Chapter 1

Introduction

1.1 Studying the Sensory Nervous System

To interact with its environment, an organism needs a mechanism with which to gather information about its environment, process that information according to its needs and experiences, and then act appropriately. The essence of sensory processing in the central nervous system is ultimately dependent on a close interplay of the anatomy, biophysical properties, and connectivity of the cells which encode the information. This translates into four principal questions: *what* conveys the information; *where* is that information sent; *what* information does each downstream relay actually receive; and *how* is that information used and processed? More specifically, it is important to consider not only the output of particular neurons to a stimulus, but also the integrative properties of the receiver/decoder cell(s) receiving that information - the elements of the output that are relevant to the nature of the neuronal "code."

To assist in this search for coding principles, any functional study of the nervous system requires identification and close examination of circuits. First, one must identify *which* cells to study; sometimes, neuroanatomy can help in identifying function as links are found to already studied pathways. The present account endeavors to further define circuits in the olfactory system of the locust, *Schistocerca americana*, which has been studied in great detail for its biophysical and computational properties in odor encoding. This thesis presents a way in which to isolate and view cells and surrounding brain structure in three-dimensions, making the anatomy of these

intricate cells more accessible. Moreover, using these data, this thesis draws parallels and demonstrates similarities between this system and other insect olfactory systems which are ideal for alternate methods of functional investigation. It is shown here that the locust is, indeed, more similar to those other insects than previously thought, and therefore, the conclusions made about the way it encodes and processes olfactory information are more likely to generalize to other organisms.

1.2 Olfaction: A General Survey

Chemosensation is perhaps the oldest sensory modality [92]. Most organisms possess some means by which to sample the chemical world around them, using any number of specialized chemosensory organs. Olfaction is a special mode of chemosensation, referring to the detection of chemical signals originating from a source that is physically removed from the sensory organ. This is in contrast to the gustatory system which requires physical contact with the chemical source [71].

Airborne odorants are complex stimuli and can be monomolecular or a mixture of different molecules, sometimes totaling several hundred [39] [63]. These compounds are small — ranging from 26 Da to 300 Da — and volatile [63]. Over 40,000 molecules and compounds are identified as sources of odor as perceived by humans, and probably many more compounds are odorous to those animals whose olfactory systems are more sensitive than ours.

In humans, olfaction is often thought of as an esthetic sense, in contrast to its crucial survival and reproductive roles for most other animals, mammals and invertebrates alike. In many organisms, olfactory cues function to guide emotional responses like fear and anger, physiological regulation like menstruation, as well as mate finding and social interaction between conspecifics [71]. Chemical signals also help in learning, memory formation, and recall [11].

Due to the fact that identifying an odor involves both the binding together of information about many different molecules as well as their relative concentrations within a mixture, the encoding of olfactory information is a complex task [38]. Fur-

thermore, the absolute concentration of an airborne odorant can sometimes alter how it is perceived; the same odorant can be perceived completely differently based on whether it is encountered at a high concentration or a low one [49]. Moreover, at some level in the central nervous system, these odor identities and concentrations have to become associated with other stimuli to be able to effect behavior. The encoding of information by olfactory systems is a fascinating computational problem.

In recent years, the scientific study of olfaction has become interesting to many neurobiologists, not only for its own sake, but also for the light it sheds on information encoding within other sensory systems. Indeed, information processing in neurons of different systems — even across different animal phyla — shares many common principles, often reflected by similar anatomical features [71] [11]. In general, studying sensory systems in mammals — olfactory or otherwise — poses some problems. Mammalian brains are extremely intricate; they contain many cells and even more connections between those cells. Even the simplest mammals exhibit a large variety of complex behaviors. Insects, however, have smaller brains and exhibit less complex behavior. Due to this reduced complexity, coupled with the fact that mammals and insects have shared anatomical similarities, insects have become a prime model for the study of sensory systems in general [30] [31]. In particular, studies have focused on the olfactory system because there tends to be greater design analogy among olfactory systems than other sensory systems [11].

The phylum Arthropoda includes the class Insecta (the insects) and the class Crustacea (the crustaceans). Using an assortment of techniques, including but not limited to electrophysiology, genetic analysis, immunohistochemisty, Golgi stains, and behavioral tests, a variety of species within the class Insecta have been rigorously examined. Of all those studied, four orders within Insecta: Diptera, Dictyoptera, Hymenoptera and Orthoptera have provided the bulk of the information currently available on insect olfaction [90]. In the following sections, a species from each of these orders will be considered: the fruitfly, *Drosophila melanogaster* (dipteran); the cockroach, *Periplaneta americana* (dictyopteran); the honeybee, *Apis mellifera* (hymenopteran); and the locust, *Schistocerca americana* (orthopteran). In section 1.3.1, a generalized

account of the insect olfactory system is given, pointing out analogous structures and organizational principles. Section 1.4 describes the particular anatomical details of the olfactory systems of each of the three species, *Drosophila melanogaster*, *Apis mellifera* and *Periplaneta americana*. *Schistocerca americana* is described in detail in section 1.5. Particular focus is placed throughout on the organizational principles of the mushroom body, the brain region that is the focus of this thesis.

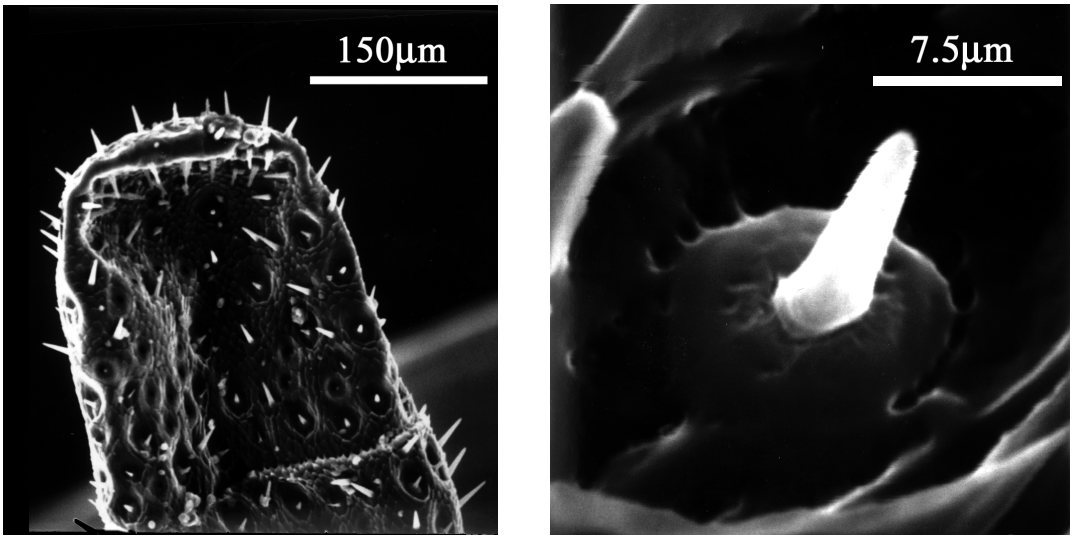
1.3 Anatomical Considerations of Olfactory Systems In Insects

1.3.1 The Antennae and Antennal Lobes

The main odorant sensing organs in insects are their antennae, which come in a variety of forms and sizes [27]. Insects that can smell possess antennae that contain the somata and dendrites of olfactory receptor neurons (ORNs). ORNs are specialized neurons containing receptors which bind odorant molecules and transmit information to the brain [27]. Depending on the species of insect, these ORN dendrites terminate in various segments of the antennae within microscopic sensilla (figure 1.1). Odorant binding proteins within the fluid in these sensilla bind odorant molecules and carry them to specialized receptors on the ORN dendrites where a signaling cascade begins [32] [9] [12].

