is not the case.[90]

In locust, however, this does seem to be the case for CII extrinsic cells. If, in fact, these cells exist at offset positions, as shown here for a pair of them, then 12 CII cells could cover the entire spiny-division of the MB β lobe, as well as the pedunculus and calyces, with their isomorphic trees (figure 5.3). Evidence from cobalt fills of these cells also shows a pair of CII cells that innervate relatively thin mediolateral planes the OC (Stijn Cassenaer, personal communication). If CII extrinsic cells are GABA-ergic cells as suggested here, then GABA immunocytochemistry should reveal GABA-ergic lamina within the lobes, and perhaps the calyx.

In general, the structure of the locust MB is striking in its bilateral symmetry. The IC regions are certainly a clear demonstration of a repeated structural unit. Also, the peripheral peduncular fibers are symmetric across the anteroposterior axis (from the frontal view). Though locust (and orthopterans in general) have been defined as possessing a single undifferentiated calyx, it is increasingly clear that the calyces of the locust are split across the midline; this perhaps is an example of an early modification that led to the fully separated calyces of evolutionarily newer species, like honeybee and cockroach, and in contrast to the fruitfly calyx which has been suggested a fused calyx structure.

5.4 Future Directions

Further studies are required for examination of how function is associated with the new divisions and cells described here.

Recording of KC responses to odors and other stimuli coupled to dye injection will clarify whether KCs innervating different calyx zones are functionally different. Other sources of innervation of the calyx divisions also need to be further delineated. Experiments that use stimulation of the β lobe will determine if there is feedback

from the extrinsic cells to the calyx for modulation of KC output, or if extrinsic cell innervation of the calyx is purely dendritic. Further examination and identification of other extrinsic cells is also necessary. Coupled recording of PNs and extrinsic cells is required to determine their relationship within the lateral horn lobe: Are PNs outputting to extrinsic cells, and if so, for which cell classes does this hold? Finally, anatomical studies of cells innervating the lateral horn lobe and the α lobe will help identify the next relay stations of the locust olfactory system, and eventually close the circuit by identifying sources of motor output - the behavioral response of the animal to the odorant.