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THE NOVEL GUANYLYL CYCLASE MsGC-I IS STRONGLY EXPRESSED IN HIGHER-ORDER NEUROPILS IN THE BRAIN OF *MANDUCA SEXTA*

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Summary

Guanylyl cyclases are usually characterized as being either soluble (sGCs) or receptor (rGCs). We have recently cloned a novel guanylyl cyclase, MsGC-I, from the developing nervous system of the hawkmoth *Manduca sexta* that cannot be classified as either an sGC or an rGC. MsGC-I shows highest sequence identity with receptor guanylyl cyclases throughout its catalytic and dimerization domains, but does not contain the ligand-binding, transmembrane or kinase-like domains characteristic of receptor guanylyl cyclases. In addition, MsGC-I contains a C-terminal extension of 149 amino acid residues. In this paper, we report the expression of MsGC-I in the adult. Northern blots show that it is expressed preferentially in the nervous system, with high levels in the pharate adult brain and antennae. In the antennae, immunohistochemical analyses show that it is expressed in the cell bodies and dendrites, but not axons, of olfactory receptor neurons. In the brain, it is expressed in a variety of sensory neuropils including the antennal and optic lobes. It is also expressed in structures involved in higher-order processing including the mushroom bodies and central complex. This complicated expression pattern suggests that this novel guanylyl cyclase plays an important role in mediating cyclic GMP levels in the nervous system of *Manduca sexta*.

Key words: guanylyl cyclase, cyclic GMP, immunohistochemistry, olfaction, mushroom body, hawkmoth, *Manduca sexta*.

Introduction

The regulation of the generation of cyclic GMP (cGMP) plays an important role in the signal-transduction processes in the nervous systems of a wide variety of organisms. It is involved in such diverse processes as learning and memory in locusts (O'Shea et al., 1998) and honeybees (Muller and Hildebrandt, 1995), adaptation in olfactory receptor cells in both insects (Ziegelberger et al., 1990) and mammals (Zufall and Leinders-Zufall, 1997), odor memory in slugs (Gelperin, 1999), control of ecdysis behavior in insects (Ewer and Truman, 1996; Baker et al., 1999) and sensitization of nociceptive neurons in molluscs (Lewin and Walters, 1999). Guanylyl cyclases [GCs; GTP-pyrophosphate-lyase (cyclizing); EC 4.6.1.2], the enzymes that catalyze the synthesis of cGMP, are usually characterized as being either soluble guanylyl cyclases (sGCs) or receptor guanylyl cyclases (rGCs). The sGCs are obligate heterodimers of an alpha and a beta subunit. Upon heterodimer formation and heme binding, the activity of this enzyme complex can be strongly stimulated by nitric oxide (Denninger and Marletta, 1999). The rGCs, in contrast, are membrane-spanning proteins thought to act primarily as homodimers, although they have been shown to form higher oligomer units (Lowe, 1992; Chinkers and Wilson, 1992). They are usually activated through interactions with extracellular ligands (Foster et al., 1999) or intracellular guanylyl-cyclase-activating proteins (GCAPs) (Olshevskaya et al., 1999) and are completely insensitive to activation by nitric oxide.

Structurally, both rGCs and sGCs have a conserved, carboxy-terminal catalytic domain. Each sGC subunit also consists of an amino-terminal heme-binding and dimerization domain followed by the carboxy-terminal catalytic domain (Zhao and Marletta, 1997). The rGCs contain an amino-terminal extracellular ligand-binding domain, a transmembrane domain, a kinase-like domain, a dimerization domain and the carboxy-terminal catalytic domain (Wilson and Chinkers, 1995).

We have recently cloned a novel GC, MsGC-I, from the developing nervous system of the hawkmoth *Manduca sexta* (Simpson et al., 1999). MsGC-I shows highest sequence identity with rGCs throughout its catalytic and dimerization domains, but does not contain the ligand-binding, transmembrane or kinase-like domains characteristic of rGCs. In addition, MsGC-I contains a C-terminal extension of 149 amino acid residues that has no sequence homology to other

proteins, although there are other examples of rGCs with C-terminal extensions, e.g. Gyc76c in *Drosophila melanogaster*. The biochemical properties of MsGC-I are equally interesting. When the MsGC-I clone was transiently transfected into COS-7 cells, it functioned as a soluble homodimer with high levels of NO-insensitive basal activity. Western blot analyses of the *Manduca sexta* nervous system, however, showed that the protein was associated with the membrane fraction *in vivo* (Simpson et al., 1999). This suggested that MsGC-I may be membrane-associated *in vivo*, perhaps through interactions with as yet unidentified membrane-spanning or membrane-associated proteins. Although MsGC-I is best characterized in *Manduca sexta*, there is evidence that GCs with similar properties exist in other organisms. Scanning through the recently completed *Caenorhabditis elegans* and *Drosophila melanogaster* genome projects, it is possible to identify one *C. elegans* GC (C06A12) and at least two *Drosophila* GCs (CG3216 and CG9786) that, like MsGC-I, have similarity to rGCs in the catalytic domain yet lack transmembrane and ligand-binding domains. The *Drosophila* gene product CG9786 also has a C-terminal extension.

While the sequence and biochemical properties of MsGC-I are extremely interesting, its function in the nervous system is unclear since its expression was detected only in a few unidentified cells in the developing abdominal nervous system (Simpson et al., 1999). In the present paper, we extend the immunocytochemical analysis of the distribution of MsGC-I to the adult brain and antennae of *Manduca sexta* and show that it is expressed extensively throughout these tissues.