Olfactory receptors are 7-transmembrane proteins, usually G-protein coupled [61]. In rats and mice, mammals with exquisitely sensitive olfactory abilities, as many as 1000 different receptor types exist to bind various odorants within the olfactory epithelium in the nose [60] [62] [7]. In *Drosophila* — probably the most studied insect with relation to ORNs — about 38 different olfactory receptor types within each antenna are encoded by as many genes [51]. It has also been shown that each ORN expresses only one of these receptors in addition to one ubiquitous OR gene [60], although each receptor type is expressed by many ORNs.

In most insects, ORNs project axons to the ipsilateral antennal lobe (AL), the

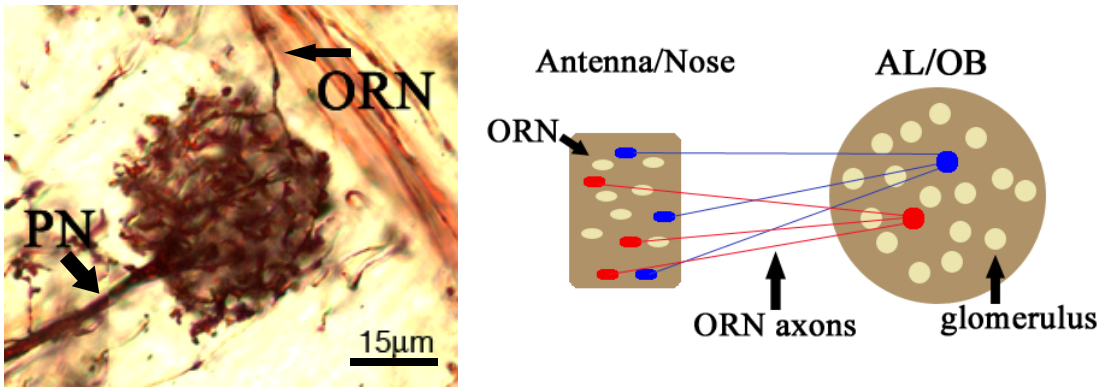


(**a**) (**b**)

Figure 1.1: (**a**) Scanning electron micrograph of the tip of an antenna of the locust, *S. americana*. (**b**) Higher magnification micrograph of an individual sensillum. Images acquired with the help of Dr. David Barsic.

equivalent structure to the olfactory bulb (OB) in mammals. At this early stage, there is a massive convergence of input as thousands of ORNs are consolidated to converge onto hundreds of AL output neurons. In both insects and mammals, the site of synaptic contact between ORNs and their target neurons are the glomeruli [28] [27] [79].

Glomeruli are defined as dense spheroid neuropillar structures that are composed of synaptic contacts between ORN axons and AL/OB neurons [27](figure 1.2). In most cases, in insects and mammals alike, one glomerulus houses contacts between ORNs expressing only one specific type of receptor. In flies where 1300 ORNs on the antennae express about 50 different receptor types, there are about 43 glomeruli per AL (figure 1.2). Glomerular numbers per AL can vary from as few as 43 in the fly to as many as 1000 in the locust [27]. The rat main olfactory bulb (MOB), by contrast, has approximately 1,800 glomeruli. Each rat MOB is itself bilaterally symmetric, containing 900 glomeruli per side. Thus, each receptor type in mammals is represented in two glomeruli, one on each side of the brain. In insect ALs, such duplication has not been observed so far [9].



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

Figure 1.2: (**a**) A Golgi-stained glomerulus of *S. americana*, showing innervation by a projection neuron (PN) and an olfactory receptor neuron (ORN). For details, see chapter 2. (**b**) Schematic of ORN convergence from the antenna/nose to the antennal lobe (AL)/olfactory bulb (OB).

In contrast to the mammalian MOB, which is specialized for detecting volatile odors, is the accessory olfactory bulb (AOB), a structure specialized for detecting pheromonal signals which receives input from the epithelium of the vomeronasal organ (VNO). Pheromonal signals provide information about the status of other individuals within the same species with regard to reproductive status, gender, and social hierarchy, and often influence behaviors by changing physiology and neuroendocrine release [52] [75]. These behaviors tend to be stereotyped or innate behaviors, rather than learned behaviors [75] [26] [6]. Neurons of the VNO, the vomeronasal receptor neurons (VRNs), display one receptor from the vomeronasal receptor gene repertoire. This repertoire is mostly likely composed of two classes of genes with 30-100 genes each. Within the AOB, VRNs synapse with downstream neurons in glomeruli; one VRN projects to about 15 glomeruli in the AOB, which are smaller in size than glomeruli of the MOB [75]. Each glomerulus receives inputs from about 17 different VRNs all having expressing same vomeronasal receptor. Within the AOB, mitral and tufted cells project dendrites to several distant glomeruli and project their axons to the amygdala and hypothalamus, bypassing the cortex completely [75].

In insects, the post-synaptic neurons which compose part of the glomeruli and project out of the AL to higher brain centers are projection neurons (PNs). In

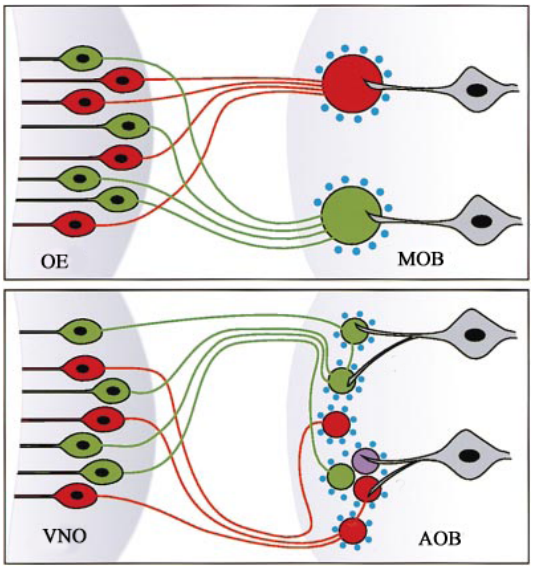


Figure 1.3: Schematic of the connections of the olfactory epithelium (OE) and the vomeronasal organ (VNO) to the main olfactory bulb (MOB) and accessory olfactory bulb (AOB), respectively. Glomeruli in the AOB are smaller; mitral and tufted cells of the AOB project dendrites to several glomeruli in contrast to the one glomerulus innervated per mitral/tufted cell in the MOB. Adapted from [75].

mammals, the analogous cells are known as mitral/tufted cells. In non-mammalian vertebrates, PNs (mitral cells) tend to be excitatory and multiglomerular, projecting dendrites to several glomeruli. In mammals, MCs are uniglomerular, and tufted cells are multiglomerular. Some insects, like the cockroach, have both uniglomerular and multiglomerular cells. In the locust as discussed below, PNs are multiglomerular, receiving inputs from as many as 20 glomeruli [28].

ALs also contain local inhibitory interneurons (LNs) that only project within the AL (see figure 1.6 inset). Immunochemistry and electron microscopy studies in insects [44] [27] have shown that LN arborizations in most insects are extremely broad, innervating the entirety of the AL and thus, potentially contacting many PNs [44]. As discussed in section 1.5.3, LNs aid in the synchronization of PN output, thus tuning the population output of the AL [40]. LNs also synapse within the glomerulus, forming connections with PNs, ORNs and other LNs [44]. In mammals, several inhibitory neuron types exist; periglomerular cells, which form reciprocal synapses with ORNs and mitral cells in the glomeruli, and granule cells, which form synapses with the

mitral cell secondary dendrites outside of the glomeruli (figure 1.4. The OB has also been shown to contain centrifugal fibers (fibers from downstream areas) contributing feedback from higher brain centers in mammals, although little information is available as to the function of these connections [79]. Such feedback is attributed to a few neurons (usually modulatory in insects).

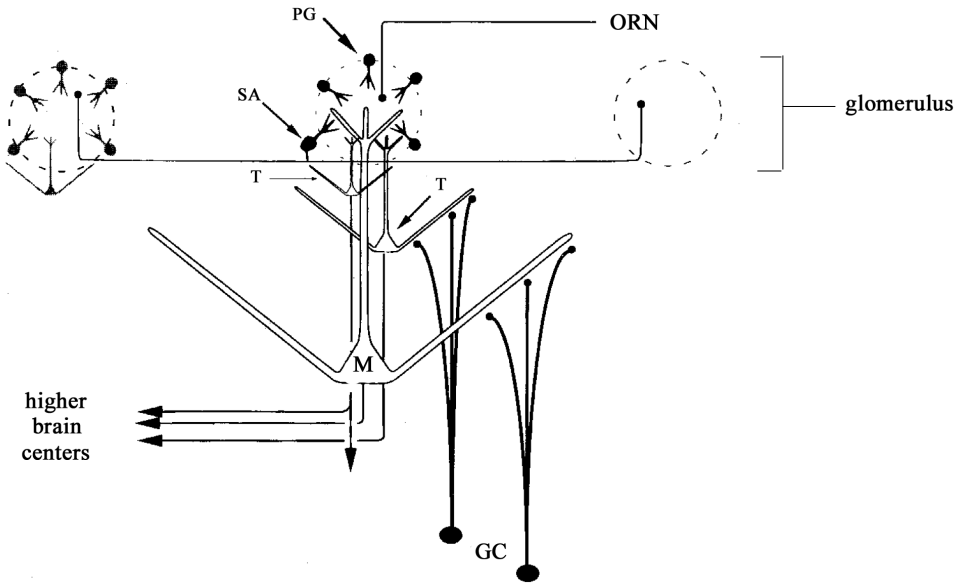


Figure 1.4: Schematic of the basic circuitry of the mammalian MOB. Periglomerular cells (PC), short axon cells (SA) and granule cells (GC)are all inhibitory cells. M; mitral cell, T; tufted cell (two types are shown, both marked by T). There are one-way and reciprocal synapses between branches of M and T cells with inhibitory neurons. Adapted from [80].

PNs of the insect olfactory system feed their excitatory axons via the antennocerebral tracts, to the second relay in the insect olfactory system, the mushroom body (MB). PN axon collaterals also project on further to the lateral protocerebrum, referred to as the lateral horn in locust and fly [27] (figures 1.5 and 1.6). From the MBs and lateral protocerebral areas, the olfactory pathway may merge with, and influence many other pathways. Cells in the lateral protocerebrum contain feedback connections to the MB itself, while the cells post-synaptic to MB neurons are com-

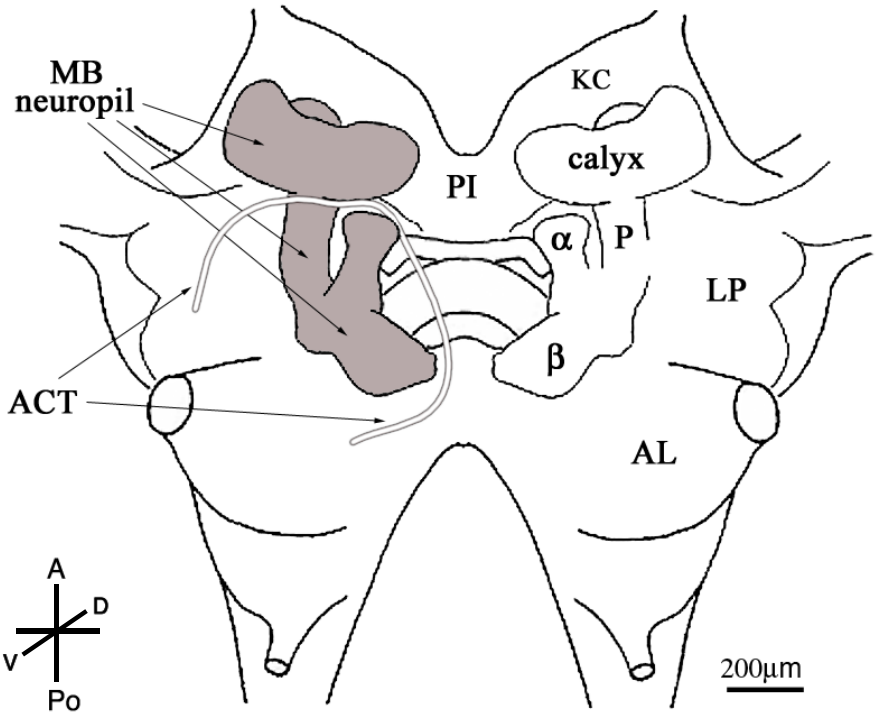


Figure 1.5: Schematic of the locust brain, showing the positions of the AL, mushroom body (MB) neuropils (the β lobe [β], the α lobe [α], pedunculus [P], and calyx), Kenyon cell somata (KC), the antennocerebral tract (ACT), the lateral protocerebrum (LP), and the pars intercerebralis (PI). Left hand corner shows the axes of the brain. A: anterior; Po: posterior; V: ventral; D: dorsal. Adapted from [8].

plex cells that innervate various parts of the protocerebrum, including the lateral protocerebrum, and areas thought to innervate descending motor pathways [35] [45] [5].

1.3.2 Mushroom Bodies

The MB is an extensively studied structure, mainly due to its long-imagined cortex-like functions. Studies done on flies [16] [76], bees [84] [53], cockroaches [97] and locusts [99], among other insects, have shown that these structures are indispensable to odor identification, sensory integration, learning, and memory storage [31]. MBs have been likened to the piriform cortex, which is the area in the mammalian cortex where mitral cells project. MBs have also been compared to the hippocampus because

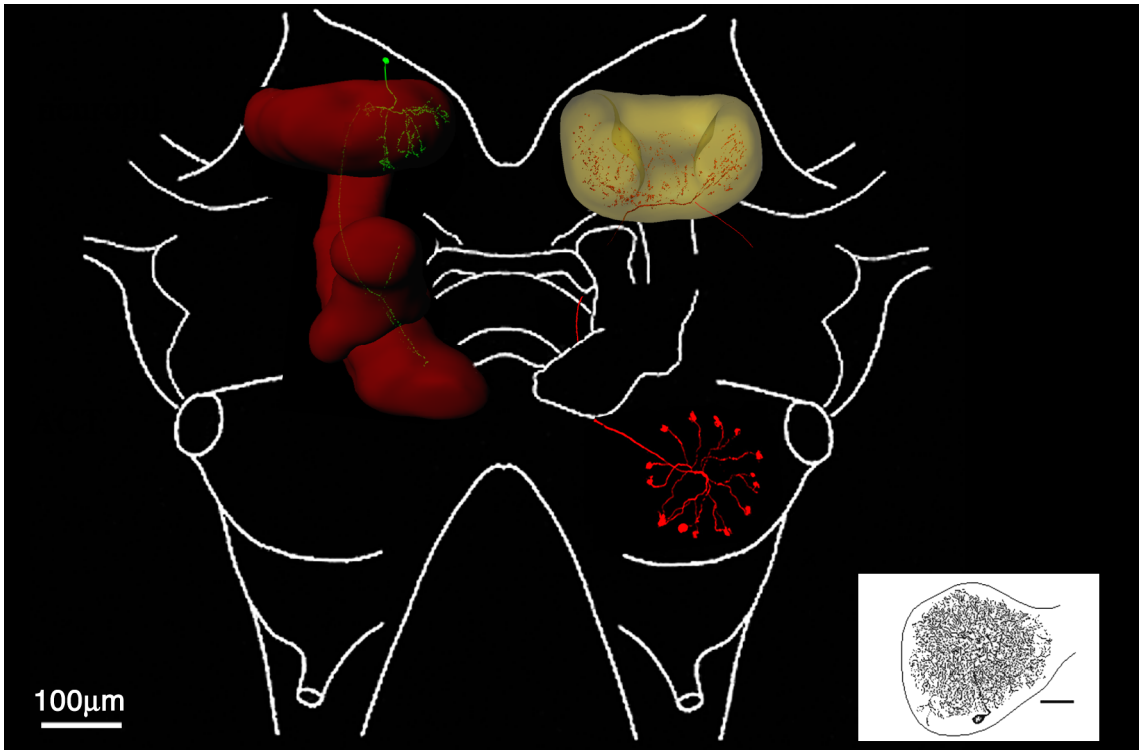


Figure 1.6: Reconstruction of a PN (see chapter 2) superimposed on right hemisphere of brain schematic from figure 1.5; scale bar is 200 μm. Left hemisphere shows a three-dimensional reconstruction of the MB and a KC (green) within it. Inset (adapted from [28]) shows a drawing of a typical local neuron in the AL. Scale bar is 100 μm.

they both play a role in learning and memory; high expressions of learning-related second messenger cascades (e.g., cAMP and related enzymes) are found in each [90] [58].

The MB is composed of a dense set of neuropils which resemble a mushroom. The cap of the mushroom is composed of the cup-like calyx whose concavity is filled with numerous somata of Kenyon cells (KCs), the so-called intrinsic cells of the MB (figure 1.6). In the more evolutionarily advanced insects like the honeybee and the cockroach, each side of the brain actually contains two of these mushroom-like caps which then become fused at the tip of their stalks (figures 1.9 and 1.7). The MB stalk is a long and mostly axonal neuropil, called the pedunculus, which covers 1/3 to 1/2 the length of the brain, projecting dorsally and posteriorly. The posterior base of the pedunculus splits into two major lobes, the ventrally projecting α lobe and

the medially projecting β lobe, and in some insects, into a third division called the γ lobe.

The KCs are one of the most numerous cell types within insects; the tiny *Drosophila* contains 2,500 KCs per MB, while the cockroach has 200,000 [27]. Within the calyx, the KCs receive input from the PNs in a massive divergence of input. In most insects, PNs number in the hundreds; thus, there is a 100 fold divergence at this level in olfactory processing. The voluminous calycal neuropil structure is the site of these synaptic contacts, as well as contacts with inhibitory neurons from other local areas. Though the calyx is mainly the site of olfactory afferent input to KCs, visual, auditory, gustatory, and mechanosensory afferent inputs are present in the calyx to varying degrees in different insect species [59] [17] [93].

Aside from calycal dendrites, each KC also projects an axon as part of a collection of dense parallel fibers down the pedunculus of the MB. Axons then bifurcate into the two lobes of the MB. In those insects with γ lobes, certain KCs with specialized clawed dendritic endings in the calyx send just one unbifurcated axon to that lobe only. Within the lobes, KCs form reciprocal synapses with cells whose somata are outside the MB and whose neurites partially or completely innervate the lobes and pedunculus.

MBs were discovered by the French scientist, Dujardin in 1850 [90]. He was the first to observe that these mushroom-like neuropils were the *corps d'intelligence* of the insect, noting that social behavior was correlated with enlarged MBs. These structures, in fact, exist in all but the most primitive insects. In 1896, F. C. Kenyon went on to describe the numerous intrinsic cells of the MB in the bee, *Apis mellifera* [36]. His pioneering work forever stamped these cells with his name, which Kenyon himself had named globuli cells. More recently, Strausfeld and Mobbs continued this legacy by further defining and classifying KCs based on their morphologies and innervation patterns within the calyx and lobe divisions [59], [89], [88], and [56].

KCs are classified in several ways in the current literature. One description is based on the area of the calyx innervated by a particular cell; these calycal divisions are based on afferent input to, and immunoreactive zones within, the calyx. For

example, in the bee there exists a certain region of calyx whose main afferent input is visual, and certain KCs can be found that also specifically innervate this division. Other classifications are based on specific morphology of KC dendrites, depending on the types and densities of pre-synaptic specializations. Still other classifications are based on the laminar organization of the pedunculus and lobes as revealed by immunohistochemistry of various neurotransmitter-like substances — a method which reveals molecular identity and possible activities of the cell processes located there. As these studies become more detailed, some of the morphological classifications can be brought together, and one can begin to see how afferent zones within the calyx are translated to efferent zones within the pedunculus and lobes. Specifically how this zonation pertains to function has yet to be delineated.

1.4 Examples from Three Insect Orders

1.4.1 Order Hymenoptera: *Apis mellifera*

Among the 120,000 species named in the order Hymenoptera, the most extensively studied are the social insects: the wasps, the ants, and the bees. Of these, the bee has been a prime olfactory model, owing in part to the fact that the worker bee — responsible for gathering nectar for the hive — can learn and perform olfactory tasks [18], [24], [53], and [54]. The fact that behavioral experiments can be carried out with relative ease makes the bee a good model organism. The honeybee was also one of the first insect species whose MB was mapped out in great neuroanatomical detail [36].

A typical worker bee has approximately 65,000 ORNs per antenna which synapse in 156 glomeruli in each AL. The AL contains 750 inhibitory LNs and 1000 excitatory PNs [27] and [22]. The MBs comprise 10% of the worker bee's entire brain volume [59], and each contains 170,000 KCs [95].

The MB of the bee is comprised of two symmetric calyces whose pedunculi fuse proximal to the calyces' posterior edge (figure 1.7). Each calyx of the bee MB can

Table 1.1: Comparison of olfactory system neuron numbers per half brain

Organism	ORNs	Glomerulus	PNs	LNs	KCs
Locust, *S. americana*	100,000	1000	800	300	50,000
Cockroach, *P. americana*	200,000	129	700	300	200,000
Honeybee, *A. mellifera*	65,000	156	1000	750	170,000
Fruitfly, *D. melanogaster*	1,300	43	200	100	2,500

be divided into three regions based on afferent input. The lip of the calyx receives input from the AL exclusively. The collar derives most of its input from the optic lobes, while the basal ring receives input from both olfactory and visual afferents [36] and [96]. Visual input to MB calyces is rare among Arthropoda in general, and large numbers of visual afferents to the calyces seem to be specific to the hymenopterans [90]. Odonats (dragonflies), for example, have no antennae, no AL, and a MB devoid of a calyx.

P. G. Mobbs further classified bee KCs into four groups on the basis of their dendritic specializations and suggested that each calycal afferent zone contains particular classes of KCs [59]. More recently, studies have suggested that the calyces, pedunculus and lobes are further subdivided into immunoreactive and non-immunoreactive zones. Based on immunocytochemical data, Strausfeld suggests that KC axons sort themselves in the pedunculus based on the neurotransmitter they are expressing, rather than their dendritic field position within the calyx [88] (figure 1.7). Thus, in the honeybee MB, three characteristics of KCs identify their type: the morphology of their dendrites; their connections with afferents as determined by KC dendritic position within the calyx; and their peptide epitope.

The honeybee has been demonstrated to have an α, a β and a γ lobe at the base of the fused pedunculus. The γ lobe has only recently been described and shown to contain the axons of a particular class of KCs [87] and [19]. Extrinsic cells within the lobes form reciprocal connections with KC axons, and some have been shown to innervate specific immunoreactive strata within the lobes [5]. Furthermore, α lobe extrinsic cells form feedback connections from specific parts of this lobe to specific

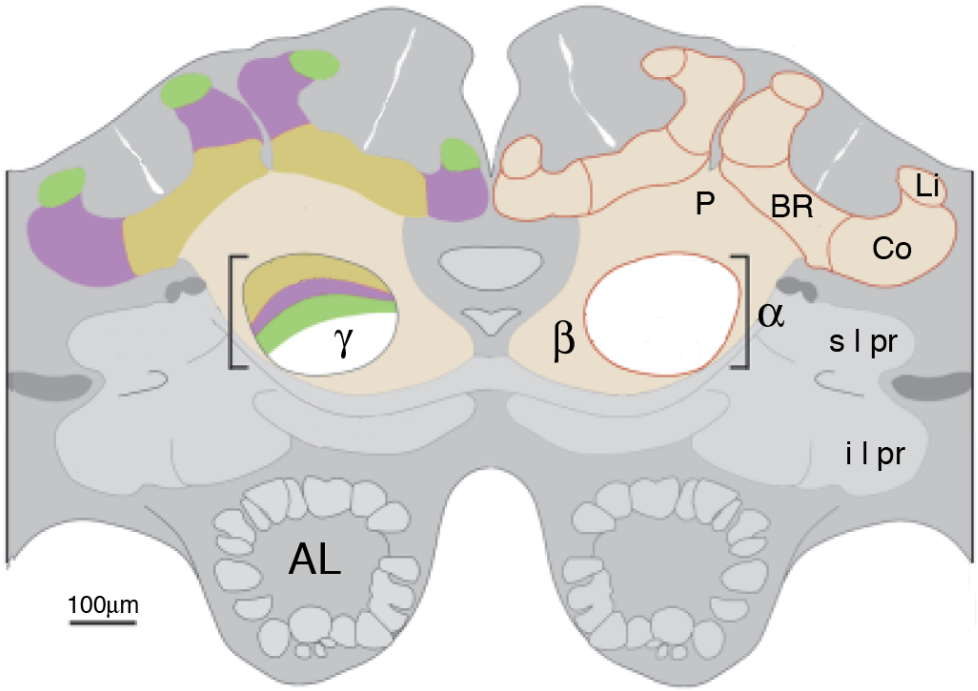


Figure 1.7: Schematic of the brain of the honeybee, *Apis mellifera*. Brain nomenclature is shown on the right hemisphere of the brain. The three divisions of each of the two calyces per hemisphere, the lip (Li), the collar (Co), and the basal ring (BR), feed into the pedunculus (P) and down into the β lobe (β), and the α lobe (α) which projects out of plane of the page. Representation of the calyx zones within the α lobe are schematized in the left hemisphere. The Li, Br, and Co feed to the colored anterior portions of the α lobe, while clawed KCs from all the calycal regions feed to the posterior γ division of the α lobe. s l pr: superior lateral protocerebrum; i l pr: inferior lateral protocerebrum. Adapted from [87].

parts of the calyx; the purely olfactory lip region receives feedback from dorsal parts of the α lobe, while the basal ring and collar receive input from the other areas of the α lobe [25].

In summary, the honeybee MB has been shown to have divisions throughout. In the calyx, immunoreactive zones are contained within the larger afferent zones. Immunoreactive divisions are also defined in the pedunculus and lobes, some corresponding to the KCs and some to the extrinsic cells. KCs have been shown to form classes based on morphology, immunoreactivity and calyx innervation; these classes correspond further to specific divisions within the pedunculus and lobes. Finally, the

extrinsic cells innervate specific zones of the calyces and lobes as well, and themselves can be differentiated by their immunoreactivity.

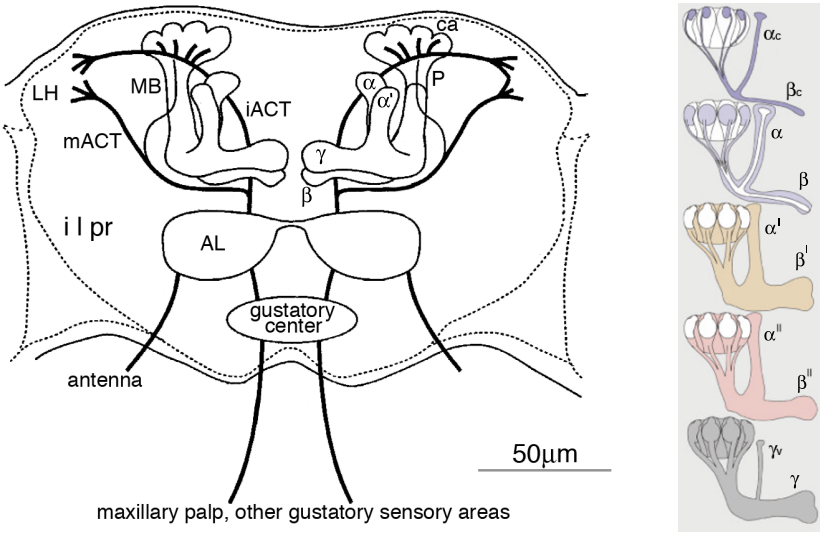
1.4.2 Order Diptera: *Drosophila melanogaster*

The dipteran order contains insects such as the true flies. The commonly known fruitfly, *Drosophila melanogaster*, is one of the best genetic models available for biological studies. Another advantage of studying fruitflies is their ability to learn olfactory associations to noxious stimuli, such as footshock [72], and various genetic studies have shown that the MBs are involved in learning and memory formation [76]. Mutant flies with malformed MBs cannot learn olfactory associations although they are otherwise normal. Targeted disruption of genes involved in MB cell signaling also leads to learning deficits [16], [55], and [76]. Recently, it has also become feasible to record from individual cells in the fly brain, and functional studies have been performed, starting with classification of PN odor responses [103].

The anatomy of the *Drosophila* olfactory system follows the general schematic outlined above for insects. 1300 ORNs from each antenna project bilaterally, synapsing in 43 glomeruli within each AL [83]. ORNs expressing specific receptors converge onto a single specific glomerulus on either side of the brain [23]. Roughly 200 PNs per side are post-synaptic to the ORNs, as well as local inhibitory neurons whose numbers are undetermined [27], but probably on the order of 100.

The fly olfactory system contains several tracts that carry PN axons to the MB calyx and lateral horn [91] and [104] (figure 1.8(a)). In the calyx, approximately 2500 KCs send dendrites to receive this mainly olfactory PN input. Up to 8 different KC types have been defined, based on dendritic and axon morphology. Each of these types innervates particular divisions of the calyx [89]. Unlike in the honeybee, the divisions within the calyx of the fly are subtle and were only recently described [89]. These calycal zones are defined based on KC dendritic "decorations" and immunochemistry with antisera against the neurotransmitters, taurine, aspartate, and glutamate (figure 1.8[b]). Since all the input to the calyces is from the AL, no afferent zones are defined.

Strausfeld has recently postulated that in *Drosophila*, each MB calyx is not a single structure, but like that of the cockroach and honeybee: made of two calyces. In the fly, the calyces are highly fused, whereas in the cockroach and honeybee, they are more visibly separated [89].



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

Figure 1.8: Schematics of the brain of the fruitfly, *Drosophila melanogaster*. (**a**) The entire brain is depicted, showing the calyces and MB lobe divisions in each hemisphere of the brain. PN axons project through two tracts: the inner antennocerebral tract (iACT) and the medial antennocerebral tract (mACT) to the calyx and the lateral horn (LH). Adapted from [35] (**b**) Representation of the calyces by the KCs within the different subdivisions of the lobes based on morphology and molecular epitope. The calyx is viewed from the dorsal surface of the brain in each of the five instances. The KCs projecting to the γ lobe innervate all parts of the calyx. Adapted from [89].

The lobes of the MB have also been thoroughly described [105]. Two major lobe divisions, with a total of 10 subdivisions are observable, based on KC axon projections [10] and immunocytochemistry citeStrausfeld03. Four sets of these divisions are actually subdivisions of the medially projecting β lobe and the vertically projecting α lobe (termed β, β', β'',β_c, α, α', α'' and α_c respectively). The fifth division, comprised of the γ and γ_c lobes, is also medially projecting and lies dorsal to the β lobe [10] and [89]. A heel region has also been described which is a lateral protuberance at the intersection of the pedunculus with the lobes.

Immunocytochemistry with a number of compounds (anti-FASII, anti DAMB, anti-DCO, anti-DRK) [10] has revealed the pattern of peduncle projections to the lobes; medial peduncle fibers project to the α, α_c,β and β_c lobes, the later peduncle projects to the γ lobe and heel, while the central pedunculus fibers project to the α', α'', β' and β'' lobes [10].

As described in figure 1.8 the immunoreactive zones in the calyx correspond to immunoreactive zones defined in the lobes, defining topographical correspondence between dendrites and axonal regions.

Different types of KCs segregate into different lobes and exhibit particular axonal morphologies there. The β and β' lobes contain axons that give rise to numerous branches and collaterals, while the β'' lobe contains KC axons that appear sparsely or completely unbranched. KCs in the γ lobes are distinguished by their "clawed" dendritic endings in the calyces; they occur in all calyx regions, unlike KCs of the other lobe regions [89] (figure 1.8).

In summary, Golgi and immunochemical studies in the fly indicate that the calyx is divided into zones based on KC dendritic morphology and immunoreactivity, and that these zones correspond to particular sections of the lobes. The lobes themselves have specific immunoreactive patterns and are innervated by different KC axonal types.

1.4.3 Order Dictyoptera: *Periplaneta americana*

The dictyopteran, *Periplaneta americana*, has been studied extensively with relation to olfaction. This insect is a nocturnal scavenger and relies critically on olfaction for survival. Numerous anatomical studies have allowed a detailed mapping of its olfactory system, and behavioral and electrophysiological studies have allowed some functional mapping as well [97] and [58].

The AL of *Periplaneta* is innervated by 200,000 receptor neurons from its associated antenna. 125 glomeruli are the site of contact between the ORNs and the 300 LNs and 700 PNs of the AL [17] and [50]. All connections in the AL occur within

the glomeruli in the following way: ORNs contact PNs and LNs; LNs contact and receive input from PNs, ORNs and other LNs [13] [14]. Action of the LNs onto ORNs is presynaptic.

PNs in *Periplaneta* come in several varieties: uniglomerular PNs innervate only one isomorphic glomerulus; multiglomerular PNs innervate several isomorphic glomeruli; and macroglomerular PNs innervate the large, sexually dimorphic macroglomerular complex (a specialized pheromonal-sensing region in the male) [93]. All PN types appear to output into the MB calyx and the lateral protocerebral area [94], [93], and [17].

As in *Apis*, *Periplaneta* MBs have two calyces, each of which fuses at the anterior end of the pedunculus (figure 1.9). The pedunculus continues posteriorly and splits into the lobes. The calyces are innervated by 200,000 KCs — the most KCs of any insect studied to date [57]. Inputs to the calyx are mainly olfactory, derived from AL PNs, but afferents from several other modalities innervate the calyx as well. Four afferent zones, I, II, III and IIIa, have been identified in the calyces (figure 1.10). These zones have been defined based on which afferent cells project to them, and not necessarily based on the particular modality represented there. All zones receive olfactory and mechanosensory inputs from different combinations of afferent cells. Only zone II also receives visual afferents [64], [81], and [93].

Aside from calycal afferent zones, KCs in *Periplaneta* have also been classified according to their patterns of peduncular and lobe projections, as well as their dendritic decorations [56], [93], and [15]. However, it is increasingly clear that cells with different types of decorations innervate all afferent zones of the calyx, but that cells in specific zones run together in specific regions of the pedunculus and lobes. Thus, afferent zones in the calyx may make up functional units of the MBs which map morphologically within the pedunculus and lobes. KC soma position is not a good indicator of cell type due to the fact that position is related to the birthdate of the cell during development, and not its pattern of neuropil innervation [20].

Zones within the pedunculus and lobes have been defined based on results of staining using various techniques. When stained for various neurotransmitters, neu-

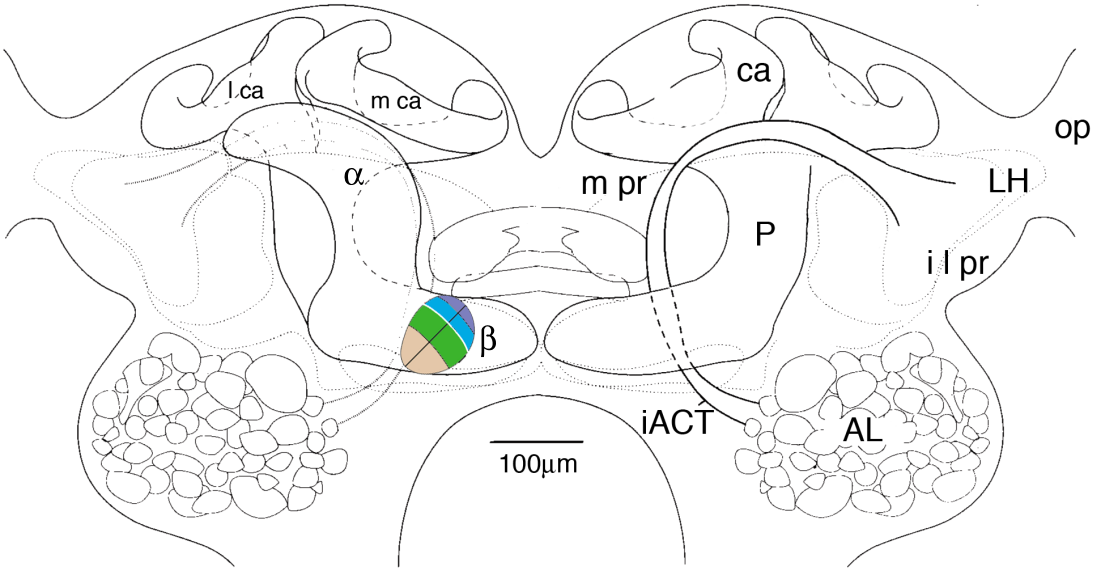


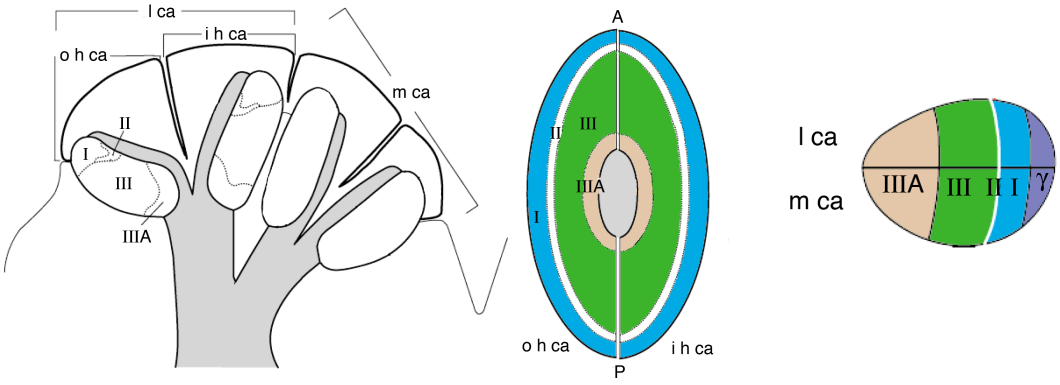
Figure 1.9: Schematic of the brain of the cockroach, *Periplaneta americana*. AL PNs project to the calyces via the iACT and terminate in the LH. On the left hemisphere, a sagittal section of the β lobe is shown in colors that are described in the figure 1.10. op: optic lobe; l ca: lateral calyx; m ca: medial calyx. Adapted from [58].

romodulators, and with reduced silver techniques, these regions are seen to have a laminar arrangement corresponding to bundles of KC axon fibers and efferent neuron branches [81] and [57].

Efferent MB neurons of *Periplaneta* are very complex. They can be broad, innervating many lamina in both lobes, as well as the lateral and medial protocerebra, or they can innervate some portion of just one lobe, or just the calyces. These cells, when tested with a variety of sensory inputs, often show multi-modal responses. Consequently, the MB has been proposed to act as a sensory association center [46] and [57].

1.4.4 Comparison Between the Three Orders

Looking at the sum of these studies, some organizational principles of MBs emerge. It is clear that in all three species, KCs can have several distinct types of dendritic and axonal specializations. Barring a few exceptions, however, KC classes based on



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

Figure 1.10: Schematics showing the calycal divisions within the cockroach brain and their representation within the β lobe. (**a**) On the left is a frontal (viewed from ventral surface) representation of the two calyces of one MB showing their various divisions. Each calyx (l ca and m ca) is further subdivided into the outer hemicalyx (o h ca) and the inner hemicalyx (i h ca). Within each hemicalyx is shown the three afferent divisions of the calyx, zones I-IIIA. On the right, the same divisions are viewed from the top (anterior surface) of the brain, stretched along its anteroposterior axis (A: anterior; P: posterior). Adapted from [93]. (**b**) A sagittal cross-section of the β lobe as described in figure 1.9, showing the representation of the calyces as defined by immunochemistry and Golgi stains. The lateral and medial calyces are represented on the anterior and posterior portions of the lobe, respectively. Further, the zones are represented in order from one side to the other. The γ lobe contains clawed KCs from all calycal divisions. Adapted from [94].

these features reveal little about MB organization and function.

More telling classifications rely on macroscopic features such as spatial divisions of the calyx. These groupings of KCs are therefore based on input modality. This kind of zonation is seen in both *Apis* and *Periplaneta*; in *Apis*, two of three zones are multimodal, while in *Periplaneta* all four zones are multimodal. In *Drosophila*, the entire calyx appears to be exclusively olfactory.

This zonation has been further refined and correlated to immunoreactivity; in all three orders, areas of the calyx can be delineated that stain for particular neurotransmitters and neuromodulators. In some cases, these reflect staining of KCs and in others, staining of afferent and extrinsic cells. Further, these zones of immunoreactivity in the calyx can be mapped onto corresponding, ordered zones of

immunoreactivity in the lobes.

Besides immunoreactive regions, KC-defined and morphologically-defined lobe subdivisions have been identified in *Apis*, *Periplaneta* and *Drosophila*. In the former two, α and β lobes are divided along their long axes; in the latter, subdivisions occur in the form of physically distinct lobes: the α, α', β, and β' lobes. Identification of the γ lobe of all three species is based on KC morphology. This lobe contains KCs with "clawed" dendritic specializations and these KCs are found in all parts of the calyx.

Thus, although differences occur between these species of insects, many organizational similarities can also be found. In the following chapters, these organizational principles will be compared to those found in the locust, *Schistocerca americana*, based on the present available literature (below) and on the results of this thesis (following chapters).

1.5 A Detailed Look at Order Orthoptera: *Schistocerca americana*

The order Orthoptera includes grasshoppers and locusts. Of these, the olfactory systems of the locusts, *Schistocerca americana* and *Schistocerca gregaria*, are those most comprehensively studied. Anatomical studies done to date on locusts have been relatively few and carried out more extensively in *S. gregaria*. The olfactory system of *S. americana* is the best studied for its biophysical and physiological properties. A range of electrophysiological experiments on AL and MB intrinsic neurons has been conducted, and much is known about the coding of olfactory information by these neuronal populations [41], [86], [85], and [39]. So far, the literature indicates that no significant differences have been seen between the two species of *Schistocerca* [82], and thus, data available from one species will be used interchangeably for data obtained from the other throughout this account.

1.5.1 Olfactory Anatomy: The Antennal Lobes

The locust brain is bilaterally symmetric, with an average size of $6mm^3$. A conservative estimate puts the total number of cells within the brain at 360,000 [8] and [17]. Between 50,000 - 100,000 ORNs of each antenna innervate the ipsilateral AL which contains about 1,000 glomeruli [29] and [17]. Each ORN axon terminal of the locust can innervate between one and three glomeruli [17], and each glomerulus receives processes from more than one ORN [1]. Whether the ORNs and glomeruli are unireceptor as in the fly is yet to be determined.

The number of glomeruli in locust is indeed among the highest of any insect studied to date. The glomeruli are also considerably smaller (figure 1.2), measuring about 25 μm in diameter [17] [1]. According to immunocytochemical studies, synapses in the locust AL only occur within these glomeruli [44], and Ernst[17] proposed that up to eight cells, including ORNs, PNs and LNs synapse within a single glomerulus in locust.

Besides the axons of ORNs, the locust AL contains roughly 800 PNs and 300 LNs [8], [27], and [41]. All PNs in the locust are multiglomerular, each innervating between 10 and 20 glomeruli (figure 1.6). According to the above numbers it is plausible to assume that each glomerulus is innervated by 8-16 PNs, although according to the literature to date, that has not been established. The relationship between specific ORNs and PNs is still unclear, and so is that of individual receptor types to PNs. A recent study by Moureaux and Laurent (in publication) shows that different dendrites of a single PN within the same glomerulus respond differently to odors, suggesting that more than one type of receptor is represented within one glomerulus. There are varying estimates of the number of different receptor types that are represented in the locust, and it is unknown whether more than one receptor type is expressed by any given ORN [29].

Information about synapses in the locust comes mainly from electron microscopy studies, specifically one which visualizes a stain against the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [44] and [98]. This reveals that LNs are a

GABA-ergic population of cells, while the processes of PNs and ORNs are not. This staining also reveals the patterns of cell to cell contacts: PNs synapse onto LNs and possibly onto other PNs, ORNs synapse onto LNs and PNs, and LNs synapse onto ORNs, PNs and other LNs, not unlike the other insect species already discussed. PNs and ORNs are generally assumed to be cholinergic because of strong staining for the antibody acetylcholinesterase [33] within the antennocerebral tracts where PN axons project, as well as within the AL.

1.5.2 Olfactory Anatomy: The Mushroom Bodies

The afferent neuropil of the locust MB is composed of a primary cup-like ventral calyx, and a dorsally situated accessory calyx. Each locust MB has been described as having a single calyx, as in the original descriptions of the MB in *Drosophila*, and in contrast to the duplicated calyces of honeybees and cockroaches. The calyx is described by Weiss [101] as having a central ring through which many of the KC processes pass on their way to the pedunculus. 50,000 KC bodies fill the calyx cup and spill over, covering the entire cap of the MB. KCs project dendritic trees into the calyces, and an axon down the pedunculus which bifurcates into the medially projecting β lobe and the ventrally projecting α lobe [27] and [8] (figure 1.5).

The input to the turban-like locust calyx has so far been established as primarily olfactory. Axons of the PNs ascend anteriorly to the MB calyx through the antennocerebral tract, which runs from the dorsal side of the AL, ventral to the central body and up to the calyces. PN collaterals broadly innervate the primary calyx and proceed on to terminate in the lateral horn (LH) (also referred to as the lateral protocerebrum) [42] and [8]. The accessory calyx is the site of gustatory input from neurons of the globular lobe. This lobe is situated just dorsal to the AL and receives the axons of gustatory sensory cells of the maxillary palp (mouth parts) [17].

The primary calyx also receives other neurites, though none have been established as direct sources of sensory input (e.g., visual). Lateral horn interneurons (LHIs) have been identified as one set of feedback cells [70]. This group of neurons has

cell bodies close to the lateral horn and sends axons that branch broadly within the calyx. LHIs receive input (directly or indirectly) from the PN lateral horn branches [70]. As described below, several other neurons cells have been identified through immunocytochemistry that innervate the calyces, as well as the pedunculus and lobes, but none of these has been studied for their responses or effects on MB function.

Weiss [101] described the pedunculus as having three columns of separate fiber bundles of which one is derived from accessory calyx processes and two from the primary calyx. KC axons are packed densely within the pedunculus, and are often spiny throughout [42] and [44]. KCs are seen to form en-passant synapses with one another down the length of the pedunculus, as well as synapses with GABA-ergic and non-GABA-ergic processes belonging to other cell types. Extrinsic innervation of the pedunculus is more extensive in the locust than has been described in other insects. Large diameter fibers run up the sides of the pedunculus, parallel to KC processes while small diameter fibers run perpendicularly at intervals through the length of the pedunculus [44] [47].

The so-called extrinsic cells of the MB include both efferent cells carrying information away from the MB, and feedback cells that send information from pedunculus and lobes or through cells of the LH to the calyx. Besides the LHIs, several other extrinsic cells have been identified, mostly on the basis of immunocytochemistry. The neurotransmitters and neuromodulators examined pharmacologically in the locust occur in all the insects so far discussed, and in most insects in general.

In insects, dopamine has been implicated in the modulation of endocrine activities and some behaviors (e.g., flight), as well as olfactory memory formation. Dopamine immunocytochemistry has highlighted four neurons from the antennal motor and mechanosensory centers in the tritocerebrum that innervate the pedunculus and lobes densely [102]. Other unidentified neurons also innervate the lobe and pedunculi. However, there is no analysis of whether dopamine immunopositive areas within the pedunculus and lobes form lamina or zones analogous to immunoreactive zones in *Apis* and *Periplaneta* for neurotransmitters (e.g., glutamate).

In the locust, GABA has been established as an inhibitory neurotransmitter [44]

and [41]. As mentioned above, GABA immunochemistry has identified LHIs which project from the lateral horn to the calyx. Such immunochemistry has also identified a large inhibitory neuron which innervates calyx, pedunculus and lobes. LHI cell bodies are situated in the LH and are thought to act as delayed feedfoward inhibitors from the PNs' lateral horn projections to the calyx. The cell body of the so-called giant inhibitory cell is also located in the lateral horn. Other unidentified GABA-positive processes have also been seen in the pedunculus and lobes [44]. The neuromodulatory peptide, mas-allatotropin, co-localizes with GABA in the giant inhibitory neuron and other perpendicular peduncular processes. Mas-allatotropin is also seen to stain the dense processes of projection neurons from the globular lobe within the accessory calyx [34].

Staining for NADPH-diaphorase reveals the sites of synthesis of nitric oxide, a gaseous intercellular signaling molecule [66], [67], and [68]. Nitric oxide has been implicated in learning and synaptic plasticity in nervous systems [4]. In the locust, this is the only staining that has revealed stratification of the MB, comparable to that of the other species so far discussed. Within the center of the pedunculus runs an immunoreactive tube, such that the center and edges of the pedunculus are immuno-negative. In the α lobe, six tubes of presumably extrinsic cell origin run parallel to the lobe's long axis. In the β lobe, the core is densely stained, but the outer edges remain negative [65] and [66] [67].

Thus far, immunostaining and reduced silver staining in the locust have not led to the observation of anything that could be construed as functional units, analogous to those described for honeybee, cockroach and fly. Except for the broad division between primary and accessory lobes, the calyx has not generally been thought of as having divisions. Up to now, the pedunculus has only been shown to have diaphorase positive "tubes", and the three-column structure described by Weiss. The β lobe too has not been described as being organized in a laminar fashion. Finally, the α lobe is the only MB area that has structure reminiscent of the lamination described in other insects. A brief account of what is known about olfactory coding in the locust system follows.

1.5.3 Olfactory Coding

In the locust, the processing of olfactory information has been extensively studied in the AL PNs and LNs, as well as the KCs [43]. These data have also been modeled using computational methods [74], [2], [3], and [73]. When ORNs are activated by odors, they in turn excite sets of PNs and LNs in the AL. This gives rise to a global AL phenomenon: oscillatory synchronization in subsets of PNs at 20-30 Hz [42]. This activity can be recorded as a local field potential (LFP) from PN axons within the primary calyx of the MB. Synchronization is set up and maintained by LN inhibitory activity, which is reflected in the LFP [100] and [48]. Each cycle of the oscillation is made up of a different combination of PNs, and the identity of PNs that participate in each cycle is odor dependent [99] and [40]. It has been shown in honeybee [84] that abolishing oscillatory synchronization by blocking GABA A receptors in the AL using a Cl channel blocker diminishes the ability of the organism to perform fine odor discrimination tasks. PN firing statistics do not change with Cl current block; PNs are merely kept from synchronizing their activities [48]. In zebrafish, it has been shown that odor representations within the olfactory bulb become decorrelated over time, so that if at the beginning of an odor response, two mitral cells have similar spiking patterns, over the evolution of the odor response, they will come to have different patterns [21].

To show how the information from PNs is read out by KCs, Perez-Orive et al. [70] presented data showing that KCs respond only when excited by multiple PNs during a narrow time window of an LFP oscillation. This cycle-pattern sensitivity is set up by the feed-forward inhibition of PNs to LHIs to KCs. Each KC receives at any cycle of the LFP a certain number of excitatory post-synaptic potentials (EPSP) from pre-synaptic PNs. If threshold is reached, the KC fires. Because of the IPSPs induced by the LHIs that occur shortly after PN EPSPs, there is a narrow time window to integrate EPSPs, during which spike threshold can be reached. Since activated PN identity is changing from cycle to cycle, the population of KCs receiving EPSPs also changes. Further, since the KC firing threshold is high [69], the KC population

response is very sparse compared to that of the PNs. For a puff of odor, 60% of PNs may respond with an increase or decrease in firing, while for the same odor puff only 10% of KCs fire, and then only one or a few precisely timed spikes at a particular cycle of the LFP.

The overall hypothesis proposed by Laurent et al. [39] and [70] is that these essentially temporal codes allow the separation of odors — similar and otherwise — at the AL level in olfactory processing. Further, the nature of the connections between PNs and the MB and LH, and KC intrinsic properties allow these odors to be represented in a simplified manner by the KCs [37] and [39].

The output of the KCs has been investigated as well. In 1998, Macleod et al. [47] characterized some properties of locust extrinsic neurons (then called β lobe neurons, but here, referred to as extrinsic neurons as they innervate not only the β lobe, but the α lobe, pedunculus, and LH as well). In relation to odor tuning and specificity, of the extrinsic neurons tested, many responded to multiple odors, and half showed significant phase locking to the MB LFP. The odor responses were stimulus specific, though less complex than those seen in PNs. When PN input was desynchronized by picrotoxin injection in the AL, KCs became more promiscuous [70] and some but not all of the extrinsic neurons' odor responses were affected as well [47]. Changes included decreases in baseline activity, changes in temporal patterning of the response, combinations of decreases in spontaneous activity, and increases in odor response, as well as new responses to odors.

Just as it was necessary to study how KCs connected to and decoded information transmitted by the PNs and through the LH, it is further necessary to study how information from the KCs is transmitted to extrinsic cells. Several aspects must be considered in order to decode KC spikes, some of which can be addressed by studying the neuroanatomy of the system: What, if any, inputs do KCs receive in the calyx in addition to PN axons? Are KCs arranged into functional groups in the pedunculus and lobes? To which extrinsic cells are specific KCs connected? What are the regions of extrinsic cell input and output? What is the nature of any feedback to the calyx?

1.6 Specific Goals and Layout of this Study

This study focuses on the architecture of the locust MB. One goal is to characterize the KCs based on their morphology, dendritic extent and positions within the calyx, as well as their axon morphologies and lobe innervation. The extent of the anatomy of PN calycal output is also shown. In addition, extrinsic cells are classified and described with relation to MB innervation. To complete the picture, MB structure is studied to identify morphological features that can direct KC classification. All of these data are then compared to existing literature to find similarities and significant differences between the locust system and other insects.

As laid out in Chapter 2, the main methods used to stain cells for anatomical study were Golgi stains and fluorescent dye fills of individual cells using intracellular iontophoresis. Confocal microscopy was used to image these populations, as well as to examine brain structures using the tissue's own intrinsic fluorescence. To analyze images of intracellularly filled cells, software was developed to trace out filled cells in digital images and extract them from the background. These outlines were then reconstructed along within their brain structures in three dimensions. This effectively presents the anatomical data in a more tractable way than has been done previously in studies of this kind.

Chapters 3 through 4 present the data, Chapter 3 presents an overall view of the most important anatomical observations and their implications based on all the relevant data including KCs, PNs, extrinsic neurons and general MB structure. Chapter 4 presents a more detailed look at individual cells, including their morphologies and innervation patterns. This chapter is an atlas of KCs, PNs and extrinsic cells. To date, this is the most extensive anatomical study of these cells in the locust, using single cell fills.