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Global and Local Modulatory Supply to the Mushroom Bodies of the Moth *Spodoptera Littoralis*

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Abstract

The moth *Spodoptera littoralis*, is a major pest of agriculture whose olfactory system is tuned to odorants emitted by host plants and conspecifics. As in other insects, the paired mushroom bodies are thought to play pivotal roles in behaviors that are elicited by contextual and multisensory signals, amongst which those of specific odors dominate. Compared with species that have elaborate behavioral repertoires, such as the honey bee *Apis mellifera* or the cockroach *Periplaneta americana*, the mushroom bodies of *S. littoralis* were originally viewed as having a simple cellular organization. This has been since challenged by observations of putative transmitters and neuromodulators. As revealed by immunocytochemistry, the spodopteran mushroom bodies like those of other taxa, are subdivided longitudinally into discrete neuropil domains. Such divisions are further supported by the present study, which also demonstrates discrete affinities to different mushroom body neuropils by antibodies raised against two putative transmitters, glutamate and γ -aminobutyric acid, and against three putative neuromodulatory substances: serotonin, A-type allatostatin, and tachykinin-related peptides. The results suggest that in addition to longitudinal divisions of the lobes, circuits in the calyces and lobes are likely to be independently modulated.

Keywords

Insect brain; mushroom body; functional organization; immunocytochemistry; chemical neuroanatomy

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1. Introduction

In insects, the paired mushroom bodies (MB) are thought to play cardinal roles in learning and memory, sensory integration, and context-specific sensory processing (for reviews see Strausfeld et al., 1998; Heisenberg, 1998; 2003; Farris, 2005a). However, most classical and many recent studies persist in viewing the organization of these neuropils as being rather simple. Recent studies have manifestly demonstrated that this is not so, at least for the mushroom bodies of Hymenoptera and Blattoidea (Strausfeld and Li, 1999; Ehmer and Gronenberg, 2002; Strausfeld, 2002; Farris et al., 2004; Sinakevitch et al., 2001). However, might these two taxa be exceptional? With some exceptions, interpretations of *Drosophila* mushroom bodies, for example, have generally been comfortable with viewing these centers as having straightforward rather than elaborate organization. How simple, then, is a “simple” mushroom body?

Here we address this question using the moth *Spodoptera littoralis*, the mushroom body of which, like that of the fruit fly, is superficially uncomplicated. Compared to Hymenoptera with similar sized brains, viewed with conventional silver stains the spodopteran mushroom body is seen to comprise relatively few intrinsic neurons (Kenyon cells) and its calyx is not obviously divided into concentric zones. Like those of other moths, its lobes are not perceptibly partitioned down their length, as they are in honey bees and cockroaches. However, in addition to sensory interneurons from the antennal lobes that carry information to the mushroom bodies' peripheral neuropils (the calyces), these centers are supplied by neurons that can be individually labeled by antisera raised against a variety of putative transmitters and neuromodulatory peptides. The existence of such neurons suggests a more complex circuitry than might be suggested by afferent supply alone, and their distribution in the mushroom body's neuropils suggests that circuits at different levels in the mushroom bodies are independently modulated.

The present account is the third in a series describing the organization of the mushroom bodies in *S. littoralis* (Sjöholm et al., 2005, 2006), a member of the genus *Spodoptera* that includes several pest species in both the Old and New World. The Mediterranean climbing cutworm, *Spodoptera littoralis*, is found in Mediterranean Europe and Africa, where it is destructive on subtropical agricultural plants. In Central and Northern Europe, *S. littoralis* is recognized as an imported pest species that invades environmentally appropriate nurseries and greenhouse environments. Its resistance to many pesticides requires novel approaches to insect control mechanisms. Knowledge of the spodopteran central nervous system and brain, in particular areas that may support olfactory processing and memory, may assist in such endeavors.

Here we focus on the distribution of five substances that are thought to participate in olfactory discrimination and sensory associations within these higher centers. Mushroom bodies receive inhibitory and putative modulatory innervation by neurons that are immunoreactive to γ -amino butyric acid (GABA), as well as other amino acids, biogenic amines, and peptides (e.g. Dacks et al., 2005; Homberg et al., 1987; Homberg and Hildebrand, 1989; Homberg et al., 2004; Kim et al., 1998; Sinakevitch et al., 2005). GABA is a major inhibitory neurotransmitter in invertebrates (Kerkut et al., 1969; Usherwood,

1978), having an inhibitory effect at the neuromuscular junctions (Sattelle, 1992; Richmond and Jorgensen, 1999). GABA-mediated inhibition is thought to play a role in the processing of olfactory information both at the level of the antennal lobes (Christensen et al., 1998; Sachse and Galizia, 2002; Wilson and Laurent, 2005) and the mushroom bodies (Mizunami et al., 2005; Leitch and Laurent, 1996; Perez-Orive et al., 2002). GABA-immunoreactive neurons supplying terminal processes to the mushroom body calyces originate from dendrites situated either in the mushroom body lobes or in circumscribed regions of the lateral protocerebrum including the lateral horn (Homberg et al., 1987; Yamazaki et al., 1998; Bicker, 1999; Strausfeld and Li, 1999; Grünewald, 1999a). These regions receive the telodendria of output neurons from the mushroom body lobes (Strausfeld and Li, 1999).