Materials and methods

Animals

Male *Manduca sexta* (Lepidoptera: Sphingidae) were reared on an artificial diet as described previously (Sanes and Hildebrand, 1976; Prescott et al., 1977). Animals were staged according to external morphology as described by Tolbert et al. (Tolbert et al., 1983).

Isolation of RNA

Adult brains, abdominal nervous systems, indirect flight muscles and antennae were dissected from male moths. Total RNA from each tissue was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD, USA). Poly(A^+) RNA was obtained from total RNA using oligo(dT) cellulose columns (Gibco-BRL).

Northern blot analysis

Poly(A^+) RNA (5 μg per lane) from each tissue was separated on a formaldehyde/1 % agarose gel. Ethidium bromide staining showed approximately equal levels of loading of the RNA in each lane. The gel was then blotted onto a Zetaprobe membrane (BioRad, Hercules, CA, USA) and hybridized overnight at 42 °C. The hybridization buffer contained 50 % formamide, 5× SSPE (1× SSPE is 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Na_2HPO_4, 0.005 mmol l^{-1} EDTA), 5× Denhardt's solution (1× Denhardt's solution is 0.1 % bovine serum albumin, 0.1 % polyvinylpyrrolidone, 0.1 % dextran sulfate), 1 % SDS, 10 % dextran sulfate, 100 μg ml^{-1} sheared salmon sperm DNA and 10^6 cts min^{-1} ml^{-1} ^{32}P-labeled random-primed probe prepared from gel-purified full-length MsGC-I cDNA using a Decaprime labeling kit (Ambion).

Origin of the primary antiserum

The generation of the polyclonal anti-MsGC-I antiserum is described (Simpson et al., 1999). Briefly, this antiserum was generated against a bacterially expressed glutathione *S*-transferase (GST) fusion protein containing the unique carboxy-terminal domain of MsGC-I.

Western blot analysis

Westerns blots were performed essentially as described (Simpson et al., 1999). Nervous tissue was homogenized [10 brains in 450 μl in phosphate-buffered saline (PBS) containing protease inhibitors; Complete Cocktail (Boehringer Mannheim)], and a sample was removed for protein assay. SDS sample buffer was added (final concentration 20 mmol l^{-1} Tris-HCl, pH 6.8, 1.6 % SDS, 4 % β-mercaptoethanol, 4 % glycerol and 20 mg ml^{-1} Bromophenol Blue), and the samples were boiled for 5 min. The proteins (100 μg) were separated on a 10 % polyacrylamide gel and transferred to a PVDF membrane. Primary antiserum was used at a dilution of 1:5000 and detected using a 1:10 000 dilution of horseradish-peroxidase (HRP)-conjugated goat anti-rabbit antibodies (Jackson Laboratories) and visualized with chemiluminescence and a digital imaging system (ChemiImager, Alpha Innotech).

Immunocytochemistry

Immunohistochemical analyses were performed on 15 different animals. Patterns of staining were essentially the same from animal to animal.

Brains

Pharate adult male brains were dissected in PBS, then fixed in 4 % paraformaldehyde overnight (16–20 h) at 4 °C. Using a vibratome sectioning protocol adapted from Davis et al. (Davis et al., 1997), fixed tissue was washed for several hours at room temperature in PBS containing 0.5 % Triton X-100 (PBST), then washed in PBST at 4 °C overnight. The tissue was embedded in 5 % agarose (Sigma Type I-A) and cut into 100 μm sections in the presence of PBST. Sections were blocked for 1 h at room temperature in PBST containing 10 % normal goat serum and 0.1 % sodium azide as a preservative. Primary antiserum was added to the blocking solution at a dilution of 1:2000, and the tissue was incubated at room temperature overnight. Following incubation, the tissue was washed thoroughly in PBST and blocked for 1 h at room temperature. Secondary antibody (goat anti-rabbit Alexa 546) was added to the blocking solution at a dilution of 1:250, and the tissue was incubated at room temperature overnight. Sections were mounted in 80 % glycerol. Images were gathered

using a Nikon PCM 2000 laser-scanning confocal microscope. Preimmune serum was used at the same concentration as the primary antiserum as a control in each experiment. No visible staining was ever seen in these controls when viewed at the same confocal settings as the experimental preparations.

Antennae

Antennae from pharate adult males were dissected into five-annulus segments in PBST, then fixed in 4% paraformaldehyde at 4 °C overnight. Fixed tissue was washed briefly in PBST. The tissue was cryoprotected for frozen sectioning in a solution containing 10% sucrose in PBS for 3 h at 4 °C, then in 20% sucrose in PBS and finally in 30% sucrose in PBS overnight. Tissue was embedded in OCT (TissueTek) and cut into 20 µm sections at −22 °C with a Zeiss HM500 cryostat. Sections were mounted on positively charged slides and baked at 42 °C for 48 h. The tissue was blocked for 1 h at room temperature in PBST containing 10% normal goat serum. Primary antiserum was added to the blocking solution at dilutions of 1:2000 and 1:3000 and the tissue was incubated at 4 °C for 72 h. Following a brief wash in PBST, the tissue was blocked again for 1 h at room temperature. Secondary antibody (goat anti-rabbit Alexa 546) was added to the blocking solution at a dilution of 1:250, and the tissue was incubated for 2 h at room temperature. Sections were mounted in ShurMount (Electron Microscopy Sciences). Images were gathered using Nikon PCM 2000 and Bio-Rad MCR 600 laser-scanning confocal microscopes.

Pre-absorption controls

A GST fusion protein of MsGC-I was prepared as described by Simpson et al. (1999). To pre-absorb the primary antiserum, 2.2 mg of MsGC-I GST fusion protein was added to 500 µl of a 1:10 000 dilution of primary antiserum and allowed to incubate for 48 h at 4 °C. Immunocytochemical staining was performed using the protocol described above, with the exception that primary antiserum was used at a dilution of 1:10 000. Images were gathered using a Nikon PCM 2000 laser-scanning confocal microscope. The gain and neutral density settings were kept identical for both the pre-absorbed and non-pre-absorbed images.

Results

We examined the adult tissue distribution of MsGC-I mRNA using northern blots. A strong single band of 2.6 kilobases (kb) was detected in mRNA isolated from antennae, and a weaker single band of the same size was detected in brain (Fig. 1A). The single band of 2.6 kb was the same size as that detected in the developing nervous system (Simpson et al., 1999), suggesting that size or splice variation is not changed during development or in different regions of the nervous system. Although the differences in expression were not quantified, the focus of MsGC-I expression in the adult was clearly in the antennae and brain. Note that this result does not rule out the possibility of low-level expression in the ventral nerve cord similar to that seen in the developing nervous system (Simpson et al., 1999). Levels of mRNA expression in the adult brain are relatively low, while the immunohistochemical staining shows a widespread and strong expression of the MsGC-I protein (Figs 2, 3, 4). This suggests that there may be low-level expression in the ventral nerve cord that might not be detectable with the antibody.

The size of the MsGC-I protein product in the adult nervous system was examined using western blots. A strong single band at 55 kDa was detected in pharate adult brains (Fig. 1B). The predicted size of MsGC-I is 55 kDa, suggesting that the antiserum recognizes the same protein in both adult and prepupal nervous systems (Simpson et al., 1999) and that the size and probable structure of MsGC-I are the same in both prepupal and adult nervous systems. As an additional test for the specificity of the antiserum, we compared the immunocytochemical staining pattern of the antiserum with that of a sample of antiserum that had first been pre-absorbed against the MsGC-I protein. The pre-absorption significantly decreased the level of staining (see Fig. 3A,B) in all areas of the brain. These results suggest that the same isoforms of MsGC-I mRNA and protein are expressed in the nervous system of both developing and adult animals and that the MsGC-I antiserum can be used specifically to localize MsGC-I in the adult.

MsGC-I is highly enriched in the central brain

To investigate the pattern of MsGC-I expression in the adult brain, we performed immunocytochemistry on serial frontal

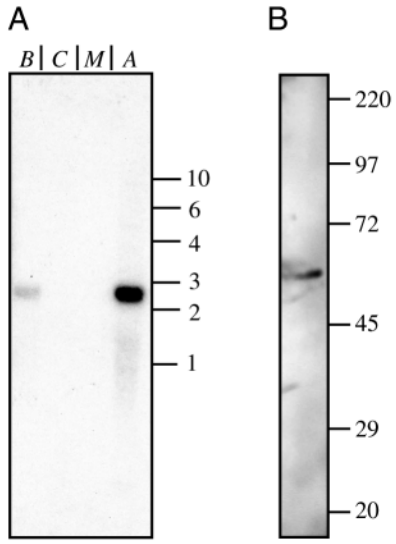

Fig. 1. Adult tissue distribution of the novel guanylyl cyclase MsGC-I. (A) Northern blot analysis showing that a single 2.6 kb transcript for MsGC-I is expressed in brain (*B*) and strongly expressed in antennae (*A*) while being undetectable in muscle (*M*) or abdominal nerve cords (*C*) isolated from adult *Manduca sexta*. Molecular mass markers are in kb. (B) MsGC-I antiserum recognizes a 55 kDa protein in the adult. Protein isolated from pharate adult brains was probed with MsGC-I antiserum. A single band of 55 kDa is detected. Molecular markers are in kDa.

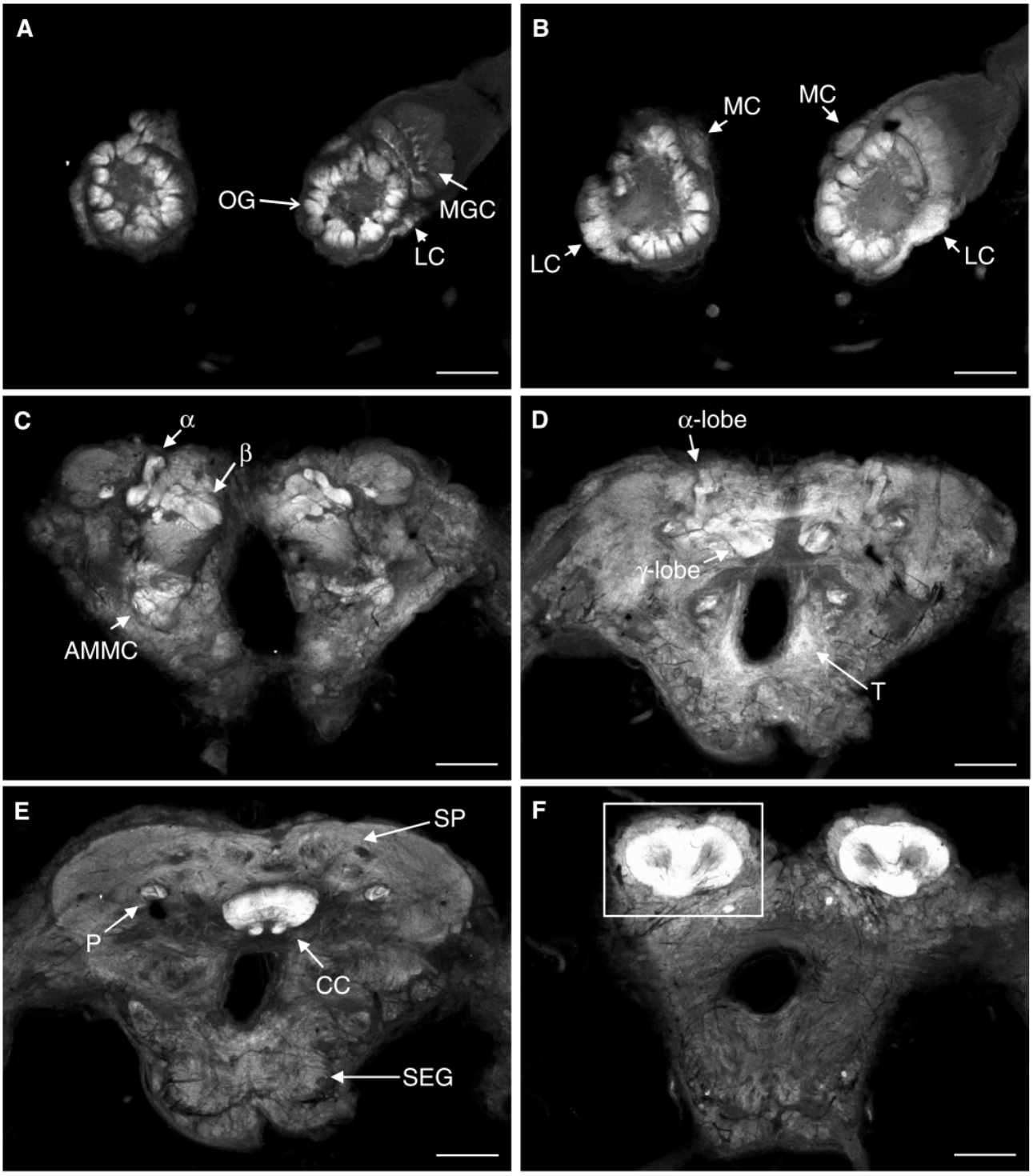

Fig. 2. MsGC-I protein is localized throughout the adult brain. (A–F) A series of low-magnification views of frontal sections through the brain of an adult male in an anterior (A) to posterior (F) direction. Scale bars, 220 μm. (A) MsGC-I is highly expressed in the antennal lobes, including the ordinary glomeruli (OG) and individual cells in the lateral cell body cluster (LC). Staining can also be seen in the macroglomerular complex (MGC). (B) A slightly more posterior section through the antennal lobes. Note the staining of both the lateral (LC) and medial (MC) cell body clusters. The staining of the LC is much more intense. (C) At this level, the protocerebrum can be seen, with strong staining of the alpha (α) and beta (β) lobes of the mushroom bodies. The antenno-mechanosensory center (AMMC) also stains strongly. (D) Deeper in the protocerebrum, the alpha (α) and gamma (γ) lobes of the mushroom bodies and the tritocerebrum (T) can be seen. (E) At this level, the central complex (CC) and the peduncles of the mushroom bodies (P) can be seen to stain strongly. Weak and diffuse staining can also be seen in the subesophageal ganglion (SEG) and the superior protocerebrum (SP). (F) Extreme posterior section showing strong staining of the calyces of the mushroom bodies (outlined by a white box).

vibratome sections through the brain of male *Manduca sexta*. The resulting staining pattern was both widespread and dramatic. Starting at the anterior part of the brain, MsGC-I staining is strongly present in the cell bodies and neuropil of the antennal lobes (Fig. 2A,B). More posteriorly, we find strong staining of the antennal mechanosensory and motor center neuropil and even stronger staining of the alpha, beta and gamma lobes of the mushroom bodies (Fig. 2C). This strong staining of the lobes of the mushroom bodies can be seen to extend into the next stack of sections, where there is also lower-level diffuse staining of the lateral protocerebrum and the remains of the antennal mechanosensory and motor center. The tritocerebrum (Fig. 2D) can also be seen to stain strongly. The next stack of sections shows strong staining of the central complex and the peduncles of the mushroom bodies. Finally, there is also strong staining of the cell bodies and neuropil of the calyces and the accessory calyces at the very posterior portion of the brain. Thus, in the central brain, MsGC-I can be found concentrated in the central complex, in all portions of the mushroom bodies, in the antennal mechanosensory and motor center and in the antennal lobes. In addition, there is somewhat fainter staining in the tritocerebrum and in the inner antenno-cerebral tract and diffuse staining throughout the lateral protocerebrum.

The central complex is a relatively invariant neuropil present in all insect orders. It is known to respond to multimodal sensory stimuli (Homberg et al., 1999; Ilius et al., 1994) and is thought to be involved in the higher-order processing of information (Strausfeld, 1976). It is also shows structural plasticity, changing volume depending on experience (Barth and Heisenberg, 1997). The central complex consist of the central body, the ventral bodies and the protocerebral bridge. Examining MsGC-I expression in this neuropil more closely, we find that the MsGC-I antiserum stains the components of the central body very strongly but does not stain the ventral bodies or the protocerebral bridge. The central body consists of the ellipsoid body, the fan-shaped body, the superior arch and the noduli. Staining of both the ellipsoid body and the superior arch can be seen in Fig. 3A. Staining of the fan-shaped body and the noduli is shown in Fig. 3C. Note that the noduli are stained especially strongly. There is also a diffuse, lower-level staining in the neuropil of the superior protocerebrum just dorsal to the central body (Fig. 3C).

The mushroom bodies are a pair of complex structures that are also thought to be involved in the processing of multimodal sensory information (Strausfeld and Li, 1999; Gronenberg, 1999) and in learning and memory (McBride et al., 1999; Hammer and Menzel, 1998; Mizunami et al., 1998; Davis, 1993). These structures are mainly made up of Kenyon cells, whose dendrites form the cup-like calyces at the dorsal posterior of the brain. Surrounding the calyces are the Kenyon cell bodies. The Kenyon cells then project axons in a bundle termed the peduncle that reaches to the very anterior portion of the central brain. There, the axons branch to form the alpha, beta and gamma lobes. Staining with MsGC-I antiserum can be seen in a subset of Kenyon cell bodies and is unevenly expressed throughout the calyx (Fig. 3D). Staining is particularly strong at the base of the calyx where the peduncle begins to form. This area, sometimes referred to as the accessory calyx (Homburg et al., 1988) or the base of the peduncle (Strausfeld, 1976), is known to be rich in visual and olfactory input. Somewhat weaker staining can be seen in the subdivisions of the peduncle as it travels to the anterior of the brain (Fig. 3E). In the anterior, MsGC-I staining is again very strong in the lobes of the mushroom bodies (Fig. 3F). Note, however, that this staining pattern is not uniform, suggesting that only a subset of Kenyon cells expresses high levels of MsGC-I.

MsGC-I is expressed in the olfactory and visual systems

MsGC-I is expressed both in the antenna, where the olfactory receptor cells detect odorants, and in the antennal lobe, to which the olfactory receptor cells project their axons and where initial processing of olfactory information takes place. A cross section through a male antenna shows MsGC-I expression in a portion of the olfactory epithelium (Fig. 4A). A higher magnification shows that MsGC-I is expressed in small clusters of olfactory receptor cells whose processes can be seen to extend towards the olfactory sensilla (Fig. 4B). Small amounts of staining can be seen in the proximal axon. No staining can be seen, however, in the cross section of the antennal nerve that contains all the axons of the olfactory receptor cells (Fig. 4A). In addition to the olfactory receptor cells, a small group of four cells, positioned deep within the epithelium, are MsGC-I-positive (Fig. 4B). The identity and function of these cells is unknown.

Strong staining for MsGC-I can also be seen in the antennal lobe. The antennal lobe is both functionally and anatomically similar to the olfactory bulb (Hildebrand and Shepherd, 1997). This structure processes olfactory information, is thought to be important for the discrimination of odorants and may play a role in olfactory-based learning and memory behaviors (Stopfer and Laurent, 1999; Hammer and Menzel, 1998; Heinbockel et al., 1999). In the antennal lobe, sensory axons from the antennae and inhibitory interneurons and projection neurons whose cell bodies are present in the antennal lobe enter into complex synaptic arrangements within spheroidal neuropils termed glomeruli. Sensory afferents project to the outer margin, interneurons form dense arborizations more medially, and neurites of projection neurons occupy the basal margin of each glomerulus (Homberg et al., 1988; Sun et al., 1997). MsGC-I could be seen strongly staining the medial portions of most of the ordinary glomeruli (Fig. 4C–E). The intensity of staining among the glomeruli was not uniform. Some of the glomeruli in the lateral portion of the antennal lobe stained more intensely than glomeruli in the dorsal medial antennal lobe (Fig. 4C). In addition to staining normal glomeruli, MsGC-I is also present in the male-specific macroglomerular complex (MGC), a set of glomeruli uniquely and specifically dedicated to the detection and processing of responses to the female sex pheromone mixture (Christensen and Hildebrand, 1997) (Fig. 4D,E). The staining of the MGC

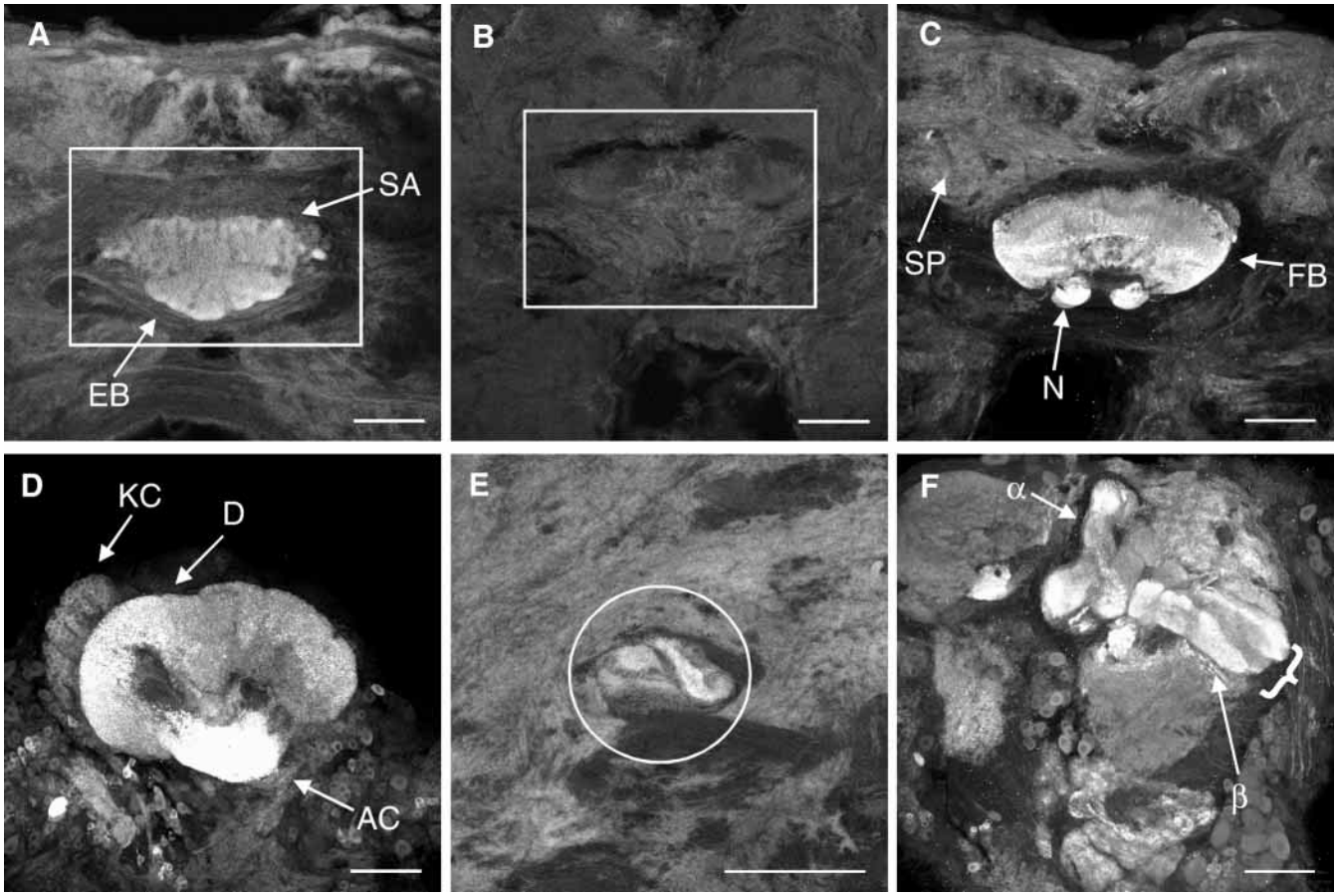

Fig. 3. MsGC-I is localized to specific portions of central brain neuropils. (A–C) Views of the central complex. (D–F) Views of the mushroom bodies. Scale bars, 100 μm. (A) High-magnification view of the ellipsoid body (EB) and superior arch (SA) of the central complex showing strong expression of MsGC-I (stained with a 1:10 000 dilution of primary antiserum). (B) Same view as A visualized with pre-absorbed antiserum showing the specificity of the MsGC-I antibodies. (C) High-magnification view of the noduli (N) and the fan-shaped body (FB) of the central complex showing strong staining for MsGC-I and weaker and more diffuse expression in the superior protocerebrum (SP). (D) High magnification of the mushroom body calyx region. MsGC-I is expressed in a subset of Kenyon cell bodies (KC) and in the dendrites (D) that make up the calyx. Note the especially strong staining in the accessory calyx region (AC). (E) Cross section through the mushroom body peduncle (outlined by white circle). Note the staining of three different subdivisions within the peduncle. (F) Frontal section showing the alpha (α) and beta (β) lobes of the mushroom bodies. Note the different layers of staining within the β lobe (outlined by a white bracket).

was generally much less intense than the staining in the other glomeruli, although it is not clear whether this is due to a decrease in the MsGC-I levels in individual neurons or to a general decrease in the density of the neuropil of these exceptionally large glomeruli.

MsGC-I is also expressed in a subset of antennal lobe cell bodies. The cell bodies of antennal lobe neurons are housed in two main clusters. The medial cell body cluster houses the cell bodies of uniglomerular projection neurons. These neurons have dendrites that innervate a single glomerulus and axons that project to higher brain regions including the mushroom bodies and the lateral protocerebrum. The lateral cell body cluster houses the cell bodies of both uni- and multi-glomerular projection neurons and inhibitory multiglomerular interneurons. MsGC-I is strongly expressed in a subset of lateral cell body cluster neurons (Fig. 4C,D). Although it is impossible to determine the exact identity of these strongly staining neurons without further double-label analysis, the strong staining of the medial portions of the glomeruli (Fig. 4E) suggests that these are inhibitory interneurons. Occasionally, cells from the medial cell body packet can also be seen to stain, although not as intensely as the cells in the lateral cell body packet (Fig. 4C). This suggests that MsGC-I is expressed in some uniglomerular projection neurons, although no staining can be seen in the fiber tracts leaving the antennal lobe, showing that MsGC-I is localized to the cell bodies and dendrites but not to the axons of these neurons. Overall, however, the focus of MsGC-I expression in the antennal lobe is in the cell bodies and processes of the antennal lobe neurons housed in the lateral cell body cluster. Although the strong staining of the medial portions of each of the glomeruli suggests that MsGC-I is expressed in a subset of inhibitory interneurons, this expression pattern cannot be confirmed without a double-label analysis.

A different pattern of staining was found in the visual system. No staining of MsGC-I was found in the retina,

showing that the sensory neurons of the visual system do not express MsGC-I. Moderate staining of MsGC-I was found in the neuropil of the optic lobes, although no staining was detected in cell bodies in the optic lobes. The optic lobe is subdivided into four neuropil structures termed the lamina, the medulla, the lobula and the lobula plate (Strausfeld, 1976).

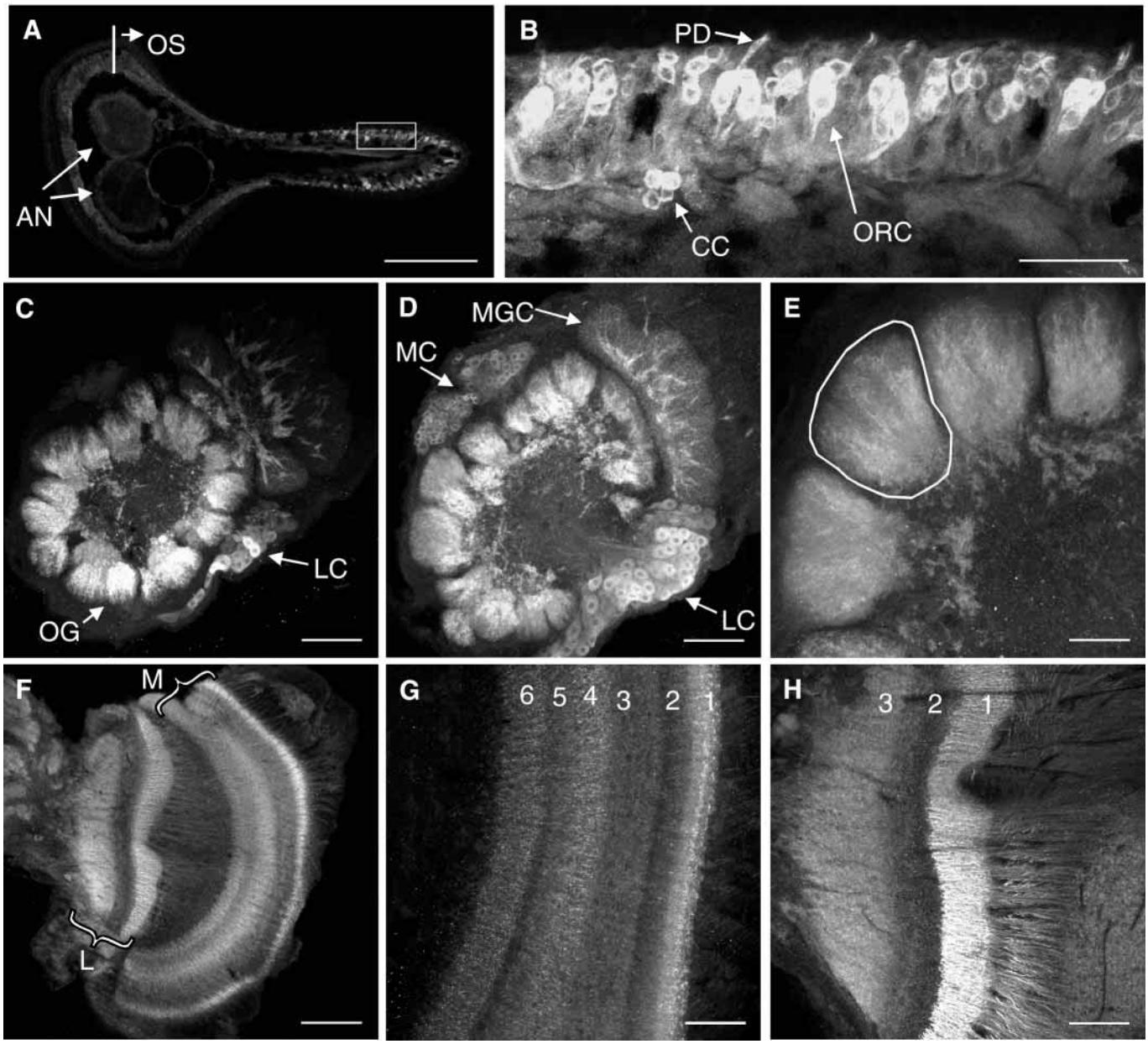

Fig. 4. MsGC-I is expressed in sensory neuropils. (A–E) Olfactory system. (F–H) Visual system. (A) Cross section through an adult male antenna. Note the lack of staining in the antennal nerves (AN). The distal portion of the olfactory sensillar epithelium (OS) can be seen to express MsGC-I. Scale bar, 200 μm. (B) High-magnification view of the MsGC-I-positive portion of the sensillar epithelium outlined in A. Note the strong staining of subsets of clusters of olfactory receptor cells (ORC). In some cases, the proximal dendrites (PD) can be seen to stain. A small cluster of cells (CC) of unknown function deep within the epithelium also expresses MsGC-I. Scale bar, 33 μm. (C) Anterior frontal section of the antennal lobe clearly showing individual cells in the lateral cell body cluster (LC) strongly expressing MsGC-I (arrow) and weakly expressing MsGC-I (above the arrow). Differential staining among the ordinary glomeruli (OG) can also be seen. Scale bar, 100 μm. (D) A more posterior frontal section through the antennal lobe showing faint staining in a subset of medial cell body cluster neurons (MC) and stronger staining in a subset of LC neurons. Staining within the processes of the macroglomerular complex (MGC) can also be seen. Scale bar, 100 μm. (E). High-magnification view of an individual ordinary glomerulus (outlined in white). MsGC-I can be seen to stain more brightly at the basal and medial portions of each glomerulus, which the interneurons are known to innervate. Scale bar, 33 μm. (F) Frontal section through the optic lobes showing staining in the crescent-shaped medulla (M) and the lobula (L). Scale bar, 200 μm. (G) High-magnification view of the medulla. MsGC-I is localized to different levels in at least six different layers within the medulla. Scale bar, 50 μm. (H) High-magnification view of the lobula. Three distinct layers can be seen, with the outermost layer (1) expressing the highest levels of MsGC-I staining. Scale bar, 50 μm.

Most retinal photoreceptors project to the lamina, the first-order neuropil of the visual system. The lamina neurons then project to the medulla, the second-order neuropil. Medulla neurons then project to the lobula and lobula plate. MsGC-I staining is absent from the lamina and lobula plate but is present in the medulla and lobula (Fig. 4F). The medulla has a columnar organization, with each column representing input from one of the retinal ommatidia. Horizontal layers can be seen within this columnar organization, representing either horizontal elements or varicosities within the columnar neurons. MsGC-I can be seen to localize to several of these layers. The strongest staining is in the very outermost layer. In the lobula, three distinct layers of staining can be seen, with the strongest staining in the outermost layer. The middle layer does not stain, and a relatively faint, diffuse staining can be seen in the innermost layer. The overall staining pattern of MsGC-I in the horizontal layers in the visual neuropils suggests that it is expressed in neurons that modify and integrate visual information.

Discussion

In our initial description of MsGC-I (Simpson et al., 1999), we described a guanylyl cyclase with unique structural properties and potentially unique mechanisms of regulation. This made MsGC-I of significant biochemical interest, but its role in regulating cGMP levels *in vivo* was unclear. Here, we have shown that the MsGC-I protein is expressed widely in the adult brain and antenna in a variety of neurons whose functions are known. Examining its expression pattern more closely, we find that MsGC-I is expressed most strongly in areas of the brain that are involved in the integration and modification of sensory information. It is strongly expressed in the mushroom bodies and central complex. Both these complicated brain structures process multimodal sensory information. The mushroom bodies have been implicated in a variety of different learning and memory paradigms in insects that include classical conditioning, operant learning and courtship learning. A large number of genes that have been identified genetically as being important for learning and memory in *Drosophila melanogaster*, including adenylyl cyclase, cyclic AMP phosphodiesterase and protein kinase A, share similar expression patterns, with strong staining of the central complex and mushroom bodies (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993). Although the expression pattern of a protein can only predict and not demonstrate function, the strong expression of MsGC-I in these structures suggests that cGMP in general, and the regulation of cGMP levels by MsGC-I specifically, might play a role in mediating neuronal plasticity in *Manduca sexta*.

In addition to its potential role in neuronal plasticity and its expression in the mushroom bodies and central complex, MsGC-I is expressed in a manner consistent with it playing a role in the processing of sensory information. In the olfactory system, MsGC-I is expressed in the cell bodies and dendrites of olfactory receptor cells, where it may mediate some of the initial events in olfactory signal transduction. It is thought that cGMP levels increase in the antennae of *Manduca sexta* in response to pheromone stimulation (Ziegelberger et al., 1990). This elevation of cGMP concentration is thought to occur in the olfactory receptor cell bodies and dendrites and may play a role in adaptation (Stengl et al., 1992). MsGC-I is expressed in an ideal location to mediate this increase in cGMP levels.

MsGC-I is also expressed in antennal lobe neurons and in the medulla and lobula. These are second-order neuropils that process visual or olfactory information before it is transmitted to higher brain centers. These are, of course, not simply relay stations. In the honeybee and the fruit fly, for example, it is clear from behavioral studies that the antennal lobes function in odorant discrimination and in simple classical conditioning (Hammer and Menzel, 1998; Zars et al., 2000).

Interestingly, MsGC-I is not localized to the same subcellular location throughout its expression range. In the olfactory receptor cells, it is restricted to the cell bodies and dendrites, while in the optic lobes it is not present in cell bodies. In the mushroom bodies, it is present in the cell bodies and dendrites (in the calyces) and in the axons (in the peduncles and alpha, beta and gamma lobes). This difference in subcellular localization might have implications for its function and the mechanisms of its regulation in different cell types.

It is also interesting to consider the regulation of MsGC-I in the context of other GCs. For example, NO-sensitive sGC subunits are expressed in antennal lobe neurons (Nighorn et al., 1998), suggesting that both NO-sensitive and NO-insensitive regulation of cGMP levels may be important in the antennal lobe. In contrast, NO-sensitive sGCs are not present in olfactory receptor neuron cell bodies and dendrites, where MsGC-I is highly expressed. The regulation of cGMP levels generally, and MsGC-I levels specifically, may be considerably different in the two different environments.

To summarize, the localization of MsGC-I suggests that it is important for the regulation of cGMP levels in the adult brain. It is strongly expressed in the cell bodies and proximal dendrites of a subset of olfactory receptor cells, where it may be involved in the initial olfactory signal-transduction events. MsGC-I is expressed in neuropils that process second-order visual and olfactory sensory information. Finally, it is also strongly expressed in the mushroom bodies and the central complex, where it may play a role in mediating the signal-transduction events that underlie the structural and functional plasticity thought to occur in these structures.

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References

Baker, J. D., McNabb, S. L. and Truman, J. W. (1999). The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3037–3048.

Barth, M. and Heisenberg, M. (1997). Vision affects mushroom bodies and central complex in *Drosophila melanogaster*. *Learning Memory* **4**, 219–229.

Chinkers, M. and Wilson, E. M. (1992). Ligand-independent oligomerization of natriuretic peptide receptors. Identification of heteromeric receptors and a dominant negative mutant. *J. