Structure and Function of the Moth Mushroom Body

Marcus Sjöholm

Faculty of Landscape Planning, Horticulture and Agricultural Science
Department of Crop Science
Alnarp

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Abstract

The mushroom bodies are paired, high-order neuropils in the insect brain involved in complex functions such as learning and memory, sensory integration, context recognition and olfactory processing. This thesis explores the structure of the mushroom bodies in the noctuid moth *Spodoptera littoralis* using neuroanatomical staining methods, immunocytochemistry and electron microscopy, and investigates how the intrinsic neurons of the mushroom body, the Kenyon cells, respond to olfactory stimulation of the antennae using whole-cell patch clamp technique.

The mushroom body in *S. littoralis* contains about 4,000 Kenyon cells, and consists of a calyx, pedunculus and two lobes, one medial and one vertical. The calyx houses dendritic branches of Kenyon cells and the pedunculus and lobes contain the axons and terminals of these neurons respectively. The calyx is doubled and concentrically divided into a broad peripheral zone, which receives input from antennal lobe projection neurons, and a narrow inner zone, which receives yet unidentified input. The lobes are parsed into three longitudinal divisions, which contain a separate subset of Kenyon cells each. The Kenyon cells are divided into three morphological classes, I-III. Class I Kenyon cells have widely branching spiny dendritic arborisations in both zones of the calyx and occupy the two most posterior subdivisions of the lobes called α/β and α'/β'. Class II Kenyon cells have narrow clawed dendritic trees in the calyx and invade the most anterior division in the lobes, called γ. Class III Kenyon cells have clawed, diffusely branching dendrites in the calyx and provide a separate system of axons and terminal branches, partly detached from the rest of the mushroom body, called the Y tract and lobelets. Kenyon cells within the classes display differential labeling with antisera against neuroactive substances. Kenyon cells make synaptic contact with one another and with other neuron types in the mushroom body. Extrinsic inhibitory and putative modulatory neurons were identified.

Whole-cell patch clamp recordings revealed that Kenyon cells exhibit broadly tuned subthreshold activation by odor stimulation and a few cells responded with action potentials to specific biologically relevant odor combinations.

Keywords: amino acids, Lepidoptera, neuroanatomy, neuropeptides, pheromone, plant odor, synapse.

Author's address: Marcus Sjöholm, Department of Crop Science, SLU, SE-23053 ALNARP, Sweden. marcus.sjoholm@vv.slu.se

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Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Sjöholm, M., Sinakevitch, I., Ignell, R., Strausfeld, N.J. & Hansson, B.S. 2005. Organization of Kenyon cells in subdivisions of the mushroom bodies of a lepidopteran insect. *Journal of Comparative Neurology* 491, 290-304.

II. Sjöholm, M., Sinakevitch, I., Strausfeld, N.J., Ignell, R. & Hansson, B.S. 2006. Functional division of intrinsic neurons in the mushroom bodies of male *Spodoptera littoralis* revealed by antibodies against aspartate, taurine, FMRFamide, Mas-allatotropin and DC0. *Accepted for publication in Arthropod Structure & Development.*

III. Sinakevitch, I., Sjöholm, M., Strausfeld, N.J. & Hansson, B.S. 2006. Glutamate-, GABA-, serotonin-, allatostatin- and TRP-like immunoreactivity in the mushroom bodies of the moth *Spodoptera littoralis. Submitted manuscript.*

IV. Sjöholm, M. & Hansson, B.S. Synaptic organization in the mushroom bodies of the moth *Spodoptera littoralis*, a TEM study. *Submitted manuscript.*

V. Sjöholm, M., Ignell, R. & Hansson, B.S. Odor-evoked sub- and suprathreshold activation in Kenyon cells in the moth mushroom body. *Manuscript.*

Objective

The objective of this thesis was to describe the structural and chemical anatomy and explore the function of the mushroom bodies in the brain of the Egyptian cotton leaf worm moth, *Spodoptera littoralis*. This was achieved by using standard neuroanatomical staining techniques, immunocytochemistry targeted toward putative neuroactive substances viewed with laser scanning confocal microscopy and transmission electron microscopy. Lastly, whole-cell patch clamping was used in order to investigate the role of the mushroom body intrinsic neurons in olfactory processing.

Introduction

The insect brain is, for its size, an amazing piece of computational tissue. An adult honey bee, *Apis mellifera*, has just under one million neurons and a fly, *Musca domestica* some 340,000 neurons in their forebrain (Witthöft, 1967; Strausfeld 1976) which consists of the fused proto-, deuto- and tritocerebra. In addition, insects possess several neural ganglia located along the ventral nerve chord, which stretches throughout the length of the body. These ganglia handle sensory-motor integration and coordination locally. The ganglia of the forebrain, on the other hand, are responsible for integrating and perceiving information detected by the principal peripheral sensory organs, i.e. the eyes, antennae, palpae and mouth parts and information about the internal state of the insect. The brain allows the insect to filter and translate a plethora of often meaningless events in its immediate environment into meaningful information such as vision, odor and taste. This information is used by the insect to deal with choice situations and to make decisions that meet the current needs of the animal. Additionally, the brain is responsible for storing information and comparing new situations with already experienced ones. Together, all these functions are used to initiate and guide adequate and essential behaviors, which help the insect to survive in an often unpredictable environment. A strong candidate within the insect brain for mediating several of these functions is a specific structure called the mushroom body (de Belle and Heisenberg, 1994; Mizunami et al., 1998b, c; Strausfeld, et al., 1998; Tang and Guo, 2001), a paired, lobed, high-order neuropil found in all insect groups but one.

   The mushroom bodies are in most insects intimately associated with the primary olfactory centers of the brain, the antennal lobes. In fact, a substantial part of the neural input to the mushroom bodies is olfactory. Olfaction is one of the most important senses to insects (Hansson 1995). Insects often rely on odors to locate and evaluate food and oviposition sites or to find a suitable mate (e.g. Visser, 1986; Baker 1989; Renwick 1989; Anderson et al. 1993). Particularly in nocturnal insects like moths, odors provide the major contribution of the sensory world. A good example of the impact the sense of smell has on insects is the great

importance of pheromones. Sexual pheromones are often crucial in order to at all detect and identify potential mates and many insects are extremely sensitive and acutely tuned to attraction to the conspecific pheromone but indifferent or directly repelled by those of other species (Hartlieb and Anderson 1999; Kehat and Dunkelblum 1990). This thesis will explore the general structure and function of the *Spodoptera littoralis* mushroom body and its involvement in olfaction.

Insect Mushroom Bodies

Discovery and early findings

In 1850, the French biologist Félix Dujardin, mostly known for his studies on protozoans, described that honey bees were equipped with two large, folded structures on top of their brains (Dujardin 1850). To Dujardin, these structures reminded of the folded cortex in mammals and he accordingly named them "lobes à convolutiones". When he compared with other insect groups he observed that these structures were larger and more conspicuous in social hymenopterans. Dujardin linked the prevalence of these cortex-reminiscent brain structures to the complex social structure and high behavioral adaptability of honey bees, and consequently ascribed them as being "corps d'intélligence". Several decades of studies followed and Dujardin's findings were confirmed in more insect groups and his ideas were further elaborated. The structures were later named according to their shape more than to their putative function: "gestielter Körper" (Leydig 1864), "Pilzhutförmiger Körper" (Dietl 1876), "mushroom bodies" (Packard 1880), "corpo fungiformo" (Bellonci 1882) (all in Kenyon, 1896b).

In 1896 F. C. Kenyon applied the newly developed Golgi method (see Box 1) on honey bee brains and could in detail describe the small, thin, tightly packed intrinsic neurons, globuli cells, that comprised the mushroom body (MB). We know them now as Kenyon cells (Strausfeld 1976). Kenyon also described converging sensory input leading to the MBs and essentially embraced Dujardin's view of the MBs as being "higher" brain centers in insects (Kenyon 1896a, b). The Golgi method has since then been applied to a wide range of insect species, including the subject of the current thesis, and they have all confirmed and elaborated the findings by Kenyon and his contemporaries (Reviewed by Strausfeld et al. 1998; Farris 2005b). The study of the insect brain and MBs and their application as models for linking neuroanatomy to function and behavior has expanded enormously and more detailed descriptions of particular findings will be provided below. In addition, MBs have also been shown to be a good substrate for studying evolution and development of the insect central nervous system.

General structure

Mushroom body-like structures, defined as lobed paired neuropils constituted by parallel axons of globuli cells, are found across divergent invertebrate taxa including Insecta, Chilopoda, Diplopoda, Chelicerata and some non-arthropod invertebrates such as Onychophora, Annelida and Platyhelminthes (Holmgren, 1916, in Strausfeld et al., 1998). A possible analogue to the MB, the hemiellipsoid

body (HB), has been proposed in decapod crustaceans. This analogy is, however, based more on vague functional similarities than on structural ones and it is highly debated whether these structures share a common origin with MBs (McKinzie et al., 2003; Strausfeld et al., 1998). In insects, MBs are found in all insect groups except one, the Archaeognata (Farris 2005b). MBs are thought to have arisen in different arthropod groups several times during evolution (Strausfeld et al., 1998) but within the Insecta the prevailing theory is that MBs are homologous structures that appeared early in the evolution of hexapod arthropods (Farris 2005b).

Although the structure of MBs is enormously variable across insect taxa, a few traits are common to all and basically provide the definition of MBs in insects (Fig. 1). The intrinsic neurons of MBs, the Kenyon cells (KC), are located at the rostrodorsal apex of the protocerebrum, according to the neural axis. In most insects, this means that the KCs in practice lie in the dorsoposterior part of the brain, since the protocerebrum and medial deutocerebrum often is tilted upwards and backwards compared to the neural axis. From here on, the description of the spatial orientation within the brain will be according to the observed situation and not according to the neural axis. The KC somata form two dense clusters of cells, one in each hemisphere and each cell extends a single neurite slightly down and forward in direction of the antennal lobes, in cases where antennal lobes are present. Proximal to the soma, dendritic branches project laterally from the neurite and, together with a number of afferent neural elements, form a dense conspicuous neuropil, often shaped like a bowl or a cup and hence termed the *calyx*. The calyx primarily receives sensory innervation from the antennal lobe via projection neurons, and it was the calyces that Dujardin (1850) first spotted in the honey bee. As the neurites exit the calyx, they assemble themselves into a densely packed bundle or stalk, called the *pedunculus*. The KC neurites within the pedunculus project forward through the brain and eventually diverge into two perpendicularly oriented lobes, one projecting vertically, toward the roof of the brain, and the other medially, reaching the midline just anteroventrally to the central complex, where it touches the tip of its contralateral counterpart in the opposite hemisphere. In *the vertical and medial lobes*, the KCs ramify with numerous terminal branches that may be both presynaptic and postsynaptic to extrinsic neurons that enter and exit the MB lobes (Fig. 1) (Ito et al., 1998; Li and Strausfeld, 1997; Mobbs, 1982; Pearson, 1971; Schildberger, 1983; Schürmann 1974; Strausfeld 1976).

MBs are in general parsed into parallel divisions (Fig. 4) (Crittenden et al., 1998; Heisenberg 1980; Larsson et al., 2004; Mizunami et al 1998a; Mobbs, 1982; Strausfeld, 2002; Strausfeld et al., 2000, 2003; Strausfeld and Li 1999b). This organization often reflects the birth order of the constituting KCs and in the calyx newborn KCs are located centrally and the distance from the calyx center increases with the age of a given KC (Cayre et al., 2000; Dufour and Gadenne 2006; Farris et al., 1999; Farris and Sinakevitch 2003; Farris and Strausfeld 2001; Malaterre et al., 2002). Thus, a concentric organization of the KCs can often be discerned in the calyx and in the pedunculus close to the exit from the calyx (Malaterre et al., 2002; Kurusu et al., 2002). In the lobes, this arrangement is substituted for a parallel organization of KCs (Fig. 4), which may be more or less strict, but the separation into age-related modules is in most cases maintained and younger cells are found posteriorly and increasingly older KCs are displaced to the anterior (Farris et al., 2004; Farris and Sinakevitch, 2003; Farris and Strausfeld 2001; Lee et al., 1999). This basic scheme is elaborated and altered in different insect taxa and some factors that may appear very different in different species are described below:
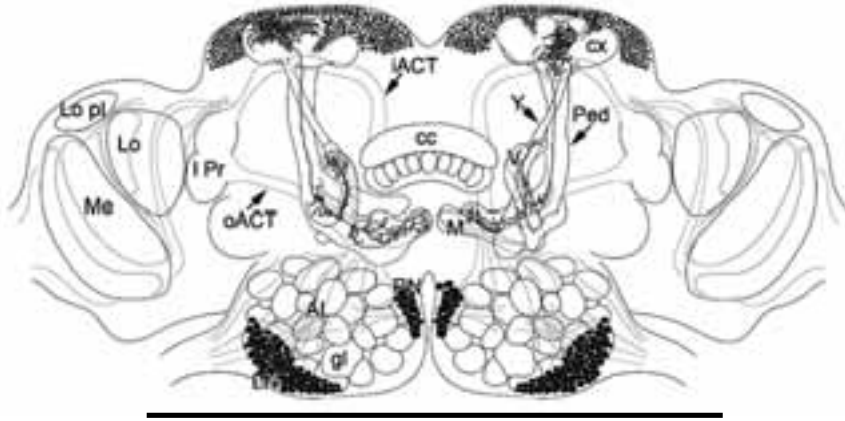
Fig. 1. Schematic overview of the insect brain, exemplified by the moth *S. littoralis*. The right hemisphere shows the mushroom body and its different components, Kenyon cell bodies (KC), calyx (cx), pedunculus (Ped), vertical lobe (V), medial lobe (M), Y tract (Y). In the mushroom bodies, two types of Kenyon cells are reconstructed. The left brain half gives examples of areas that are associated with the mushroom body: antennal lobe (AL), projection neuron somata (PN), local interneuron somata (LN), antennal lobe glomerulus (gl), inner antennocerebral tract (iACT), outer antennocerebral tract (oACT), lateral protocerebrum (l Pr). Other denoted structures: medulla (Me), lobula, (Lo), lobula plate (Lo pl), central complex (cc). Scale bar = 1 mm.

Extrinsic innervation

It is often stated that the calyx is the main sensory input region but this seems to hold true only when the insect is equipped with a functional olfactory system and antennal lobes (Strausfeld et al., 1998). In anosmic species or in insects with rudimentary antennae, like the Odonata and some aquatic insects, the calyces are absent or greatly reduced although the pedunculus and lobes may be well developed (Farris 2005b; Strausfeld et al., 1998). In these groups, as well as in calyx-equipped insects, the MB lobes and pedunculus receive multimodal afferent input originating from different parts of the brain (Ito et al., 1998; Strausfeld et al., 1998; Strausfeld and Li, 1999a; Li and Strausfeld, 1997). Amongst odor-sensitive insects, even within the same order or species, the elaboration of the calyx is highly variable and seems to be partly determined by afferent innervation (Fig. 3). Some of the most derived examples of MB calyces are found in social hymenopterans like the honey bee. The calyx of the honey bee is concentrically divided into three distinct zones: lip, collar and basal ring, each further parsed into smaller areas defined both by the input and by KC make-up (Abel et al., 2001; Ehmer and Gronenberg, 2002; Gronenberg, 2001; Mobbs, 1982; Strausfeld 2002) (also see below). Each zone is supplied by modality-specific input such that the lip receives olfactory input from the antennal lobe projection neurons, the collar is supplied by visual interneurons from the optic neuropils and the basal ring receives

combined input from the optic lobes, antennal lobe and the subesophageal ganglion (Abel et al., 2001; Ehmer and Gronenberg, 2002; Gronenberg, 2001; Schröter and Menzel 2003). In ants, the innervation pattern as well as the size of the calyx may vary with sex, age and caste within the same species (Ehmer and Gronenberg, 2004; Gronenberg, 1999; Gronenberg and Hölldobler, 1999). In the cockroach, *Periplaneta americana*, the input to the calyx is primarily olfactory but there is a spatial separation within the calyx, which differentially receive innervation originating from different parts of the antennal lobe, possibly carrying information about different odors (Strausfeld and Li, 1999a). In other species like the fruit fly *Drosophila melanogaster*, the relationship between input and KC branching seems to overlap extensively and zonation and differentiation of the calyx is less clear (Masuda-Nakagawa et al., 2005; Tanaka et al., 2004; Wong et al., 2002). Additional to sensory input to the calyx, MBs receive extensive inhibitory (Grünewald, 1999a, b; Bicker, 1999; Homberg et al., 1987; Perez-Orive et al., 2002; Strausfeld and Li, 1999a) and putative modulatory innervation, the pattern of which seems to differ between species (Dacks et al., 2005; Homberg and Hildebrand, 1989; Homberg et al., 2004; Kim et al., 1998; Nässel, 1999, 2000; Sinakevitch et al., 2005).

Intrinsic neuron types

So far, three basic classes of KCs have been described in insect MBs (Fig. 5). These classes are distinguished and defined both on morphological grounds and on their time of birth during the embryonic development (Farris, 2005; Farris and Strausfeld, 2003; Strausfeld and Li 1999b).

Class I neurons are the KCs to differentiate last during the development and are always found in the central region of the calyx. Their cell bodies normally fill the calyx cavity and the primary neurites of class I KCs line the inner surface of the calycal neuropil (Farris and Sinakevitch 2003). In the lobes, they are normally found in the posterior part. Class I KCs are usually equipped with spiny postsynaptic specializations on their dendritic branches but several morphological subtypes have been described (Schürmann, 1973; Strausfeld, 2002; Strausfeld et al., 2003; Strausfeld and Li, 1999b). Class I KCs are often the most abundant type, and in many insects, again with honey bee as the cardinal example, the dendritic trees of different class I KC subtypes occupy discrete calycal zones, often defined by the input as described above (Strausfeld, 2002). The separation seen between these KCs in the calyx is maintained in the lobes and these class I subtypes may also display varying chemical composition as revealed with immunocytochemistry, suggesting different functional properties (Strausfeld 2002; Strausfeld et al., 2000).

Class II KCs are in many insects the oldest KCs found. They are born before the class I neurons and are accordingly displaced by the class I toward the periphery in the calyx and anteriorly in the lobes as the class I cells develop although extensive rearrangement is observed in some species (Farris and Sinakevitch 2003, also see Farris et al., 2004). Class II KCs are also known as "clawed" KCs because of their dendritic morphology (Farris et al., 2004; Pearson, 1971; Schürmann, 1973;

Strausfeld, 2002; Strausfeld et al., 2003; Strausfeld and Li, 1999b), but claw-like dendrites are not exclusive to class II cells. The class II KCs occupy a separate part of the lobes, referred to as the γ lobe or γ layer, depending on the species studied (Pearson, 1971; Strausfeld, 2002; Strausfeld et al., 2003; Yang et al., 1995). This part is often morphologically distinguishable from the rest of the lobes. Differences between species are seen in the terminal branching patterns of the class II KCs. In the adult *D. melanogaster*, class II neurons only branch in the medial lobe, leaving the vertical lobe short of a γ division (Crittenden et al., 1998; Yang et al., 1995; Strausfeld et al., 2003). In the honey bee, the situation is reversed and only the vertical lobe is equipped with a thick γ lobe (Strausfeld, 2002). In both species, however, class II collaterals invade both the medial and vertical lobes during development but are secondarily pruned before eclosion (Farris et al., 2004; Lee et al., 1999). In the cockroach, the γ layer is represented in both the medial and vertical lobes. In the calyx, class II KCs branch throughout the neuropil and sample input from afferents in all calycal regions (Strausfeld 2002; Strausfeld et al., 2003; Strausfeld and Li, 1999b; Zhu et al., 2003). Both class I and II KCs seem to be very common and are found in most studied insects, however, in the scarab beetle, *Pachnoda marginata*, no spiny KCs have been found (Larsson et al., 2004).

Lastly, *class III* KCs have so far only been convincingly described in a few species, but where present they are always the first born (Farris, 2005b; Farris and Sinakevitch, 2003; Farris and Strausfeld, 2003). Class III KCs often display a peculiar branching pattern, quite different from that of the other two classes. Their dendrites do not invade the primary calycal neuropil in most species but form a separate accessory calyx, adjacent and posterior to the primary calyx. However, in Lepidoptera, putative class III KCs enter the primary calyx but from the peripheral wall and not from the inner half of the calyx like classes I and II. In the lobes, they do not fuse with the other KCs but form a separate neuropil called lobelet or satellite lobe (Farris, 2005b; Farris and Strausfeld, 2003; Pearson, 1971). In some insects, like the Lepidoptera and Neuroptera, they also form a separate tract from the calyx, the Y tract, and never enter the pedunculus (Ali, 1974; Pearson, 1971). The dendritic morphology of class III KCs is not well described and appears to be variable between species (Farris, 2005b). The reason class III KCs are not found throughout all insects might be that they are either diffusely integrated into the rest of the MB or that they are secondarily lost, possibly with developmentally younger KC classes adopting their function (Farris, 2005b).

Neuronal development

The KCs stem from groups of mushroom body neuroblasts (MBNBs) that are situated in the very center of what will be the calyx. However, the number of MBNB groups in each brain hemisphere varies between insects and as a result, the number of calyces providing each MB also varies. In the honey bee, two clusters of MBNBs are observed in each brain hemisphere of the newborn first instar larva and these clusters will give rise to two identical calyces in each hemisphere (Farris et al., 1999). The distantly related *P. americana* also has two large calyces in each MB. In the cricket, *Acheta domesticus* (Schürmann, 1973; Malaterre et al., 2002),

as well as in the basal insect firebrat, *Thermobia domestica* (Farris, 2005a), only a single primary calyx develops for each MB. *Acheta* has, however, also a large accessory calyx, called the posterior calyx, constituted by putative class III KCs. In yet other groups, like Dermaptera and Neuroptera, three MBNB clusters give rise to as many calyces (for review see Farris, 2005b). In *D. melanogaster*, only one single calyx is observed in the imago but studies using genetic markers have revealed that this single calyx derives from four original MBNBs, each hypothetically giving rise to an embryonic hemicalyx that are secondarily fused (Ito et al., 1997; Kurusu et al., 2002; Tettamanti et al., 1997; Zhu et al., 2003).

Holometabolous and hemimetabolous insects display some clear differences in MB structure. As mentioned above, KCs are deposited sequentially during development, which produces a columnar or layered appearance of the lobes. In Holometabola, with *D. melanogaster* as example, KCs are produced during the larval stages in consecutive bouts (Lee et al., 1999). First the class II KCs are born and then two types of class I, each innervating a separate division in the lobes. This results in a tripartite structure, with more morphological and biochemical subdivisions overlaid on this basic pattern (Strausfeld et al., 2003). In the honey bee, the basic structure also consists of a few large sequentially produced divisions, the γ lobe containing class II KCs and representing the whole calyx, and three class I divisions, each representing one of the calyx zones (Mobbs, 1982; Strausfeld 2002; Strausfeld et al., 2000). Each division is, however, further parsed into multiple strata with biochemically and morphologically differentiated KCs (Strausfeld, 2002) and the basic sequential pattern is further modulated by axon rearrangement during development (Farris et al., 2004). In hemimetabolous insects, new (class I) KCs are produced during each instar throughout the preimaginal development, and sometimes into adulthood, resulting in a continuously laminar appearance of the lobes. However, in the first instar, before a large laminar array of class I KCs has developed, the structure resembles that in holometabolous insects and tracing of KCs that occupy functional regions in the calyx to the lobes reveals that the cockroach MB is also parsed into a few large presumably functional divisions (Strausfeld et al., 1998; Strausfeld and Li, 1999b).

Size and shape

One obvious discrepancy between MBs in different species is the size. It ranges from extremely well developed MBs containing hundreds of thousands of KCs like in the honey bee and cockroach, to small ones consisting only of a few thousand cells, like in the fruit fly. These differences in size do not seem to be directly linked to phylogenetic relationships but rather to the behavioral ecology of the insect (Farris, 2005b). The MB size may also differ between sexes and castes or between individuals of different age and even experience within the same species, as is observed in honey bees and ants (Ehmer and Gronenberg, 2004; Gronenberg, 1999; Gronenberg and Hölldobler, 1999; Farris et al., 2001; Seid et al., 2005; Withers et al., 1993). However, age-related growth of the MB in adult holometabolous insects does not generally involve neurogenesis but is the result of growth and elaboration of neural processes. The deep convolution of the calyx seen in many behaviorally complex insects has been interpreted as a result of the
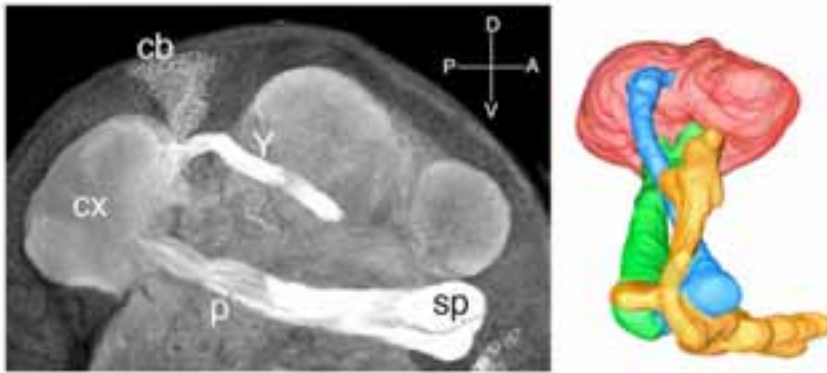
Fig. 2. Structure of the mushroom body in three dimensions. On the left a sagittal view of the *Spodoptera* mushroom body labeled with DC0-antibody. Calyx (cx), pedunculus (p), spur (sp), Y tract (Y), Y tract KC bodies (cb), anterior (A), posterior (P), dorsal (D), ventral (V). On the right a three-dimensional reconstruction of the mushroom body seen in an anterior view. Different colors denote components, calyx (red), pedunculus (green), Y tract (blue), lobes (yellow).

need for a large calycal area, and analogous to the folding of the vertebrate cortex (Farris, 2005b). This feature seems to be enhanced in generalist feeders in contrast to specialists even of closely related genera (Farris and Roberts, 2005). An increase in MB size and KC count often seems to be accompanied by elaboration of the calyx and lobes and the acquisition of functional sub-compartments like in the honey bee. However, it is possible that distinct compartments become visible to an observer only when they comprise a large number of cells. Functional segregation might be less conspicuous when the constituting cells are few. While growth and elaboration of the calyx seems important in some groups, the lobes are the more complex parts in others. In termites for example, the calyces are small and simple whereas the lobes are highly differentiated into separate neuropils (Farris, 2005b; Farris and Strausfeld, 2003).

All of the factors exemplified above are of course intimately linked to one another and cannot be completely separated or regarded solely when discussing the structure of MBs in different insects. Probably a combination of genetic and developmental prerequisites and ecological and behavioral constraints work in concert to shape and optimize the anatomy and function of MBs in every specific case.

Functions of the Mushroom Bodies

The insect MBs have been proposed to take part in a multitude of different complex functions. These include sensory integration, context recognition, generalization, choice behavior, motor control, spatial learning and olfactory discrimination (Besson and Martin, 2004; de Belle and Heisenberg, 1994; Erber et al., 1980; Hammer, 1993; Heisenberg et al., 1985; Laurent and Naraghi, 1994; Li

and Strausfeld, 1997, 1999; Liu et al., 1999; Martin et al., 1998; Mauelshagen, 1993; Mizunami et al., 1998b, c; Pascual and Préat, 2001; Perez-Orive et al., 2002; Schildberger, 1984; Stopfer et al., 1997; Tang and Guo, 2001; Tully and Quinn, 1985; Wang et al., 2003). Lately, one of the more intensely studied functions is the MBs' role in different types of olfactory learning and memory. Much of the work focused on MB function has comprised chemical or genetic ablation or disruption of the MBs and subsequent observation of how this affects specific behaviors. This may be considered as a blunt approach but with the development of novel genetic tools in the fruit fly and other insects, increasingly refined interference is possible. Another way to find out about function is to record from identified neurons, intrinsic or extrinsic to the MBs, and test how they respond to different sensory stimuli and if responses are context and memory dependent.

Learning and memory

During the early studies of genetic control of learning and memory in fruit flies, two basic approaches were employed (Mcguire et al., 2005). One approach directly screened for behavioral memory defects in chemically induced *Drosophila* mutants and another screened for mutants with structural brain defects, which secondarily were tested for memory performance. One of the first mutants identified with the former approach, *dunce* (*dnc*) (Dudai et al., 1976), was later shown to be deficient in a form of cyclic AMP (cAMP) phoshodiesterase, preferentially expressed in the MBs (Byers et al., 1981; Davis and Kiger, 1981; Nighorn et al., 1991). The latter approach produced a couple of structural mutants, *mushroom body deranged* (*mbd*) and *mushroom body miniature* (*mbm*) that exhibited severe impairment in their olfactory learning abilities (Heisenberg et al., 1985). The specific genes involved in these mutations are, however, not known. Several other memory-deficient *Drosophila* mutations with a link to the MBs have been described: *rutabaga* (*rut*) (Aceves-Pina et al., 1983) encodes a type of $Ca2+$/calmodulin-G-protein-sensitive adenylyl cyclase, which is also involved in the cAMP cascade (Livingstone et al., 1984). Local rescue of *rut*-expression in the MBs restored short-time memory (STM) functions (McGuire et al., 2003; Zars et al., 2000). Learning is often divided into temporal phases; short-term memory (STM), lasting minutes to an hour, middle-term memory (MTM) generally lasting for a few hours and long-term memory (LTM) which may last for days or the entire life of the insect. For LTM to be established several studies have shown that transcription and protein synthesis in KCs is required (Dubnau et al., 2003; Tully et al., 1994). Also, flies lacking the vertical lobe cannot form LTM (Isabel et al., 2004; Pascual and Préat, 2001). Yet anther gene *DC0*, encodes in *Drosophila* a catalytic subunit of protein kinase A (PKA), which is a downstream effector molecule of cAMP, and flies with mutated *DC0* are memory deficient (Skoulakis et al., 1993). Transient down-regulation of PKA activity in the honey bee also impaired long-term memory (Fiala et al., 1999). As seen in some of the examples above, the cAMP signaling cascade seems to be an important component of olfactory associative learning and, although an ubiquitous signaling pathway, the high expression of these identified genes in the MBs has been a major incentive for ascribing learning and memory functions to the MBs.

Non-mutagenic experimental approaches have also been used for investigating MB's role in learning and memory: Chemical ablation of the MBs by feeding *Drosophila* larvae with hydroxyurea (HU) which selectively kills MBNBs if administered at a specific time during development resulted in, otherwise seemingly normal, odor-learning deficient flies (de Belle and Heisenberg 1994). Focal cooling of specific parts of the brain in honey bees, including the MBs, transiently extinguished odor learning (Erber et al., 1980). Furthermore, studies using the temperature-sensitive dynamin mutant transgene *Shibire* to transiently disrupt synaptic vesicle recycling in *Drosophila* KCs demonstrated that neuronal transmission in the MBs is essential for retrieval but not acquisition of olfactory associative memory (Dubnau et al., 2001; Mcguire et al., 2001). A caution to studies using insects with ablated MBs is appropriate due to the observation that synaptic transmission through the MBs is necessary for odor attraction but not repulsion (Wang et al., 2003). The projection neurons originating from the antennal lobes reach several higher brain centers and it is only a part of them that feed into the MBs directly. A criterion for "normal" olfactory function in flies with corrupted MBs has often been avoidance behavior to repellent odors, which in contrast to attraction, may be independent of MBs.

Another *Drosophila* mutant, *amnesiac* (*amn*) (Quinn et al., 1979), defective in short-term memory, was shown to be linked to the expression of a putative neuropeptide, similar to the pituitary adenylyl cyclase activating peptide (PACAP), in a pair of extrinsic neurons, the dorsal paired neurons (DPM) that extensively innervate the MB lobes (Feany and Quinn, 1995; Waddell et al., 2000; see also Keene et al., 2004; Yu et al., 2005). Several other extrinsic modulatory neurons are thought to be involved in learning in the MBs. In the honey bee a large identified neuron carrying octopamine, called the ventral unpaired median neuron of maxillary neuromere 1 (VUMmx1) provides the unconditioned stimulus (US) to the MB calyx during reward-associated olfactory conditioning (Hammer, 1993; Kreissl et al., 1994). It was further demonstrated that local injection of octopamine to the calyx, but also to the antennal lobes, during conditioning could substitute for a sugar reward (Hammer and Menzel, 1998). In *Drosophila*, it has been shown that octopamine and dopamine conveys different reinforcing stimuli during training. Like in the honey bee, octopamine represents appetitive reinforcement, while dopamine mediates the US during aversive conditioning (Schwaerzel et al., 2003). Dopamine, octopamine and serotonin all have an augmenting effect on PKA activity in honey bee KCs (Müller, 1997). Other neurons associated with the MBs clearly change their response pattern during learning. One example is an identified neuron in the brain of the honey bee, termed Pe1, which has dendritic branches in the MB pedunculus and terminates in the lateral protocerebum. This neuron has been shown to selectively increase its response to a reward-paired odor but not to an unpaired one (Mauelshagen, 1993). Local inhibitory so-called MB feedback neurons also change their response to conditioned odor stimuli but not to unconditioned ones (Grünewald, 1999b). Additional to olfactory conditioning, spatial learning in cockroach has been shown to be dependent on the structural and functional integrity of the MBs (Mizunami et al., 1998c).

As is evident from the few examples given above, the mechanisms for the MB's role in learning and memory in insects is far from clear. It is not completely understood whether the crucial factors are found within the KCs (suggested by cAMP-related mutants), on the presynaptic side (exemplified by DPM and VUMmx1 neurons, as well as disruption of neuronal transmission through KCs) or on the postsynaptic side (e.g. the Pe1 and feedback neurons in honey bee) or possibly in all three locations. Furthermore, the antennal lobe has also been shown to be involved in olfactory learning (Faber et al., 1999; Farooqui et al., 2003; Hammer and Menzel, 1998; Yu et al., 2004) and neural plasticity (Devaud et al., 2001) and associative visual, tactile and motor learning in fruit fly is not dependent on MBs (Siwicki and Ladeweski, 2003; Wolf et al., 1998).

Sensory integration, context recognition, generalization, choice behavior and motor control

Somewhat in the shade of olfactory learning, several other functions have drawn researchers' attention to the MB. Most notable is the, in some species, massive multisensory convergence that takes place in the MBs. This is obvious in Hymenoptera, where the MB receives substantial visual and gustatory/mechanosensory input to the calyx in addition to olfactory innervation. It is less anatomically evident in other species like the cockroach, in which only a single visual neuron feeds into the calyx (Strausfeld and Li, 1999a). Instead, several large multimodal interneurons innervate the calyx, pedunculus and lobes from different protocerebral neuropils. These neurons have been shown to be sensitive to sound, different visual cues, odors and mechanical stimulation (Li and Strausfeld, 1997; Strausfeld and Li, 1999a). Efferent neurons in cockroach MB also respond to multisensory stimulation (Li and Strausfeld, 1997, 1999). Similar neurons have also been identified in crickets (Schildberger, 1984) and in honey bees: the Pe1 neuron, mentioned earlier, responds to visual, mechanosensory and olfactory stimuli (Rybak and Menzel, 1998).

Experiments with chronically implanted microelectrodes in the cockroach brain have revealed that neurons in the MB respond differently to self-administered and externally produced mechanical stimulation (Mizunami et al., 1998b). These recordings also showed that the MBs monitor the position and actions of various parts of the body. This suggests that the MBs can integrate sensory-motor events and recognize the contextual background to the stimuli. In *Drosophila*, animals with ablated or disrupted MBs show elevated walking activity (Martin et al., 1998), indicating that also in flies, the MBs monitor and modulate motor activity under normal conditions.

Furthermore, recent studies using contradictory visual cues have shown that MBs are involved in choice behavior and flies with the *mbm* mutation behave differently than wild type flies (Tang and Guo, 2001). MBs may also be involved in visual context generalization, as changes in the contextual background between training and testing during visual learning affects the memory in flies with manipulated MBs but not in normal flies (Liu et al., 1999). This function may be similar to the spatial learning observed in cockroaches (Mizunami et al., 1998c)

and may also be linked to a behavior known as centrophobism, which means that flies in this case avoid open spaces. This behavior is disrupted by MB interference (Besson and Martin, 2004).

Olfactory processing

Another fundamental MB research topic is the direct involvement of MBs in olfactory coding and processing. In insects, odors are primarily detected by the antennae, which carry olfactory receptor neurons (ORN) housed in sensory hairs or sensilla. The ORNs send their axons to the primary olfactory neuropil, the antennal lobe (AL), which is the functional and anatomical analogue of the olfactory bulb in vertebrates (Hildebrand and Shepherd 1997). In the AL, ORNs make synaptic contacts with local interneurons (LN) that branch only within the AL, and with projection neurons (PN), which convey the olfactory information to other parts of the brain (Fig. 1) (Anton and Homberg, 1999). These synaptic contacts are within the AL anatomically concentrated into spherical complexes called glomeruli, which contain the terminal branches of ORNs and dendritic ramifications of PNs and both terminal and dendritic collaterals of LNs (Distler et al., 1998; Sun et al., 1997). Typically, ORNs of a specific type, i.e. those carrying one specific membrane-bound odor receptor, converge into one or a few given glomerulus/i and the number of glomeruli in the AL thus roughly represents the number of ORN species found in the antenna (Gao et al., 2000; Voshall et al., 2000, but also see Goldman et al., 2005 and Larsson et al., 2004). Most of the LNs are GABAergic and inhibitory but several, both morphological and biochemical, subtypes have been described. (Anton and Homberg, 1999; Homberg and Müller, 1999). The LNs shape the pattern of activated glomeruli during odor stimulation, which results in a complex spatiotemporal pattern across activated glomeruli that evolves over time (Carlsson et al., 2005; Galizia et al., 2000; Sachse and Galizia, 2001; Wilson and Laurent, 2005). Accordingly, PNs with various degrees of qualitative tuning exhibit temporally differential response patterns, including excitation and inhibition, during an olfactory stimulus (Christensen et al., 1998, 2000; Laurent and Davidowitz, 1994; Lei et al., 2002, 2004; Perez-Orive et al., 2002; Reisenman et al., 2005; Wilson et al., 2004). Odor-evoked activity of PNs may outlast the olfactory stimulus itself, but can also be very brief (Lei and Hansson 1999). The PN axons exit the AL through several tracts, the number and trajectory of which may vary depending on the species, but typically they reach the ipsilateral MB calyx via two trajectories, one medial and one lateral (Anton and Homberg, 1999). The PNs in the medial tract, in moths called the inner antennocerebral tract (iACT), reach the calyx where they give off numerous terminal collaterals and continue laterally and ventrally to their final targets in the lateral protocerebrum. The lateral tract, in moths the outer antennocerebral tract (oACT), reaches the same targets but in reverse order.

The combinatorial pattern of activated PNs and their temporal dynamics are thought to be decoded by the KCs. The exact mechanisms for this decoding remain unclear but specific observations that may provide some clues are beginning to emerge. KCs seem to temporally sharpen the signal provided by PNs, meaning that while PNs often respond with phasic-tonic trains of action potentials that may last

for seconds, KCs respond with a short fast burst of only a few spikes (Laurent and Naraghi, 1994; Perez-Orive et al., 2002; Stopfer et al., 2003; Szyszka et al., 2005). KCs also seem to sparsen the signal, meaning that while PNs may be widely tuned, KCs are highly specific and respond only to one or a few stimuli (Perez-Orive et al., 2002; Stopfer et al., 2003; Szyszka et al., 2005; Wang et al., 2004). Perhaps contradictory to these observations is the finding that there is a massive divergence as well as convergence of the signal from PNs to KCs. PNs are contacted by many KCs and KCs receive input from numerous PNs (Perez-Orive et al., 2002; Szyszka et al., 2005).

Clearly, some highly efficient mechanisms are at work to accomplish the observed temporal and qualitative specificity in the response of KCs. One of these mechanisms may be provided by GABAergic feed-forward inhibition to the calyx via interneurons originating in the lateral protocerebrum (Perez-Orive et al., 2002). Such neurons have been found in several species and since PNs also terminate in the lateral protocerebrum these neurons are thought to mediate a delayed inhibitory input to the KCs, partly explaining the short response. Another observation is that KCs seem to have active membrane conductance properties in their dendrites (Perez-Orive et al., 2004), possibly mediated by voltage-sensitive Ca^{2+}-channels (Grünewald, 2003; Schäfer et al., 1994). This could favor detection of coinciding input events by transiently amplifying local excitatory postsynaptic potentials (EPSP) into dendritic spikelets, which elicit a response in the neuron only if they occur simultaneously, as opposed to passive graded potentials, which summate the input over a longer time. This could contribute to the sparse activation, since even if a KC receives input from many PNs, it would only respond if a given number of them fire simultaneously. Interestingly, similar active membranes properties have been suggested in parasol cells, which are the intrinsic neurons of hemi-ellipsoid bodies in crustaceans (Mellon, 2003) (see above). But why would PNs fire synchronously? From studies in locusts and honey bees, it has been suggested that part of the function of the LNs in the AL, additional to spatiotemporal shaping of the output, would be to actively drive oscillating synchronous activity in the PNs (Stopfer et al., 1997; Laurent and Naraghi, 1994; Macleod and Laurent, 1996). This is reflected in oscillations of the local field potential (LFP) in the AL, which only occurs during olfactory stimulation. By pharmacologically blocking GABA$_A$-like receptors in the AL, it was shown that synchrony and oscillations disappeared and fine discrimination between similar odors was impaired (Stopfer et al., 1997). In locusts, coherent LFP oscillations are also seen in the calyx and recordings from PNs have shown that spiking activity is phase-locked to the LFP oscillations (Laurent and Davidowitz, 1994). If these observations are universal, it would mean that information-carrying PN spikes that arrive at the KCs are phase-locked and synchronous and occur in oscillating bouts, separated by delayed, phase-shifted cyclic inhibition from the GABAergic feed-forward neurons in the lateral protocerebrum (Perez-Orive et al., 2002). However, most of the data supporting these ideas has been obtained in locusts, which have an altogether different anatomical structure of the AL compared to e.g. honey bees, flies and moths (Anton and Homberg, 1999; Ignell et al., 2001). Moreover, similar patterns of phase-locking between PN activity and LFP in the AL or the MB have not been found in the brain of the moth *Manduca sexta* (Christensen et al., 2003).

Much remains to be understood in this system and considering that not even the ultimate function and nature of the output from the antennal lobe is completely known (e.g. see Ng et al., 2002 and Wilson et al., 2004), it is difficult to draw firm conclusions about the MB's role in the coding of odors. Possibly there are general principles that apply to all insect groups but there may also be genus- or species specific differences in these mechanisms, as suggested by the vast anatomical differences in MB and AL structure. The present thesis will investigate some of these mechanisms in *S. littoralis* and compare the findings with those obtained in other species.

The model

Spodoptera littoralis (Boisd.)(Noctuidae: Lepidoptera), or the Egyptian cotton leaf worm moth, is a nocturnal insect distributed over northern Africa, the Middle East and throughout the Mediterranean countries. It is a pest on many crops and cause great damage to cotton fields and alfalfa (Avidov and Harpaz, 1969). It is a true generalist with respect to host plants and feeding larvae have been found on a wide range of plant species from over 40 different families (Brown and Dewhurst 1975). Both the male and the female are heavily dependent on olfaction in order to find resources, which for the female are suitable, healthy plants to oviposit on and for the male a receptive female to mate with. Both sexes nectar-feed when possible. *S. littoralis* is a short-lived insect and the adult dies within two weeks after eclosion.

Male *Spodoptera littoralis.*

Olfaction in *Spodoptera littoralis*

The olfactory system of *S. littoralis* is well studied, both at the periphery and in the brain. On the antennae, the male moth has about 6,000 olfactory sensilla and the female about 4,000. The higher number in the male is attributed to an increase of pheromone-sensitive sensilla, while the number of plant-odor specific sensilla seems equal to both sexes (Ljungberg et al., 1993). At least 24 specific ORN types have been described (Anderson et al., 1995), but since the number of olfactory glomeruli in the AL is roughly 60, several ORN types probably remain to be found. The glomeruli are sexually isomorphic except for a macroglomerular complex (MGC) devoted to input from pheromone-sensitive sensilla, which is only found in the male (Anton and Hansson, 1994, 1995; Ochieng et al., 1995; Sadek et al., 2002). From the AL, PNs leave through several tracts: the iACT and oACT carry axons from mainly uniglomerular PNs reaching the MB calyx and lateral protocerebrum (Anton and Hansson, 1994, 1995). The mediolateral antennocerebral tract (mlACT), carries axons from multiglomerular PNs to the lateral protocerebrum only (Anton and Hansson, 1994, 1995). While the input to each glomerulus is considered to be fine-tuned, originating from a unique ORN type, the output via PNs is more complex. Studies have reported PNs that may respond to the same stimuli although they branch in different glomeruli and PNs in the same glomerulus may exhibit different response properties (Anton and Hansson, 1994, 1995; Sadek et al., 2002). PNs also exhibit complex stimulus specific temporal response patterns that include both excitation and inhibition (Anton and Hansson, 1994, 1995; Carlson et al., 2005; Sadek et al., 2002). Much of this non-linear patterning is thought to be mediated by lateral interactions between glomeruli through the local interneurons (LN). The over-all response to odors in the AL is spatially and temporally dynamic and the pattern of active glomeruli is dependent both on the concentration and identity of the tested compounds (Carlsson and Hansson, 2003; Carlsson et al., 2002, 2005; Meijerink et al., 2003).

Odor-elicited behavior of *S. littoralis* is best studied in males with emphasis on attraction to female pheromone. The female sex-pheromone contains a blend of at least two active components, (*Z,E*)-9,11-tetradecadienyl acetate ((*Z,E*)-9,11-14:OAc) and (*Z,E*)-9,12-tetradecadienyl acetate ((*Z,E*)-9,12-14:OAc) (Campion et al., 1980; Nesbitt et al., 1973). Males have been shown to be super-sensitive to these substances and display a change in heart beat rate as a response to stimulation with minute amounts, down to a theoretical value of six molecules hitting the antenna (Angioy et al., 2003). Males increase their behavioral sensitivity to these compounds after a single previous exposure to the complete female pheromone, however without any mating experience (Anderson et al., 2003). Another interesting system has also been studied. Noctuid moths have auditory organs on their thorax, which are tuned to the frequencies emitted by an echo-locating bat (Roeder, 1969). When a flying moth hears a bat cry it will, depending on the strength of the sound, i.e. the distance to the bat, exhibit evasive maneuvers or simply stop flying and drop to the ground in order to escape

predation (Miller and Surlykke, 2001; Roeder, 1962). However, in the presence of female sex pheromone male moths are willing to take a much higher risk of being eaten than without the pheromone. An intricate trade-off behavior dictated by the relative salience of the pheromone signal and bat sound has been demonstrated (Skals et al., 2005).

In females, odor-guided behavior has been studied in oviposition experiments, showing that gravid females can distinguish between and prefer a healthy host-plant over one that is under herbivore attack (Anderson and Alborn, 1999; Anderson et al., 1993; Jönsson and Anderson, 1999). Plant-odor compounds are similarly detected by the antenna and in the brain of both sexes, suggesting that the ability to locate host-plants is not exclusive to females. (Anderson et al., 1995; Anton and Hansson, 1994, 1995). Finally, associative learning to odors is effective in both females and males (Fan et al., 1997; Fan and Hansson 2001) with one significant difference. Both sexes readily learn to associate plant odors and individual pheromone components with a sugar reward but only females, although less robustly, will learn the complete female pheromone blend, indicating partly different processing strategies of olfactory signals between the two sexes (Hartlieb et al., 1999).

Questions

- How is the structure of the MB in *S. littoralis* related to that found in other insects? What similarities and dissimilarities can be seen?

- How does the MB structure and size relate to the behavioral ecology of this moth? *S. littoralis* is nocturnal and primarily guided by odors. Is this reflected in the afferent input to the MBs? The moth is short-lived and leads, compared to many other insects, a rather simple life with a hypothetical low demand on elaborate learning and memory or social competence. Is this reflected in a small or simple MB or is the structural complexity seen in many other species a general feature necessary for normal basic functions?

- How do moth KCs respond to odor stimulation of the antennae? Are they highly selective like those KCs previously described in e.g. locusts and honey bees? Do moth KCs sparsen the signal coming from AL PNs?

Summary of Results

Mushroom Body Structure in *Spodoptera littoralis*

Gross morphology of the moth mushroom body (Papers I-IV)

Reduced silver staining (Bodian, 1937) clearly showed the position and size of the MBs relative to other identifiable structures in the protocerebrum. It also revealed the different parts of the MB, namely: the calyx, pedunculus, Y tract, vertical and medial lobes and the Y tract bulbs or lobelets (Fig. 1). The calyx is composed of two partly fused cups, or calyces proper, one medial and one lateral. The double calyx, about 200 μm across, is located in the dorsoposterior part of the protocerebrum and multiple KC somata cover its dorsoposterior surface and also invade the cavities formed in the center of each calyx (Fig. 3). The calyces consist of fine, glomerular neuropil and from each calyx, a thick bundle of KC neurites, the pedunculus necks, emerges and projects anteriorly and ventrally. Immediately after the exit from their respective calyx, the necks merge to form the pedunculus, which travels down and forward in the direction of the dorsal margin of the AL. The pedunculus reaches a length of about 300 μm before it swells into a small bulbous structure, the spur, and bifurcates into two perpendicularly oriented neuropils, the vertical and medial lobe (Fig. 2). The vertical lobe curves backwards and up through the surrounding brain tissue until it reaches the dorsofrontal surface of the brain. The medial lobe reaches toward the brain midline, bending slightly down and backwards until it touches its contralateral partner in the opposite hemisphere. Additional to the pedunculus, a thinner second tract, the Y tract, emerges from the calyx' dorsolateral margin and passes diagonally in front of the pedunculus on its way to the base of the medial lobe, where it terminates in a pair of club-like bulbs, here called lobelets. The vertical and medial lobes are clearly heterogeneous and silver-staining preparations allowed us to describe the clear separation and organization of three longitudinal divisions of each lobe in detail for the first time in a moth (Fig. 4). These divisions were named after similarly oriented divisions in the MB of previously described species. Accordingly, the most anterior division of both lobes is called γ (originally used in *Sphinx ligustri*, Pearson, 1971). The most posterior division is in the vertical and medial lobes called α and β respectively, and the intermediary division α′ and β′ respectively (these terms were adopted from *Drosophila melanogaster*, Heisenberg, 1980; Crittenden, 1998; Strausfeld et al., 2003). The divisions of the lobes lie adjacent to one another as they diverge from the pedunculus, but as the distance from the pedunculus base increases the α division separates from the two anterior divisions and bends backwards. The two remaining divisions of the vertical lobe stay close together and toward the surface of the brain, the γ division envelopes the tip of the α′ (Paper II). In the medial lobe the divisions stay attached throughout their lengths. Further, silver staining resolved the spatial relationship between the medial lobe and the Y tract bulbs, revealing that the ventral lobelet penetrates through the medial lobe neuropil and emerges on its ventral surface (the term Y tract was adopted from Pearson's (1971) study of the moth *Sphinx ligustri*).
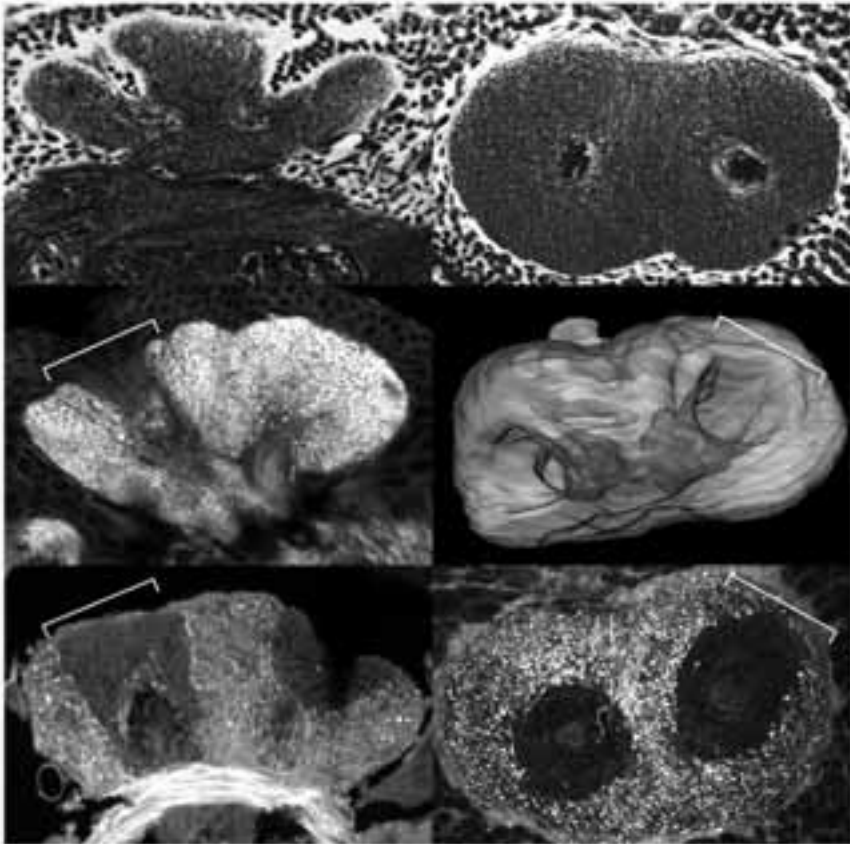
Fig. 3. Organization of the calyx. The top row shows Bodian silver stained preparations, horizontal section to the left and posterior view on the right. The calyx neuropil has a fine glomerular structure. Note KC somata in the calyx cavities. The middle row shows on the left a phalloidin stained calyx and on the right a three-dimensional reconstruction made after a phalloidin stained brain. Note the separation into an outer zone and an inner rim (bracket). Bottom row shows brains with dye-filled antennal lobe projection neurons, horizontal on the left and posterior on the right. Note the lack of antennal innervation in the inner rim.

Fluorescent staining with phalloidin, which selectively binds to F-actin (Rössler et al., 2002), confirmed the observations of the silver stained preparations but also revealed that each calyx is further parsed into two concentric neuropils, a broader peripheral ring containing a central thinner one, the latter referred to as the rim (Fig. 3) (Paper I). The double concentric arrangement in the calyx and the tripartite (omitting the Y tract) organization in the lobes present a clear mismatch. How do the calycal subdivisions relate to the ones in the lobes? And how are the two calyces, lateral and medial, represented in the lobes?