Glutamate is a common neurotransmitter at the neuromuscular junction of insects (Jan and Jan, 1976; Johansen et al., 1989) and glutamate-like immunoreactivity has been ascribed to subsets of Kenyon cells in honey bees (Bicker et al., 1988) and crickets (Schürmann et al., 2000). It is expressed transiently in the most recently generated Kenyon cells. These are in the downgrowth lamina of the developing cockroach mushroom bodies (Sinakevitch et al., 2001; Brown and Strausfeld, 2006), in the core neuropil of the alpha and beta lobe of *Drosophila melanogaster* (Strausfeld et al., 2003), and in basal ring neuropil of honey bees (Strausfeld et al., unpublished observations; Farris et al., 2004). Local release of glutamate in the honey bee mushroom body has been demonstrated to improve odor learning (Locatelli et al., 2005) and several different types of putative glutamate-receptors have been proposed to be present in the insect central nervous system (Funada et al., 2004; Xia et al., 2005; Völkner et al., 2000).

Serotonin (5-hydroxytryptamine, 5-HT), a biogenic amine that can act as a neurotransmitter, neuromodulator, or as a neurohormone (Edwards and Kravitz, 1997; Nässel, 1987), is found in widely branching neurons in the insect brain, some supplying the mushroom bodies (Schürmann and Klemm, 1984; Homberg and Hildebrand, 1989; Dacks et al., 2006). Evidence suggests that it may influence olfactory learning in honey bees (Mercer and Menzel, 1982) and modulate the sensitivity and excitability of projection neurons in the antennal lobes of the moth *Manduca sexta* (Mercer et al., 1995; Kloppenburg et al., 1999).

Tachykinin-related peptides (TKRPs) have been ascribed many diverse functions in insects including myomodulation, hormone-release and, possibly, they exist as a circulating hormone (Nässel, 1999; Nässel et al., 1995). There is evidence that TKRP may have a direct influence on odor sensitivity in fruit flies (Winther et al., 2006). TKRPs are widely distributed in the brain but preferentially expressed in the antennal lobes, central complex, and mushroom bodies (Nässel, 1999; 2000).

A-type allatostatins are a family of neuroactive peptides found in many insects (Nässel and Homberg, 2006). Their suggested functions include roles in the control of gut peristalsis, heart rate, circadian rhythms, and production of juvenile hormone (Hernández-Martínez et al., 2005; Duve et al., 1999, 2000). The distribution of immunoreactivity revealed by antibodies raised against A-type allatostatins has been investigated in the frontal ganglion of lepidopteran larvae (Duve and Thorpe, 2003; Duve et al., 2000) and in olfactory and higher centers of the insect brain where it has been shown to often colocalize with GABA

(Schachtner et al., 2005). On this basis it has been suggested that A-type allatostatin may play a role as a co-transmitter in GABA-containing circuits. However, its function in the insect central nervous system remains obscure except in *Drosophila* where it has been shown to modulate olfactory perception and locomotion (Winther et al., 2006); a commonality that may be ascribed to the segmentally homologous nature of the antennal lobes and leg neuromeres. Here, the identification of putative A-type allatostatin in the mushroom body calyces suggests it may play a role in the modulation several candidate systems, including GABA immunoreactive processes.

2. Material and Methods

Adult *Spodoptera littoralis* were reared on a semi-artificial diet (Hinks and Byers, 1976) at the division of Chemical Ecology, SLU, Alnarp Sweden, under a 16/8 h light/dark regime in 20°C and 70 % relative humidity. As in the previous studies (Sjöholm, 2005, 2006), individuals used here were 2-3 days old males. Immunolabeling was performed on vibratome sections ranging from thickness of 15-40 µm. Methods describing glutamate immunocytochemistry are described in Sinakevitch-Pean et al. (2001) and Sinakevitch and Strausfeld (2004). Antibody specificity of both the polyclonal and the monoclonal glutamate antibodies are described in Chagnaud et al. (1989) and Campistron (1990). GABA antiserum raised in rabbit was obtained from GEMAC, (Talence, France) and its specificity described in Seguela et al. (1984). Methods for antiglutamate and antiGABA immunolabeling are described in Sinakevitch et al. (2001, 2003; see also Strausfeld et al., 2003). To simultaneously reveal glutamate and taurine immunoreactivity, sections were incubated overnight with monoclonal glutamate antibodies (1:100) together with polyclonal taurine antiserum (GEMAC, 1:500) in Tris-HCl-Tx. After washing in buffer secondary antibodies were applied: goat anti-rabbit immunoglobulin conjugated to Texas Red (Jackson Laboratories, West Grove, PA) and Alexa 488 goat anti-mouse immunoglobulin conjugate (Molecular Probes, Eugene, Oregon).

Specificity of taurine immunolabeling, described in Sinakevitch et al., (2001), was tested when a working dilution of taurine antiserum was preadsorbed against taurine (Sigma, T9931), L-aspartic acid (Sigma, A-8949), and L-glutamic acid (Sigma, G-6904), each of which were each conjugated to bovine serum albumin (BSA) by glutaraldehyde (Geffard et al., 1984). After adsorption of the taurine antiserum with Tau-G-BSA, sections showed no staining after treatment with the secondary antibody but did after adsorption with aspartate or glutamate conjugates. Antibody against TKRP (tachykinin-related peptide in *Leucophaea maderae*) was a gift from Dr. Dick Nässel (Stockholm University). It was raised in rabbit and is referred to as anti-LemTKRP-1 (code K9836; Winther et al., 2003; Winther and Nässel, 2001). Specificity controls are described in Winther et al. (1999) and Winther and Nässel (2001). The antibody against A-type allatostatin was raised in rabbit against a lepidopteran peptide (ARGYDFGL-NH₂), a representative of the Y/FXFGL-NH₂ superfamily of allatostatins (Duve and Thorpe, 2003). This antibody was a gift from Dr. Alan Thorpe (University of London). Specificity and cross absorption tests are given in Duve and Thorpe (2003). Identification of serotonin-like immunoreactivity employed anti-5-HT raised in rabbit (Sigma: product no. S 5545. LOT: 091K4831) used at a concentration of 1:500 in phosphate buffer (pH.7.0), employing standard methods for incubation and secondary

labeled. Lack of cross-reactivity of 5-HT antisera to 5-hydroxytryptophan or 5-hydroxyindole, 5-hydroxyindole-3-acetic acid, or dopamine has been demonstrated elsewhere (Beltz and Kravitz, 1983). For serotonin, A-type allatostatin, and TKRP immunostaining, brains were fixed overnight in 4 % paraformaldehyde in 0.01M phosphate buffered saline (PBS; pH 7.3), washed in buffer, embedded in 8 % agarose and sectioned at 20-60 μm on a Leica vibratome. After washing in PBS containing 0.3% Triton X100 (PBST) sections were preincubated in buffer with 10 % normal swine serum (Dako corporation, Carpinteria, CA) and then incubated in the corresponding antiserum in 1:500 dilution overnight at room temperature. After washing in PBST goat anti-rabbit immunoglobulin conjugated to Texas Red (1:250, Jackson Immuno Research Laboratories, West Grove, PA) was applied overnight. Sections were mounted and coverslipped under Elvanol (Rodriguez and Deinhardt, 1960). A Zeiss LSM Meta confocal microscope was used for data acquisition comprising 20-50 serial images of 512 $\times$ 512 and 1024 $\times$ 1024 at 8-bit or 12-bit color depth, scanned through a 20 $\times$ /0.5 Neofluar or 40 $\times$ /1.0 oil Iris or 63 $\times$ /1.40 oil iris plan-apochromat objectives. Scans were made at 0.2 – 1.0 μm steps through depths of between 1 and 20 μm . Procedures for phalloidin staining and rhodamine dextran injections were conducted according to standard procedures described in Sjöholm et al. (2005).

3. Results

3. 1. General organization and concentric zonation of the calyces

The arrangement of one mushroom body in one half of the brain of *Spodoptera* is shown in Fig. 1A. Its intrinsic neurons (Kenyon cells: KCs) have their cell bodies located dorsoposterior to its peripheral neuropil, the calyx, which is composed of contributions from a variety of cell types. Most populous are the dendrites of KCs, which are visited by terminals from axon collaterals of antennal lobe projection neurons that also invade protocerebral regions lateral to the calyces. The calyces additionally receive other processes, some of which carry GABA and others modulatory substances. In *Spodoptera*, as in most other insects that possess calyces, there are three classes of KCs, the structures of which and their relationships with lobe subdivisions have been documented in two previous accounts (Sjöholm et al., 2005, 2006). These cell types, which are defined by the morphologies of their dendritic branches, are known in other species to be generated in a specific sequence during post-embryonic development (Lee et al., 1999; Farris et al., 2004), with class III generated first, then the clawed class II and lastly spiny class I cells (Farris, 2005b). In *Spodoptera*, Class I and II KCs send their axon-like processes into the underlying pedunculus that extends ventrally (according to the neuraxis) through the protocerebrum to bifurcate into two lobes, one projecting medially just above the antennal lobes (the medial lobe), the other (the vertical lobe) projecting rostrally according to the brain's neuraxis (Sjöholm et al., 2005, 2006). The axons of class III Kenyon cells leave the calyx through a distinct satellite structure, called the Y-tract (Pearson, 1971; Sjöholm et al., 2005), that provides a separate pair of small satellite neuropils (called lobelets; Farris and Strausfeld, 2003) alongside the vertical and medial body lobes.