These questions were partly answered by applying different antisera that differentially labeled specific parts of the MB (Papers II and III). Antibodies
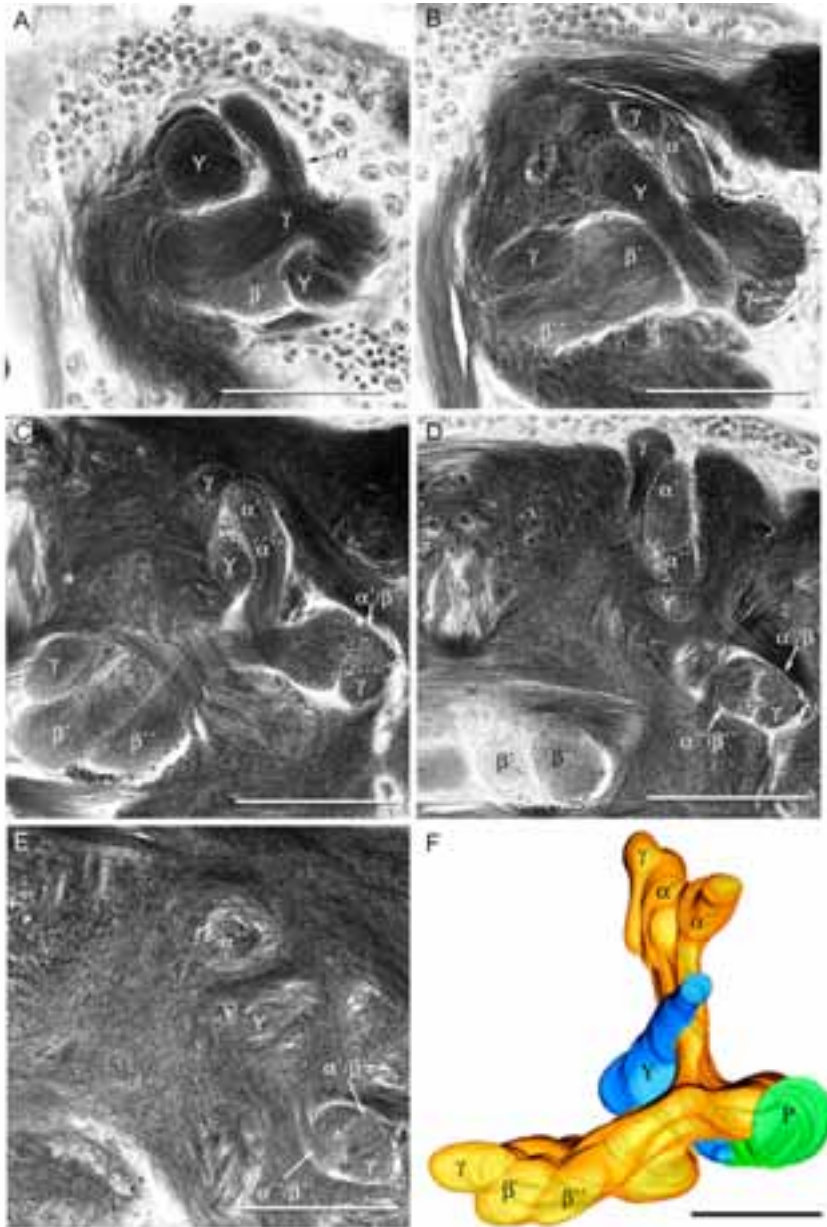
Fig. 4. Organization of mushroom body lobes and their subdivisions. **A-E:** Selected images from a series of reduced silver-stained frontal sections, from anterior to posterior in the brain showing the different divisions of the MB lobes and their three-dimensional organization. α/β-, α′/β′-, γ-lobes, dorsal and ventral lobelets (d lobl, v lobl) and Y-tract (Y) are indicated in each section. **F:** Posterior view of a 3D-reconstruction of a phalloidin-stained mushroom body to illustrate the relative locations of mushroom body subdivisions. Pedunculus (Ped; green), Y-tract (Y; blue). Scale bars = 100 μm. Reprinted with permission from Wiley-Liss, Inc.

against the peptide Mas-allatotropin only labeled the two posterior divisions selectively, thus facilitating tracing the KC bundles supplying these lobes through the pedunculus to the calyx. This labeling revealed that the two bundles supplying the α/β and α′/β′ divisions merge about half way to the calyx and fibers from each bundle mix diffusely. Closer to the calyx two new bundles, the pedunculus necks, emerge and enter the calyx neuropil. This indicates that there is an equivalent contribution from the lateral and medial calyces to each of the lobe divisions and this was further substantiated in a Golgi-impregnated preparation. The correspondence between the concentric arrangement in the pedunculus necks and the lobes was further established by immunolabeling with an antiserum against the amino acid taurine. This antiserum labeled the γ division and the posterior margin of the α/β divisions, in other words the most anterior and the most posterior portions of the lobes respectively. In the pedunculus necks labeling was observed in the very periphery (corresponding to the γ division) and in the core of each neck (corresponding to α/β). Additionally, the Y tract and lobelets were strongly labeled by taurine antiserum. In the calyx these three labeling patterns overlapped and only the stronger labeling belonging to Y tract neurons could be discerned from the background (see summary in fig. 8).

In the central core of each neck and at the very edge of the α/β, a minute non-taurinergic region was observed. In previous studies, KCs in this position have been shown to be immunopositive to antibodies against the amino acid glutamate (Sinakevitch et al., 2001; Strausfeld et al., 2003). These KCs are probably the youngest neurons since developing KCs generally grow out through the core of the pedunculus (Farris and Sinakevitch, 2003). To see whether this pattern also applies to moth we used a glutamate antibody, which indeed labeled central core fibers leaving the calyx, confirming observations in other species (Paper III). An antiserum against the neuropeptide FMRFamide revealed differential labeling within the α/β and α′/β′ divisions such that the anterior margins of both divisions were labeled. The entire γ division was also immunoreactive to this antiserum (Fig. 8). Lastly, two antibodies labeled the entire MB more or less indifferently. These were anti-DC0 and anti-aspartate, both previously found to ubiquitously label MBs in several insects (Farris, 2005b; Farris and Strausfeld, 2003; Sinakevitch et al., 2001; Skoulakis et al., 1993; Strausfeld et al., 2003).

The gross morphology of the MB in *S. littoralis* superficially resembles examples from other Lepidoptera (Pearson, 1971; Strausfeld et al., 1998) but the present description is more detailed than previous accounts. Outside the Lepidoptera, the most reminiscent MB organization is probably found in flies (Strausfeld, 1976; Strausfeld et al., 2003). The arrangement in the lobes with clearly separated parallel neuropils is very similar, although large differences exist. For example, the conspicuous Y tract and resulting lobelets are absent in flies, as in most other insects for that matter. In addition, flies have a simplified calyx that lacks the double cup arrangement seen in the moth. Another closely resembling group might be the Neuroptera, which also possess a Y tract but this group has not been thoroughly studied (Farris, 2005b). Our results confirm that the relationship between centrally located and peripheral KCs in the calyx is maintained in the lobes and represented by posterior and anterior KCs respectively. As in other

insects, it is likely that this arrangement in the moth reflects a sequential generation of KCs during development.
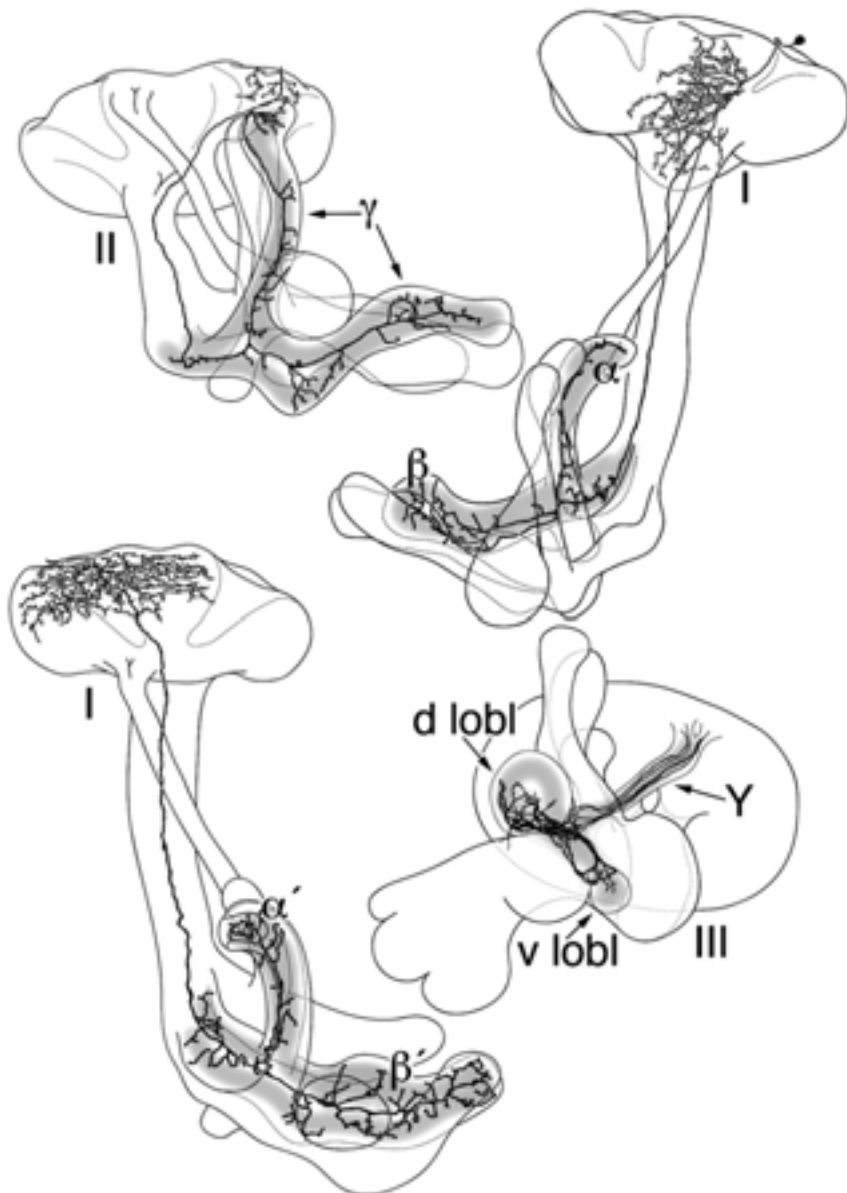
Intrinsic neuron types in the moth mushroom body (Papers I-IV)

The total number of KCs in each hemisphere was estimated to 4,060, with 3,400 sending their neurites through the pedunculus and 660 through the Y tract (paper IV). This is low compared to a similar-sized insect, ~170,000 in honey bee (Witthöft, 1967) but similar to that in *D. melanogaster*, ~2,500 (Technau and Heisenberg, 1982). The KCs were categorized into four groups according to their dendritic morphology and branching pattern in the calyx and lobe divisions. These qualities were examined by using Golgi impregnation and dye-injection in single KCs. Within these groups, subsets of neurons occupying specific zones within the divisions were identified according to the differential affinity of several antibodies to these zones as described above (Paper II-III).

Alpha/beta Kenyon cells

KCs supplying the α/β have their cell bodies located centrally over each calyx and the primary neurites line the inner surface of the rim neuropil of the calyx before they enter through the bottom of the calyx cups and join the pedunculus necks. From the primary neurite, several dendritic branches reach into the calycal neuropil, penetrating through both the inner rim and into the outer wall (Fig. 5). These branches are decorated with numerous stubby spines and radiate in one main direction from the calyx center. In the pedunculus, the α/β KCs travel through the core bundle and may give rise to a single short collateral about half way toward the lobes. At the base of the pedunculus, the neurites send out a few short collaterals in the spur region, and then bifurcate giving rise to one collateral invading the vertical α division and one invading the medial β. The lobe tributaries are equipped with terminal varicose branches along their lengths (Paper I). These KCs are, by their morphology and location, defined as class I KCs (Farris and Sinakevitch 2003). As described previously, these neurons are ubiquitously immunoreactive to anti-aspartate, anti-DC0 and anti-Mas-allatotropin. Subsets within this group show immunoreactivity toward anti-taurine, anti-glutamate and anti-FMRFamide (Paper II-III).

Alpha´/beta´ Kenyon cells

KCs occupying the α´/β´ division of the lobes share many characteristics with the α/β KCs and also fall under the class I definition. Cell bodies of these neurons are found over the calyces and their primary neurites enter through the calyx cavity. The shape of the dendritic tree differs somewhat from the α/β and forms a large umbrella-shaped canopy of spiny branches radiating from the primary neurite in all directions (Fig. 5). The dendritic ramifications of a single α´/β´ KC cover a

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Fig. 5. Kenyon cells in *S. littoralis*. Roman numerals I-III denotes their assumed class. Target divisions in the lobes are shaded grey for clarity. Top left a class II KC branching in the γ divisions of the lobes. Note the narrow dendritic tree with clawed specializations. Top right a class I KC branching in the α/β divisions. Bottom left a class I KC in the α´/β´ divisions. Note the differential dendritic branching patterns in the two class I KCs. Bottom right a camera lucida reconstruction of KCs projecting through the Y tract and branching in the lobelets. Their dendritic arbors could not be resolved in this preparation.
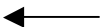
substantial part of one calyx and may reach over to the neighboring one. In the pedunculus necks, these cells occupy the space adjacent to the α/β fibers, however displaced toward the periphery. Like α/β KCs, α′/β′ neurons display occasional single short branches in the pedunculus. In the spur, these cells ramify extensively and subsequently diverge into their respective aspects of the vertical and medial lobes. In the lobes they give rise to second-order branches decorated with small swellings. In the medial β′ division, a bulbous structure is formed about half-way toward the tip by particularly long side branches (Paper I). The immunoreactive labeling pattern among α′/β′ KCs is similar to that of α/β KCs except for reactivity to anti-taurine and anti-glutamate, which is absent in the α′/β′ division (Paper II). The number of dendritic spines on class I KCs was estimated to 550. Spines on other KC classes were not counted.

Gamma Kenyon cells

In the calyx, KCs supplying the γ division have long and narrow dendritic trees that are sparsely equipped with short branches that radially extend into the calyx neuropil (Fig. 5). Their branches are equipped with clawed specializations, denoting these as class II KCs (Farris et al., 2004; Strausfeld, 2002; Strausfeld et al., 2003). The γ KC somata are displaced toward the periphery of the KC body mass (Paper II) and their primary neurites pass trough the calyx wall rather than via the calyx cavity. These cells emerge from each calyx as a ring-like sheath surrounding the neck of the pedunculus (Paper I-II). The axons then converge to form a tight bundle before they bifurcate from the pedunculus and grow into the medial and vertical lobes. These cells do not branch in the spur. γ KCs have thick axon-like processes that, after they bifurcate from the pedunculus, extend to the tip of both the medial and vertical γ divisions. In the lobes, these processes have sparse short side branches, which are more frequent toward the endings of their lobes. The fact that these neurons branch in both aspects of the lobes separates the moth from other holometabolous insects. In both honey bee and *Drosophila*, class II KCs are rearranged before eclosion to invade only one lobe in the adult (Farris et al., 2004; Lee et al., 1999). These neurons were strongly immunoreactive to anti-aspartate, anti-DC0, anti-FMRFamide and anti-taurine (Paper II).

Fig. 6. Relationship between antennal lobe projection neurons and Kenyon cells. **A:** Dextran filled projection neurons clearly show three tracts from the antennal lobe, the inner, outer and mediolateral antennocerebral tracts (iACT, oACT and mlACT respectively). The iACT and oACT innervate both the calyx (cx), and lateral protocerebtum (L Pr), whereas the mlACT only innervates the L Pr. **B:** Dextran filled PNs (red) branch extensively into the outer zone of the calyx and make contacts with Kenyon cells (green). **C-D:** Increasingly higher magnifications of the projection neuron boutons (red) and the Kenyon cell dendrite spines (green) (see text). **E:** Electron micrograph of the calyx neuropil showing microglomeruli (outlined) constituted by extrinsic boutons (b) surrounded by numerous small-diameter profiles. **F:** A clear-vesicle bouton (cvb) is seen making multiple synapses (arrows) onto small KC profiles and onto a larger round profile (asterisk), which serially synapses onto a KC profile. Three medium-size, round profiles (stars) are involved in reciprocal synapses with the bouton.
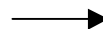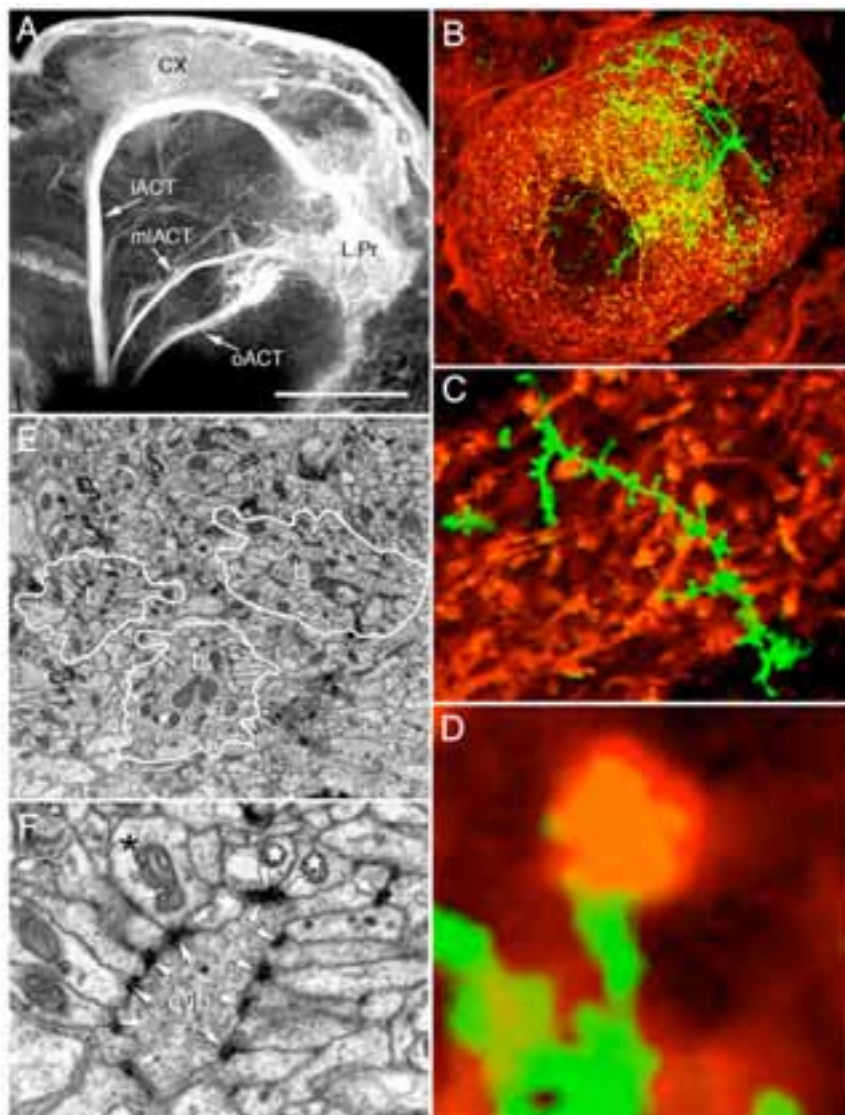
Y tract Kenyon cells

The fourth and most deviant KC type was the Y tract intrinsic neurons. Their cell bodies lie in a dorsolaterally shifted cluster farthest from the calyx. Their primary neurites extend along the outer surface of the calyx for a short distance before dividing, one tributary entering the neuropil where it divides into several branches. These branches, equipped with compact and clenched claw-like specializations, are sparse but widely spread throughout the calyx. However, they seem concentrated in the anterodorsal part of the calyx. The second tributary extends to the dorsolateral surface of the calyx where it enters the Y tract. Each of these processes projects behind the base of the vertical lobe, where they divide at least

once to send tributaries into the dorsal and ventral components the Y lobelets (Fig. 5). The projection of these tributaries is less orderly than those of the vertical and medial lobes and the same tributary may extend back and forth between the two Y lobelets. The lobelet tributaries appear "naked," lacking obvious terminal specializations, while instead being heterogeneously swollen. Tributaries supplying the Y lobelets are thicker than their axons in the Y tract and Y tract axons are about twice as thick as axons in the pedunculus. These neurons exhibit strong immunoreactivity only to two antisera: anti-taurine and anti-aspartate. They are also slightly immunoreactive to anti-DC0 but less than seen in neurons in the lobes. By analogy with the general age-dependent displacement of KCs toward the periphery in the calyx, the distant location of the Y tract KC somata suggests that these cells are the oldest in the moth MB (Farris and Sinakevitch, 2003). Their baroque organization into a separate accessory peduncle and a pair of independent terminal bulbs, reminds of the organization of class III KCs in cockroach and termites (Farris and Strausfeld, 2003). These characteristics allow us to infer the Y tract KCs to be class III KCs.

The discrimination of three classes of KCs, divided into four groups is based on their morphology and relative location (Fig. 5, 7). To ascertain their true identity and homology with corresponding KC types in other insects, developmental studies that dissect the sequential differentiation and organization of these neurons are needed.

Extrinsic cell types in the moth MB (Papers I, III & IV)

Sensory input to the calyx

By applying a fluorescent tracer dye to the AL, we could follow the trajectory of AL PNs and show how the olfactory innervation by PNs is distributed in the calyx. This method revealed a clear functional segregation of the already recognized anatomical divisions of the calyx (Fig. 6). AL PN collaterals invade the outer wall of each calyx but PN innervation is completely absent in the inner rim. In the outer wall, the PN processes are equipped with numerous large blebbed varicosities, here called boutons, presumed to be presynaptic to KC dendrites. By combining massive staining of PNs, and labeling single KCs with a different dye, we could demonstrate the precise relation between these two neuron types. KCs contact PNs with a single or a pair of dendritic spines per bouton (Fig. 6).

The identity and origin of the secondary afferent input to the inner rim has not been identified in this thesis. A clue was provided by applying tracer dye to the optic lobe, which generated a single stained fiber entering the calyx (Paper I). This does, however, not fully explain of the function of this region. We can only speculate about the identity of its additional afferents, but by comparison with other species, it may be either gustatory, visual or multimodal (e.g. Ignell et al., 2000; Mobbs 1982; Strausfeld and Li 1999b) As discussed previously, hearing is an important sense for avoiding predation by bats. Neurons convey the auditory information from the tympanic organs on the thorax to the brain but the ultimate target for these neurons within the brain remains elusive (Roeder 1969). Possibly,
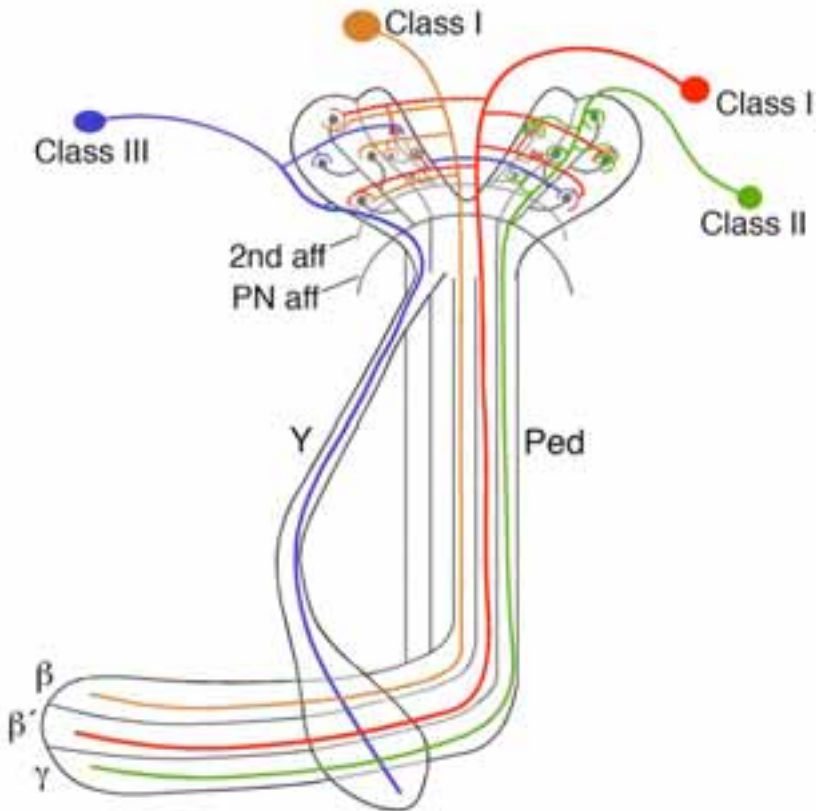
Fig. 7. Schematic diagram showing the branching patterns of the KC classes (I-III) and the differential afferent innervation to the calyx by antennal lobe projection neurons (PN aff) and secondary innervation (2nd aff).

this information could reach the MBs, perhaps indirectly via multimodal protocerebral interneurons, providing a substrate for the cross-modal integration between olfaction and hearing observed in the behavior of male moths. With the current knowledge this is, however, pure speculation.

GABAergic input to calyx, pedunculus and lobes

Additional to the excitatory input to the calyx by AL PNs (Yasuyama et al., 2002; Bicker, 1999), GABAergic inhibitory innervation has been shown to provide a major contribution to the calycal neuropil and the remaining MB in different insects (Grünewald 1999; Bicker 1999; Homberg et al. 1987; Perez-Orive et al. 2002; Yasuyama et al. 2002; Ganeshina and Menzel 2001; Leitch and Laurent 1996). We applied antibodies against GABA to investigate its distribution in the moth MB. The calyx is densely innervated by GABAergic processes, decorated with beaded or blebbed varicosities (Fig. 9). The size of these varicosities was
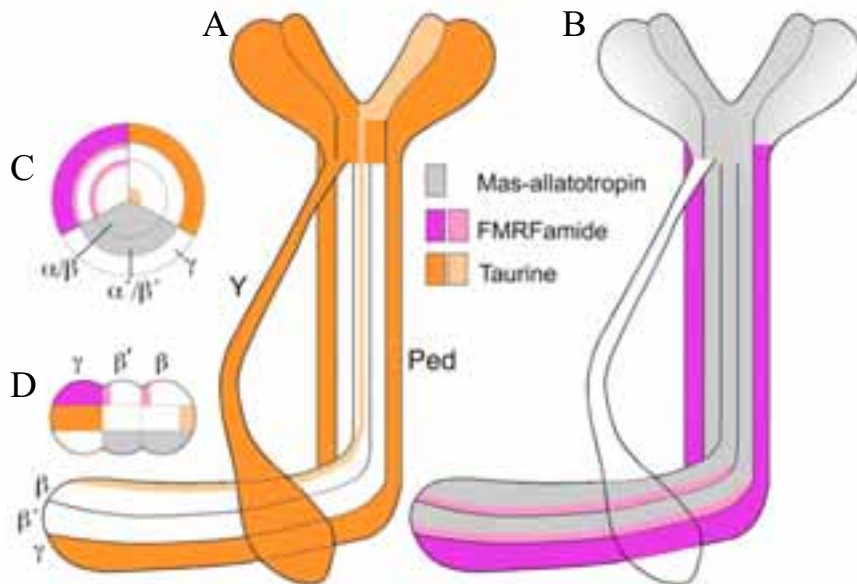
Fig. 8. Summary of the immunoreactivity to FMRFamide, taurine and Mas-allatotropin in the mushroom body and schematic diagram of the mushroom body organization in *S. littoralis*. For clarity, the vertical lobe, the dorsal lobelet and one of the calyces and necks are omitted. **A:** Schematic diagram of immunolabeling by taurine antiserum. Strong labeling is seen in the calyx, γ division and in the Y tract (Y). Faint labeling is seen in the central core of the pedunculus (Ped) and the posterior part of the α/β lobes. **B:** Diagram of immunolabeling by FMRFamide antiserum and Mas-allatotropin antiserum. FMRFamide antiserum labels the γ division and the anterior margins of the α/β and α′/β′ divisions. Mas-allatotropin antiserum labels the calyx and the α/β and α′/β′ divisions. **C:** Diagram of the immunolabeling in a cross-section through one pedunculus neck. **D:** Diagram of the immunolabeling in a cross-section through the medial lobe.

smaller than the PN boutons. Both calyces and both the outer compartment and inner rim had equal distributions of GABAergic processes. The origin of this innervation could not be determined but at least some of it arrived at the calyx via the lateral branch of the iACT, suggesting an origin in the lateral protocerebrum. This type of GABAergic innervation has previously been identified in a number of species and is thought to mediate feed-forward inhibition to the calyx during olfactory stimulation, as discussed in a previous chapter. Not only the calyx but all parts of the MB displayed GABA-immunoreactivity. Importantly, KCs were not immunopositive to anti-GABA, so all labeling must belong to other cell types. Fine-branched processes ramified diffusely throughout the pedunculus and lobes and seemed to virtually provide every conceivable space with GABAergic innervation. Additional to the protocerebral feed-forward neurons, local GABAergic cells have been found that are thought to provide feedback inhibition from the lobes to the calyx and pedunculus, and to engage in local microcircuits

(Grünewald 1999; Yasuyama et al. 2002; Ganeshina and Menzel 2001; Leitch and Laurent 1996). We could not discern such neurons specifically in *S. littoralis*, but we find it likely that the GABAergic immunolabeling observed represents several different neuron types innervating the moth MB.

Putative modulatory input to the MB

To investigate what other neuron types may be present in the spodopteran MB we used a number of antisera that detect putatively neuromodulating substances, namely one biogenic amine, serotonin, two types of neuropeptides, allatostatin and tachykinin-related peptide (TRP) and the previously mentioned amino acid, glutamate (Fig. 9). Some of these substances have previously been found in neurons innervating MBs of other insects (Bicker et al., 1988; Homberg and Hildebrand, 1989; Kim et al., 1998; Nässel, 1999; 2000; Schürmann and Klemm, 1984; Schürmann et al., 2000).

Serotonin antiserum revealed a fine web of immunolabeled processes equipped with small beaded swellings that ramified
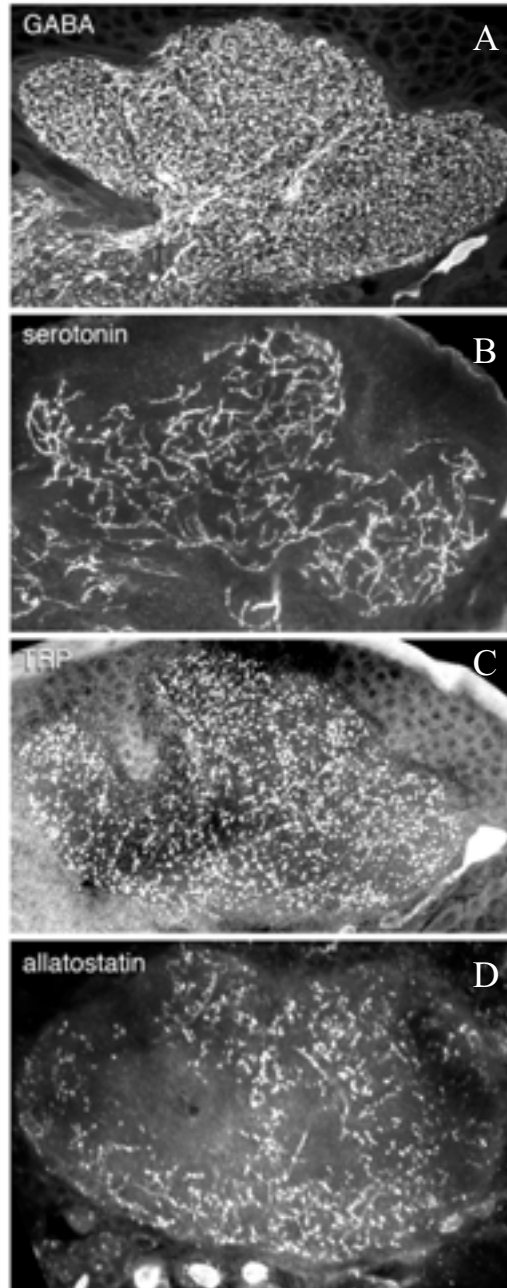
Fig. 9. Inhibitory and putative modulatory input to the calyx. **A:** GABA-like-immunoreactive collaterals invade the entire calyx. The shape of the GABA-like immunoreactive terminal endings is similar but smaller than PN boutons. **B:** The calyx is evenly supplied with serotonin-like immunoreactive neural ramifications. **C:** The calyx is richly supplied with blebby TRP-like-immunoreactive fibers. **D:** Allatostatin-like immunoreactive fibers are concentrated to the outer wall of each calyx.

throughout the calyx. In the lobes and lobelets, labeling was denser than in the calyx but consisted of very thin, filamentous processes with numerous granular specializations. This labeling is reminiscent of some extrinsic neurons that previously were Golgi-impregnated in the calyx and lobes (Paper I). Anti-serotonin labeling in *S. littoralis* resembles that in *M. sexta*, where serotonin-like immunoreactivity in the calyx originates from a single neuron that has its soma in the contralateral posterior median protocerebrum. A second pair of serotonergic neurons that branch in the ipsilateral superior protocerebrum innervates the lobes (Homberg and Hildebrand, 1989).

Allatostatin-like immunoreactivity is pronounced in the outer wall of the calyx suggesting a function of this peptide in olfactory processing. The main part of allatostatin-like-immunopositive innervation reaches the calyx via a single or a few fibers that extend through the lateral branch of the iACT. Labeling is also observed in a single neurite projecting through the Y tract. No anti-allatostatin labeling was observed in the MB lobes.

TRP-like immunoreactivity is evenly distributed within the entire calyx and found in some cell bodies surrounding the calyx. Between four and five intensely immunopositive large cell bodies are seen medial to the calyx and a group of about four small, faintly immunolabeled cells lateral to the calycal neuropil are interpreted as being intrinsic to the MBs. In addition, a group of eight large and strongly labeled, probably neurosecretory, cell bodies are found between the clusters of KC perikarya. Which of these types of immunopositive neurons provide labeling in the calyx could not be determined. TRP has been shown to co-localize with GABA (Nässel, 2000; Nusbaum et al., 2001; Ignell, 2001) suggesting another possible origin of this labeling.

Lastly, anti-glutamate labels, apart from a few KCs as described above, a number of large-profile processes that invade the lobes and the lobelets in particular. Similar labeling has been observed in the cockroach and the fruit fly (Sinakevitch et al., 2001; Strausfeld et al., 2003) but the identity of these neurons is unknown.

The specific roles of these substances in the MB is largely unknown, but a number of studies provide examples of functions they might be involved in: serotonin has been demonstrated to influence olfactory learning in honey bees (Mercer and Menzel, 1982) and to modulate the sensitivity and excitability of projection neurons in the antennal lobes of the moth *M. sexta* (Mercer et al., 1995; Kloppenburg et al., 1999). A function for TRP in the insect CNS has not been established although a recent study demonstrated that TRP may have a direct influence on odor sensitivity in fruit flies (Winther et al., 2006). Glutamate is a common neurotransmitter in the CNS of both vertebrates and invertebrates (Riedel et al., 2003; Roberts and Glanzman, 2003) and several different types of putative glutamate receptors have been reported to be present in the insect central nervous system (Funada et al., 2004; Xia et al., 2005; Völkner et al., 2000). Local release of glutamate in the MB has been demonstrated to improve odor learning in honey

bee (Locatelli et al., 2005). The function of allatostatins in the insect CNS remains unclear.

As previously discussed, additional modulatory substances, e.g. octopamine and dopamine have been found to exert important effects on MBs in other species. These substances are likely to be found also in *S. littoralis*, but this has not yet been specifically investigated.

Synaptic organization in the moth mushroom body (Paper IV)

Transmission electron microscopy of different parts of the MB reveals several interesting features of the intrinsic organization of the moth MB. The glomerular structure in the calyx neuropil represents microglomeruli composed of a central large presynaptic swelling, here called bouton (Schürmann 1974), surrounded by numerous smaller profiles of various sizes (Fig. 6). The large central bouton is likely to belong to afferent sensory interneurons and the size and shape of these boutons resembles that of the AL PN varicosities (see above). This has previously been confirmed with immuno-EM against acetylcholine related molecules in *Drosophila* (Yasuyama et al., 2002). The smaller of the surrounding postsynaptic profiles are putative KC dendrites and these are devoid of synaptic vesicles. The degree of divergence from a single bouton onto KC profiles is very high and we estimate that each bouton contacts up to 40 dendrites belonging to individual KCs. Other medium-sized profiles, presynaptic to both KCs and boutons, as well as postsynaptic to boutons, are inferred to comprise inhibitory and/or modulatory neurons that innervate the calyx (Ganeshina and Menzel, 2001; Leitch and Laurent, 1996; Yasuyama et al., 2002). The high amounts of dense core vesicles in some of them further suggests this, since dense core vesicles are thought to reveal the presence of peptides or biogenic amines (Ganeshina and Menzel, 2001).

In the pedunculus, KCs are organized in tightly packed fascia, separated by thin layers of glial substance. Within these fascia, KCs often engage in en-passant synaptic contacts with other KCs or with large extrinsic profiles. KC-KC synapses are often complex and involve several KC profiles connected through serial, converging and diverging synapses. KCs exhibit varying contents of dense core vesicles, possibly representing the differential distribution of peptides and other substances in groups of KCs as revealed with immunolabeling (Paper II-III). This is also seen in the lobes, where the content of both dense core- and clear vesicles is high in putative KCs. However, the identification of specific neuron types is significantly more difficult in the lobes than in the calyx and pedunculus. In the lobes, complicated chains of serial and converging/diverging synapses are observed and they probably involve several different neuron types that could not be separated on morphological grounds.

The Y tract contains axon-like profiles similar to the KCs in the pedunculus but they are of slightly larger diameter and individually separated by glia in the upper part of the tract. In the lobelets, the same kind of intricate synaptic arrangements are observed as in the lobes. One clear difference between the Y tract KCs and KCs invading the medial lobe is that the former contain more and larger

mitochondria. This could easily be seen as a darker shade in the lobelet compared to the medial lobe in a low-power micrograph. The reason for this difference remains elusive but previous studies have suggested a relationship between a high continuous neuronal activity and a high content of taurine, which may have an excitotoxicity-protective function (Discussed in Sinakevitch et al., 2001). A high content of large mitochondria could suggest an elevated neuronal activity in these KCs and as we have shown already, these neurons are intensely labeled with taurine antiserum.

The over-all impression of the synaptic organization in the *S. littoralis* MB is that it is very similar to previously studied insects. The microglomerular arrangement in the calyx resembles that in flies, bees and locusts, although immuno-EM is needed to ascertain the profile identity (Ganeshina and Menzel 2001; Leitch and Laurent 1996; Yasuyama et al. 2002). The tight packing of KCs into fascicles and abundance of en-passant synapses in the pedunculus confirms observations in locust, honeybee, cricket and cockroach (Leitch and Laurent 1996; Schürmann 1970; Shürmann 1974).

Our estimation that each PN bouton contacts roughly 40 dendrites each belonging to a unique KC suggests a high degree of divergence, but how high? Does a given PN synapse onto only 40 KCs? PNs normally branch widely in both calyces, implying that there must be a divergence higher than 1:40. How high is the convergence onto KC? Do these 40 hypothetical KCs only sample this one PN? Each class I KC has about 550 dendritic spines. In *M. sexta*, the PNs have about 100 boutons each (Homberg et al., 1988), meaning that even if a given KC contacts every bouton on a single PN there is still 300-400 "empty" dendrites. The widely branching dendritic tree of α′/β′ KC suggests that it may sample PNs over a large area in the calyx, further suggesting a higher convergence. There are in *M. sexta* about 500-600 PNs reaching the calyx (Homberg et al., 1988), thus providing some 60,000 boutons, each with 40 synaptic appositions, resulting in some 2,400,000 synaptic contacts. In *S. littoralis*, ~4,000 KCs provide 550 dendrites each, which gives a total number of some 2,200,000 synapses. The distribution of these synapses, with respect to how the signal diverges and converges, is difficult to speculate about but it could be very high in both aspects.

The TEM data also showed that KCs may be both pre- and postsynaptic throughout the pedunculus and in the lobes. This confirms the view that the MB is by no means monopolar in the respect that it receives information at the calyx and transmits it through the lobes, but must instead be regarded as a highly integrative neuropil not only in the calyx, but throughout the length of the KCs.

Olfactory Response and Electrophysiology of Kenyon Cells (Paper V)

To answer questions about how moth KCs respond to odor stimulation of the antennae, we employed whole-cell patch clamp on KC somata in an *in situ* head preparation of the moth (see Box 2). This allowed us to test the

electrophysiological membrane properties of the KCs, record odor-evoked activity and to visualize the recorded neurons by including a tracer in the recording electrode. An advantage of whole cell patch-clamp recording via the soma compared to e.g. intracellular recording with sharp electrodes in the axon is the possibility to monitor both sub- and supra-threshold activity and not only action potentials.

Membrane properties of Kenyon cells

In order to evaluate the membrane properties we recorded from KCs both in voltage-clamp and current-clamp mode. In voltage-clamp mode the net current through the cell membrane was measured while stepwise depolarizing the membrane. In current-clamp mode, increasing steps of depolarizing current were injected into the cell and the resulting depolarization of the membrane potential

was monitored. Depolarizing the membrane elicited voltage-activated inward- and outward currents of different types. Injection of depolarizing current evoked active membrane potentials, both action potentials and smaller spikelets, which were hypothesized to represent local voltage-activated currents (Perez-Orive et al., 2002, 2004), possibly mediated through voltage-activated Ca^{2+} channels, which have been identified in honey bee KCs (Grünewald 1999).

Odor-evoked activity of Kenyon cells

For testing the olfactory responses of KCs we used a wide range of odors, applied both singly and in blends to the ispilateral antenna while recording the activity in single KCs in current-clamp mode. The odors tested comprised different plant- and flower odors as well as pheromone, known to elicit responses in the antenna and AL. Odor stimulation elicited a short, phasic excitatory postsynaptic potential (EPSP) with spikelets riding on top of it in 5 out of 28 recorded KCs (Fig. 10). This was interpreted as sub-threshold activation caused by insufficient or "incorrect" presynaptic signaling by PNs and the qualitative specificity of this activation was very low. One single KC responded with action potentials to stimulation with behaviorally relevant complex odor mixtures (Fig. 11). The temporal characteristics of both sub-threshold activation and transmitted response was very brief and with a fast onset. The activation typically lasted ~200 ms. Odor-evoked responses in PNs have previously been shown to last ~500 ms under similar experimental conditions, although large variation is seen in the temporal characteristics of PNs in *S. littoralis* (Anton and Hansson, 1994, 1995; Sadek et al., 2002). The temporal sharpening of the KC response could suggest feed-forward inhibition to take place shortly after the response, as proposed by studies in locust and honey bee (Perez-Orive et al., 2002; Szyska et al., 2005). In some of the KCs that exhibited sub-threshold activity, an additional short depolarization was observed about 800 ms after the response, suggesting that release of feed-forward inhibition allowed lingering PN activity to once again depolarize the membrane.

If it is a general property of KC odor processing that excitatory input is intercepted by periodic inhibition, this could be a way for the system to reset itself and prepare for new information. Odors have, under natural conditions, a complex structure and appear as small packages or filaments of odor-laden air intercepted by clean air (Baker et al., 1988; Vickers et al., 2001). Being able to accurately resolve the temporal structure of odor stimuli could be important in order to detect

Fig. 10. Odor-evoked subthreshold activity. KC # 32 displayed EPSP and spikelets by stimulation with all odors. This cell was tested with all blends, the single odorants in blends 1-3 and the main pheromone component before the contact was lost. The blend of all plant odors evoked the largest EPSP (blends 1-5, grey bar). This KC also exhibited a slight depolarization around 800 ms after stimulus onset in most of the traces (vertical grey box). One spontaneous spikelet is seen preceding the stimulus in the magnification of the pheromone trace (purple bar). This neuron was filled and stained and branched in the α′/β′ divisions. Current injection and stepwise depolarization caused spikes and spikelets and fast inward currents, respectively.

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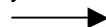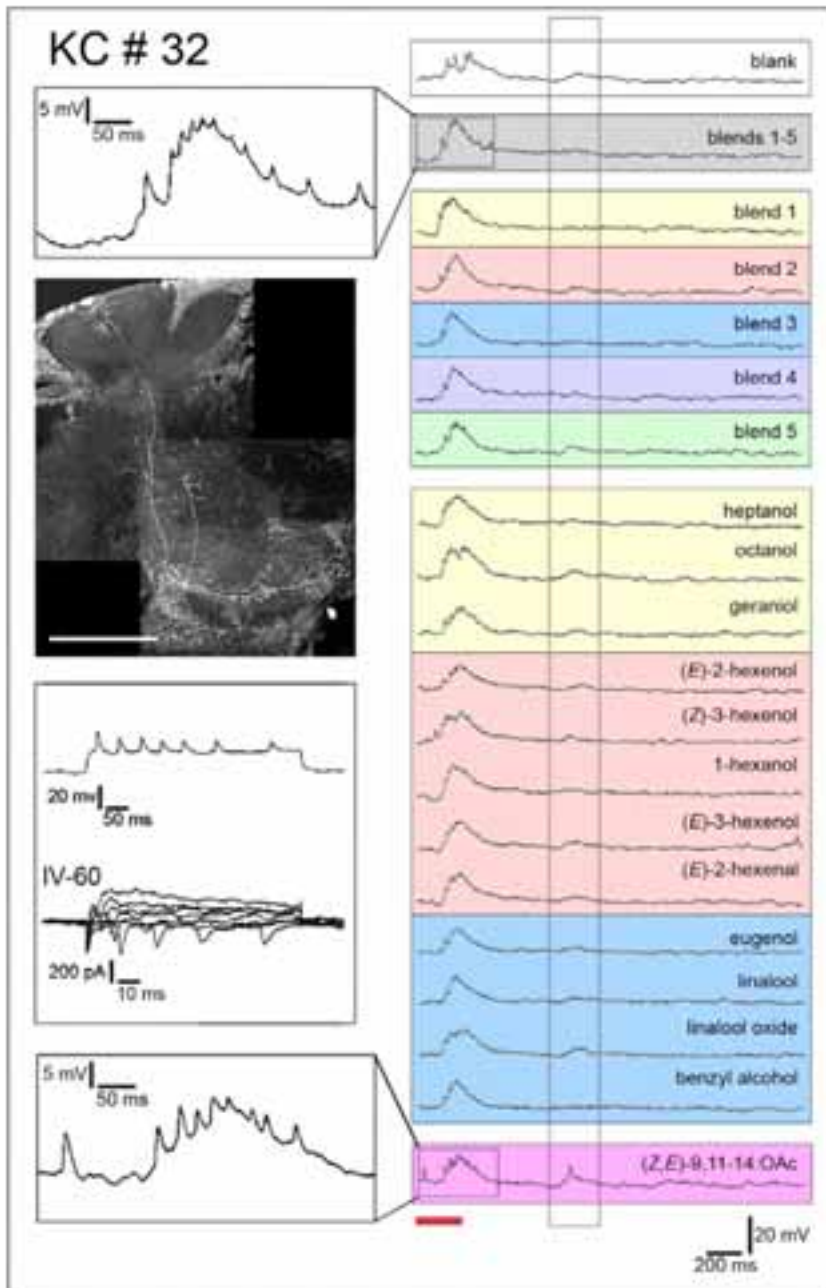
concentration differences and gradients, since the rate of odor filaments hitting the peripheral olfactory organs reflects the density of these filaments and thus the concentration of the odor. The suggested temporal sharpening could be a means to

detect the fine temporal signature of olfactory stimuli (Also see Lei and Hansson, 1999).

The identity of the voltage-activated currents observed and the active membrane potentials needs to be confirmed with pharmacological interventions. However, if the assumptions about spikelets are correct, they may provide the KCs with a tool for coincidence detection, in that they translate presynaptic events into temporally distinct activation quanta that only elicit a response if a given number of them occur simultaneously (Perez-Orive et al., 2002, 2004; also see Gulledge et al., 2005). But do moth PNs fire synchronously? In *M. sexta*, attempts to demonstrate coherent LFP oscillation and phase-locked PN spiking activity, as reported in locust and honey bee, have failed (Christensen et al., 2003). Could there be other mechanisms underlying PN synchrony? A concept rarely brought into the discussion of synchronous PN spiking is ephaptic interactions. This is the prediction that adjacent neurons may interfere with one another to the extent that one neuron may trigger action potentials in neighboring cells just by dissipation of potential across the extracellular space between the neurons (Bell 1981; Barr and Plonsey 1992; Krnjevic 1986). However, a prerequisite for this to occur is that the neurons are un-insulated, i.e. that no glial- or myelin sheath part the neurites. Vertebrate neurons are generally myelinated, but an exception to this is the olfactory nerve and modeling studies have shown that ephaptic interactions are likely to occur there (Bokil et al 2001). In the insect brain the situation is similar. Myelin insulation is absent and most neurites are "naked". Glia is abundant (Hähnlein and Bicker 1996) but normally only separate bundles of axons and individual neurons remain non-insulated and lie membrane-to-membrane. Thus, ephaptic interactions could theoretically cause synchronization of firing PNs if they lie adjacent to one another. Possibly, functionally related PNs are spatially organized in the ACTs so that their activity can be synchronized. The specificity could be further sharpened by lateral interactions by the AL local interneurons (LN) (see e.g. Lei et al., 2002). The strategy of bringing functionally coupled neurons spatially close together could be a way to reduce noise in an inherently leaky, i.e. non-insulated, system.

The promiscuous sub-threshold activity observed in KCs could functionally reflect the high degree of convergence suggested by the anatomical data. It is possible that KCs receive input from a major portion of the PNs but only respond if the temporal characteristics are correct. An additional implication of high divergence and convergence is possible. If KCs theoretically are receptive to a wide range of inputs, it should be easy to change the profile of a given KC from non-responding to a given input, to responding by modulating its integrative properties or increasing its overall excitability. This could be accomplished by extrinsic modulation or by changing intrinsic properties (for review see Frick and Johnston, 2005) and this could provide a key feature of the MB's role in associative learning.
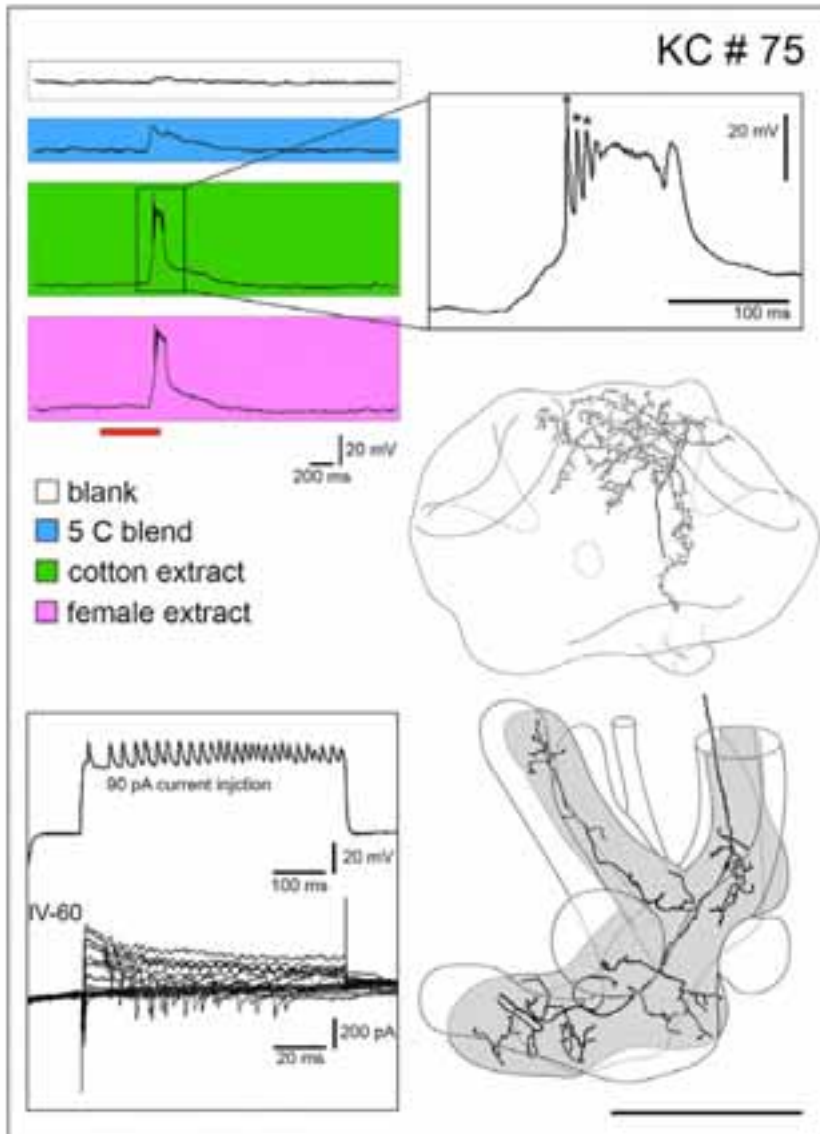
Fig. 11. KC responding to odors with EPSP and action potentials. KC # 75 was slightly depolarized by stimulation with a five-component blend (5 C blend) but responded with action potentials to cotton extract and female pheromone extract. The response was fast and brief and only 2-3 spikes (asterisks in magnified trace) were elicited during the stimulus. This neuron exhibited action potentials when injected with depolarizing current and multiple voltage-activated currents upon stepwise depolarization of the membrane. This neuron was visualized and reconstructed in the calyx and the lobes. It branched widely in the calyx and in the α′/β′ divisions of the MB lobes (shaded grey). Scale bar = 100 μm.

Conclusions

The results of the investigations of the moth mushroom body presented here answer some of the questions posed earlier in this thesis. However, they also, as is often the case, raise several new questions.

Functional Implications of Mushroom Body Structure

The small size of the MB in *S. littoralis* (~4000 neurons) can possibly be correlated to the short and behaviorally simple life of this moth. Crucial sensory cues are probably stable and reliable and the need for elaborate learning and for making new associations between stimuli seems limited. Nevertheless, the organization of the MB of *S. littoralis* is complex and seems to comprise all the basic components found in MBs across insects and the blueprint at a cellular and system level is conserved. Each calyx is anatomically and functionally divided into two concentrically arranged regions. The marginal region receives input from antennal lobe projection neurons and the central region receives yet unidentified innervation. The double concentric organization in the calyx is translated into a tripartite arrangement of parallel divisions in the vertical and medial lobes. Each of these parallel divisions contain a morphologically discernable KC species and within these KC categories, further differentiation into biochemically diverse subtypes is seen. A fourth type of KC provides a separate system partly detached from the rest of the MB.

Questions about how the structural organization might relate to function arise: Do separate divisions of the spodopteran MB support different functions? Our results show that input in to the calyx is separated but how this relates to the lobe divisions is not clear. Extensive overlap between dendrites from different KC groups is evident, but the specific combination of input to each division could be unique. Each of the lobe divisions contains biochemically different KCs and the differences in dendritic branching pattern between KC classes and subtypes suggest that they integrate their input differently, not only with respect to modality but also to activation threshold and temporal characteristics of firing (for reviews see Gulledge et al., 2005; Kath, 2005). There is extensive crosstalk in form of synapses between KCs in the pedunculus and lobes. Glial sheets separate bundles of KCs but how the arrangement of these insulated fascia relates to divisions or biochemical differentiation among KCs is unknown. Moreover, KCs are postsynaptic to extrinsic neurons that probably modulate the activity in the KCs further. It is likely that the divisions comprise parallel functional units that each processes a unique combination of inputs through a number of computational regimes by different KC subtypes, and lateral interactions within the MB and extrinsic modulation influences the MB activity further.

Olfactory Responses in Moth Kenyon Cells

Moth KCs respond sparsely to relevant odor stimulation with a temporally narrow burst of spikes. This is in line with findings in other insects and may represent a general property of KCs across insect taxa. However, subthreshold activation of KCs is odor-nonspecific, which provides a functional evidence of high convergence of olfactory signaling onto KCs. Our experiments suggest that KCs may possess active dendritic membrane properties, which could make them prone to detecting synchronous input from AL PNs. This might be a way to sparsen the converging signal but could also be involved in response plasticity of KCs.

One important issue not specifically addressed in this thesis is the output from the KCs. Previous studies have shown that the dendritic arborization of efferent neurons in the lobes may vary from limited, covering only one or few divisions to very large, sampling the majority of or all KCs (Ito et al., 1998; Li and Strausfeld 1997, 1999; Rybak and Menzel, 1998; Schildberger, 1984; Strausfeld, 2002). How do these efferent neurons "perceive" KC signaling? Indeed, what kind of information do KCs transmit? The olfactory signal changes in the calyx from a phasic-tonic, frequency-modulated signal in PNs to a brief all-or-nothing signal in KCs. How is KC activity integrated by the downstream neurons? Is the activity across assemblies of KCs temporally structured? Can ephaptic interactions take place in the pedunculus and what information do KC-KC synapses transmit? MB efferents, which sample a large part of the KCs, like the Pe1 neuron in honey bee, can alter their response during olfactory conditioning (Mauelshagen, 1993). Is this accomplished by summating the response from additional responding KCs or is the change taking place downstream of KCs (Menzel and Manz, 2005)? How does input and output relate to the multitude of KC subtypes, modal, morphological and biochemical, and to the high degree of lateral interactions between KCs that obviously is taking place?

Future Directions

Further investigation is needed to explain how information is transmitted through the MB. For example, the relationship between input and activation must be further elucidated. What criteria must be met in order for a KC to respond? The lateral integration between KCs must also be explored. What kind of information is transmitted through KC-KC synapses? How is the KC activity read by efferent neurons? Some of these questions can possibly be answered by simultaneously recording activity from multiple neurons at different synaptic levels of the odor-signaling pathway.

The insect MB provides a truly fascinating and most challenging neural structure, the function of which we are only beginning to unveil. A deeper knowledge about how MBs work might aid the understanding of higher brain functions in other animals, including mammals and humans. The low cell number and relative

simplicity of the moth MB, combined with a fairly large size, which facilitates experimental investigation, promotes the moth MB as a suitable model for studying MB function in detail in the future.

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