The two calyces of the mushroom body are partially fused, receive afferents from the antennal lobes (Fig. 1B), and comprise many thousands of synaptic complexes, called

microglomeruli (Yasuyama et al., 2002) that are revealed by phalloidin staining (Fig. 1C and inset). Each of the conjoined calyces is, however, clearly divided into discrete zones or territories, an outer zone, inner zone, and a central zone (Fig. 1B-G). These concentric divisions are demonstrated by filling rhodamine-dextran dye into the severed axons of the medial antennoglomerular tract, which carries the axons of antennal lobe projection neurons from antennal lobe glomeruli (Fig. 1B). Axon collaterals from these second order neurons extend into the calyx whereas their main axons by-pass the calyx to terminate in the lateral horn of the ipsilateral protocerebrum. Axon collaterals from antennal lobe projection neurons supply branched terminals to a broad outer zone of each of the two calyces but omit entirely the inner and central zone (Fig. 1B). Phalloidin staining demonstrates the presence of the inner zone (Fig. 1C), which is thought to receive a separate afferent supply, most likely from the visual system (Sjöholm et al., 2005). These concentric zones in the calyces are further revealed by immunolabeling with antisera raised against A-type allatostatin (Fig. 1G) and glutamate.

3. 2. Glutamate-, GABA-, and serotonin-like immunoreactivity

Although glutamate-like immunoreactive cells that are interpreted as glial somata surround the calyx (Fig. 2A), these are distinct from neurons within the central depression of each calyx (central zone), which contains clustered cell bodies of Kenyon cells. There is at the bottom of the depression a small population of glutamate immunopositive Kenyon cells (Fig. 2A, B). The axon-like processes of these neurons leave the calyx at the neck of the pedunculus to subsequently enter the core neuropil of the pedunculus and lobes (Fig. 2B). Small bead-like glutamate-immunoreactive profiles are seen scattered throughout the calyx (Fig. 2A) although the extremely slender dendritic processes that give rise to them are usually not resolvable. Similarly, glutamate-like immunoreactivity is seen in small profiles that are distributed within the α/α' , β/β' and γ divisions of the lobes and are best resolved at the spur, where the pedunculus divides into the vertical and medial lobes (Fig. 2C). Observations on other species suggest that these glutamate-immunoreactive profiles originate from parallel processes situated in the core of the lobes (see Discussion). Additionally, a few large-diameter fibers invade the medial lobe and the dorsal lobelet of the Y-tract, where several large strongly glutamate-immunoreactive profiles are observed (Fig. 2C, indicated by arrowhead).

GABA-like immunoreactivity is observed throughout the calyces. Immunoreactive fibers are generously equipped with small bleb-like varicosities (Fig. 1C). A part of the GABA supply to the calyces reaches this neuropil through a lateral tributary of the inner antennoglomerular tract (Fig. 3A, B, E). However, it is likely that the GABA-immunolabeling seen in the calyx of *S. littoralis* derives from at least two origins. This is suggested by the observation of occasional GABA-immunoreactive fibers that reach the calyx via the Y-tract (Fig. 3A), which is otherwise poor in GABA immunolabeling in contrast to the pedunculus (Fig. 3C, D). In the pedunculus, GABA-immunoreactive processes run approximately parallel to the axon-like projections of KCs, with numerous swellings along their lengths (Fig. 3D, E). This immunolabeling morphology within the pedunculus (Fig. 3D) is most reminiscent of a group of large diameter “exotic” intrinsic neurons that have been described from Golgi impregnation of the mushroom body of the scarab beetle *Pachnoda marginata* (Larsson et al.,

2004). In the distal part of the pedunculus, intense GABA immunolabeling is observed in the spur region, behind the point at which the medial and vertical lobes branch off from the pedunculus to form the α/α' and β/β' divisions. Although present, there appear to be relatively fewer and less intensely labeled GABAergic fibers in the γ lobe (Fig. 3F). However, viewed as a whole, GABA immunolabeling of the lobes clearly demarcates their longitudinal subdivisions, suggesting that each is innervated separately. This distribution also raises the possibility that a GABAergic feedback neuron originating from any one division of the lobes correspondingly supplies a domain in the calyx that provides that particular division with its Kenyon cell processes.

Serotonin (5HT)-immunoreactivity is apparent only in the outer zone of the calyces (Figs. 1E, 4A) and in the vertical and medial lobes (Fig. 4C), but not in the pedunculus. This distribution contrasts to the rich GABA-like immunoreactive supply to the entire mushroom body. Immunoreactive fibers with beaded swellings ramify widely throughout the outer zone of the calyx (Fig. 1E, 4A). This distribution compares favorably with that in the sphingid moth *Manduca sexta*, in which serotonin-like immunoreactivity in the calyx originates from a single neuron that has its soma in the contralateral posterior median protocerebrum and a few terminal branches in the inferior median protocerebrum of both hemispheres (Dacks et al., 2006). In *S. littoralis*, 5-HT labeling in the lobes is also comparable to the innervation of the mushroom body lobes in *M. sexta* by a second pair of serotonergic neurons that branch in the ipsilateral superior protocerebrum (Homberg and Hildebrand, 1989; Dacks et al., 2006).

3. 3. Tachykinin-related peptide- and A-type Allatostatin-like immunoreactivity

TKRP-like immunoreactivity is evenly distributed within the entire calyx of *S. littoralis* (Fig. 1F, 5A, B) and in some of the cell bodies surrounding the calyx. Between four and five intensely immunopositive large cell bodies are seen medial to the calyx (Fig. 5A). These lie adjacent to the calyx neuropil and send their neurites tangentially along the calyx surface and then downwards, parallel to the pedunculus. A group of about four small and faintly immunolabeled cells, lateral to the calycal neuropil, are interpreted as belonging to TKRP-neurons that are possibly intrinsic to the mushroom bodies (Fig. 5B). In addition to these two cell types, a group of eight large and strongly labeled cell bodies are situated at the pars intercerebralis between the clusters of Kenyon cell perikarya supplying the two mushroom bodies each side of the brain (Fig. 5C). These eight cells are here interpreted as neurosecretory cells that have previously been shown to be immunoreactive to TKRP in the developing brain of the South Pacific-Australian species, *Spodoptera litura* (Kim et al., 1998). Which of the observed types of immunopositive neurons provides labeling in the calyx in *S. littoralis* has not been ascertained, however.

A-type allatostatin-like immunoreactivity is pronounced in the calyx other than in its inner and central zones, the latter associated only with glutamate immunoreactive processes of last-born Kenyon cells (Fig. 1G). The main contribution of A-type allatostatin-like immunopositive innervation reaches the calyx by at most very few fibers that extend through the lateral branch of the inner antennoglomerular tract (Fig. 5D). Labeling with this antiserum is also observed in a single neurite projecting through the Y tract (Fig. 5E). In this respect, the pattern of A-type allatostatin-immunoreactive distribution in the calyces

resembles that of GABA-immunoreactive fibers. However, as discussed below, it is significant that no A-type allatostatin-like immunoreactivity has been observed in the mushroom body lobes (Fig. 5F, Table 1).

4. Discussion

The present observations support previous studies showing that in addition to distinct morphological types of neurons, the mushroom bodies of *S. littoralis* possess an elaborate chemical organization that reveals both global and local systems of modulatory elements (Table 1). Global systems, that is, distribution of arborization from a common origin in both the calyces and lobes include the distribution of glutamate-like immunoreactive neurons and GABA-immunoreactive elements. This contrasts with the three other described systems, the processes of which are clearly restricted to either the lobes or the calyces. Whereas the glutamate immunoreactivity is, in the main, ascribed to Kenyon cells it is likely to serve distributed functions throughout the mushroom bodies. By analogy with studies that have related cell body location and cell birth date (Farris and Strausfeld, 2003), glutamate-immunoreactive KCs that extend into the pedunculus are the last to have been generated during postembryonic development: thus, the basal ring Kenyon cells in the honey bee mushroom body, and Kenyon cells supplying the core neuropil of the adult fruit fly (Farris et al., 2004; Strausfeld et al., 2003). And although the most recently generated Kenyon cells at any instar are glutamate immunoreactive, in adulthood this expression persists only within the core or downgrowth zone of the pedunculus and lobes (Sinakevitch et al., 2001; Brown and Strausfeld, 2006). It is also conceivable that glutamate-positive neuron cell bodies observed in *S. littoralis* may include Kenyon cells generated in the adult, as is known to occur in the moth *Agrotis ipsilon* (Dufour and Gadenne, 2006). One interpretation of the sparse distribution of glutamate immunoreactive profiles in the calyces of *S. littoralis* is that these denote the swollen components of dendrites that belong to the very small population of glutamate immunoreactive Kenyon cells of the pedunculus core. While these specializations are resolvable, the delicate dendritic branches giving rise to them are usually not. Likewise, the extremely small glutamate immunoreactive profiles in the lobes are also likely to belong to these core Kenyon cells. In *Drosophila*, for example, Kenyon cells in the core neuropil are glutamate-immunoreactive and Golgi impregnations of these neurons show that they give rise to collaterals that extend laterally to intersect processes of other types of Kenyon cells in divisions that surround the core (Strausfeld et al., 2003). The presence of occasional large diameter and glutamate immunoreactive processes entering the medial lobe and lobelets is reminiscent of stout glutamate immunoreactive processes in the cockroach gamma lobes that are interpreted as extrinsic neurons (Sinakevitch et al., 2005).

GABA immunoreactivity is ascribed to recurrent or feedback neurons to the calyces, but there are clearly GABAergic systems that belong to the pedunculus and lobes, independent of the calyces. It is of interest that GABA-immunoreactive innervation to the calyces in *S. littoralis* originates from several different sources: from the protocerebrum entering the calyces via the lateral antennoglomerular tract, from the Y-tract, and from within the lobes and pedunculus. The first type of innervation was previously shown in *Manduca sexta*, where GABA-immunoreactive neurons innervating the calyx via the inner antennoglomerular tract have dendritic branches in the lateral horn (Homberg et al., 1987).

This organization suggests homology with similarly disposed GABA-immunoreactive neurons in cockroaches that mediate odor-evoked feed-forward inhibition to the calyx in that taxon (Strausfeld and Li., 1999); or in locusts, where they are suggested to contribute to signal sparsening in the mushroom body (Perez-Orive et al., 2002).

Whereas in *Periplaneta* and *Schistocerca*, both of which are hemimetabolous insects, GABA-immunoreactive neurons supply the calyces from the regions of the protocerebrum (Strausfeld and Li, 1998; Perez-Orive et al., 2002; Leitch and Laurent, 1996), in flies and honey bees, both holometabolous insects, the calyces receive their major contribution of GABA-immunoreactive processes from dendrites situated within the MB's vertical lobe or pedunculus. In honey bees, these neurons have been proposed to function as an inhibitory feedback loop (Grünewald, 1999b; Ganeshina and Menzel, 2001). Such differences between hemi- and holometabolous insects regarding the dendritic placement of GABA-immunoreactive neurons suggest that there may have been an evolutionary change from the basal (hemimetabolous) condition, where dendrites of GABA-immunoreactive neurons supplying the calyces were originally located in protocerebral areas outside the pedunculus and lobes. In the Holometabola, these dendrites appear to have shifted to a novel location to within the pedunculus and lobes. The presence of GABA-immunoreactive neurons in *S. littoralis* that correspond to both of these arrangements, suggests either a partial retention of the hemimetabolous condition or a novel acquisition of GABAergic afferents to the calyces from the protocerebrum in this recent crown group (see Kjer, 2004).

GABA-immunoreactive neurons are likely to provide intrinsic modulation by feedback from the mushroom body's lobes to the calyces, or from lateral horn neuropils of the protocerebrum (into which outputs from the lobes terminate). However, GABAergic profiles, which are not ascribable to dendritic arbors, are abundant in the lobes as well. This suggests that other inhibitory elements may independently invade the mushroom bodies at these levels. Likewise, the distinction of two serotonin immunoreactive systems, one innervating the calyces the other the lobes, suggests that these two neuropils may be independently modulated by 5HT. Independent modulation of circuits in the calyces but not the lobes is also suggested by the distribution of TKRP-like immunoreactivity. And although TKRP has been shown to be co-localized with GABA (Nässel, 2000; Nusbaum et al., 2001; Ignell, 2001), from the observed distribution of TKRP-immunoreactivity in *S. littoralis* we can safely assume that if TKRP is co-localized within GABA-immunoreactive neurons it is restricted to just those that supply the calyces (Fig. 1D, F, Table 1). The suggested role of TKRP in odorant sensitivity (Winther et al., 2006) would be compatible with the presence of this peptide within the calyces because it is these neuropils that are most closely associated with the olfactory pathway, receiving collaterals in the outer zone from antennal lobe projection neurons. Modulation by TKRP is thus likely to target these afferents. However, because both GABA- and TKRP-immunoreactivity invade into both outer and inner zones, they may be also involved in modulating sensory input to the inner zone, which has been suggested to receive afferents from the optic lobes (Sjöholm et al., 2006). Similarly, it has been suggested that A-type allatostatin functions as a co-transmitter with GABA (Schachtner et al., 2005). However, it is of interest that this peptide relates only to the outer zone of the calyx; that is, the part receiving olfactory afferents. Furthermore, A-type allatostatin cannot have an ubiquitous role as a co-transmitter with GABA because it is only

part of the calyces, not the lobes, that are supplied by A-type allatostatin-like immunoreactive neurons (Table 1).

Further studies are obviously needed to ascertain the identity and connections of uniquely identifiable neurons that exhibit specific affinities to neuroactive substances. However, the fact that what appears at first sight in *S. littoralis* to be a simple mushroom body, turns out to be one endowed with quite an elaborate chemical organization (Table 1; see also Sjöholm, 2006). This organization is at least as intricate as that described for the fruit fly *Drosophila* (Zhu et al., 2003; Strausfeld et al., 2003), though less so than that of the honey bee where Kenyon cells themselves may possess neuroactive substances and where the calyces are divided into more than a dozen concentric zones, each of which corresponds to a division of the lobes (Ehmer and Gronenberg, 2002; Strausfeld, 2002). The present results therefore suggest that the chemical neuroanatomy of the mushroom body of this moth may define at least three subdivisions of the calyces and lobes. Conceivably these might support some of the functional roles that have been ascribed to the more complicated mushroom bodies of other species, such as the honey bee and cockroach.

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Fig. 1.

Mushroom body organization in *S. littoralis* and the principal sensory, inhibitory and putatively modulatory inputs to the calyx. A: Summary drawing of the mushroom body, seen from a frontal aspect (midline to the left) with an example of a class II (left in calyx) and class I (right in calyx) Kenyon cell. The inner antennoglomerular tract (iAGT) provides collateral olfactory input from the antennal lobe (AL) to the mushroom body calyx (Ca) en route to the lateral protocerebrum (L Pro), which is the primary target neuropil of the inner and outer antennoglomerular tract (oAGT). Denoted parts of the mushroom body are: calyx (Ca), pedunculus (P), Y tract (Y), vertical lobe (V), medial lobe (M) and lobelet (lobl). Other abbreviations denote the fan-shaped body of the central complex (FB), medulla, lobula and lobula plate (Me, Lo, Lo P) of the optic lobes, antennal lobe (AL), and antennal nerve (AN). B: Top-down view of the two conjoint calyces shown by rhodamine-dextran injection into the antennal lobe projection neurons (AL). Injections fill axons and collaterals of antennal

lobe projection neurons (ALPNs) to show their supply to the outer zone of the calyx but not to the inner zone (bracketed) or the central zone (bracket, asterisk). C: Frontal view of a phalloidin-stained calyx showing the division into an outer and an inner zone (brackets) and the dense organization of bleblike microglomeruli (outlined in inner calyx, enlarged in inset). D: GABA-like-immunoreactive collaterals invade the entire calyx. GABA-like immunoreactive terminal endings are coarsely beaded and generally of larger diameter than the boutons of projection neuron collaterals (in panel B). E: The outer zone of each calyx is supplied with serotonin-like immunoreactive processes. The non-innervated inner zone is bracketed. F: Both inner and outer zones (bracketed) of the calyx are richly supplied with small beaded TKRP-like immunoreactive specializations. G: The characteristically varicose A-type allatostatin-like immunoreactive fibers typically omit the inner zone (bracketed) of the calyces. Scale bar in A = 100 μm , in B = 100 μm (also applying to D-F). Scale bar in C = 100 μm , in G = 50 μm .

Figure 2.

Glutamate-like immunoreactivity in the mushroom body of *S. littoralis*. A: Kenyon cell bodies labeled with glutamate are situated in the center of the calyx (arrow). Elements interpreted as glial cells that line the calyx rim exhibit glutamate-like immunoreactivity (arrowheads). Throughout the calycal neuropil there is sparse glutamate-immunoreactive labeling. Small bead- and varicose-like specializations are seen (arrow heads in inset) occasionally arising from extremely thin processes (e.g. double arrows in lower panel of inset). B: Antiglutamate (green) specifically labels scattered profiles throughout the calyx. Taurine immunopositive (magenta) cross sections of Kenyon cells (filled arrowheads) eventually supply the α/β divisions of the vertical and medial lobe. In the center of these taurine-immunoreactive bundles there are a few glutamate immunoreactive fibers (arrowed) that belong to the Kenyon cell somata shown in A. A ring of taurine-immunoreactive Kenyon cell processes (seen in cross section) that will supply the γ lobes is indicated by the open arrowhead. C: Glutamate-like-immunoreactivity in the lobes is mostly of extrinsic origin and labeling is seen in α/β , α'/β' , and the spur (sp). The lobelets (here the dorsal lobelet, d lobl) receive stout processes (arrowhead in Fig. 2C) that show glutamate-like immunoreactivity. Confocal stacks comprising panels A, B, C represent a depth of 1-3 μm . D: Control immunostaining of the calyx with glutamate antibodies after preabsorption with 10-4 M glutamate conjugated to BSA. Scale bars = 100 μm in A (also applies to panels C, D); 50 μm in B.

Figure 3.

GABA-like immunoreactivity in the mushroom body of *S. littoralis*. A, B: GABA-labeled processes invade the entire calyx (Ca), here seen from a frontal (A), and a top-down (posterior) aspect (B). Most GABA-immunoreactive arbors originate from large-diameter recurrent fibers (open arrowheads in A) although one or two fibers enter the calyx from the Y tract (panels A, B) and occasional GABA-immunoreactive processes are seen in the iAGT (panel B). C: Cross section through the pedunculus and Y tract showing abundant GABA-immunoreactive processes in the pedunculus and in the surrounding peripeduncular neuropil of the protocerebrum. D: In the pedunculus (sectioned aligned parallel to its long axis), GABA-immunoreactive processes run mostly parallel to the axon-like processes of Kenyon cells (not shown) and provide extensive ramifications that are equipped with varicose swellings. E: Reconstruction of confocal stacks from three thin sections (parallel to the pedunculus long axis) showing GABAergic immunolabelling in the calyx (Ca), pedunculus (P) and spur (sp) of the mushroom body and surrounding brain areas. Some GABA-immunoreactive fibers are seen within the lateral branch of the inner antennoglomerular tract (iAGT lb). The fan-shaped body of the central complex (FB) contains sparse GABA-immunoreactive fibers whereas the ellipsoid body beneath is densely packed. F: Frontal section at the level of the peduncular divide showing clear immunolabeling in the spur (sp) and in the α , α' and β , β' divisions and in the dorsal lobelet (d lobl). GABA immunoreactive

fibers are less abundant in the γ lobe. G: Detail of the medial lobe showing parallel organization of GABA immunoreactive profiles that then provide many branches within the swollen tip of the lobe. Scale bars = 100 μm in A (applies to B), E, F; 50 μm in C (applies to D), G.

Figure 4.

Serotonin-like immunoreactivity in the mushroom body of *S. littoralis*. A: Frontal confocal stack showing serotonin-like immunoreactive fibers with bead-like swellings ramifying evenly in both calyces. Serotonin-like immunoreactivity is absent in the pedunculus (P) and in the iAGT. B, C: Oblique frontal sections showing serotonin immunoreactive fibers that densely invade all parts of the mushroom body lobes with fine filamentous branches decorated with small varicose-like specializations. Scale bar = 100 μ m (applies to A - C).

Figure 5.

Frontal views showing TKRP-like (A-B) and A-type allatostatin-like immunolabeling (D-F) in the mushroom body calyx, and immunopositive somata (C). A: a group of 4-5 large, strongly labeled cell bodies (three shown in A, B, indicated by arrows to the right of panel) is seen medial to the calyx neuropil. Their neurites extend along the medial perimeter of the calyx and later parallel to the pedunculus. B: More ventral confocal image of the same section as in A. Here, a small cluster of about four cell bodies lateral to the calyces (left arrow) displays weak immunoreactivity to the TKRP antiserum. These are tentatively interpreted as a small subset of KCs. C: Intense anti TKRP immunolabelling is seen in eight of the large medial neurosecretory cells (MNC) located between the calycal perikarya in the two hemispheres of the brain. D: Immunolabeling in the calyx with antisera raised against A-type allatostatin. A single or a few immunolabeled fibers enter the calycal neuropil via the lateral branch of the iAGT (indicated by arrow). The pedunculus neck (asterisks) is not

labeled. E: A single immunoreactive fiber is seen projecting through the Y tract (arrow). Labeled cell bodies of unknown identity are scattered in front of the calyx. F: Immunolabeling with antisera raised against A-type allatostatin is absent in the mushroom body lobes. Abbreviations as in previous figures. Scale bars = 100 μm in A (applies to B), D, E, F; 50 μm in C.

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Table 1

Global and local distribution of neurons labeled by putative transmitters and/or neuroactive peptides in the calyces, and vertical and medial lobes (Y-lobe omitted). Asterisks indicate data from Sjöholm et al. 2006. Although serotonin immunoreactive profiles are present in both the calycal outer zone and in the vertical and medial lobes, evidence suggests that these are two independent patterns of innervation. Likewise, GABA immunoreactive neurons supplying both zones of the calyces are likely different from those invading the lobes.

Global		
(calyces + V, M lobes)		
Glutamate		
Aspartate*		
Taurine* (except in γ -lobe)		
Mas-allatropin*		
Local		
(calyx inner zone)	(calyx outer zone)	(V, M lobes)
GABA	+ GABA	GABA
----	Serotonin	Serotonin
TKRP	TKRP	----
----	A-type Allatostatin	-----
----	----	FMRFamide*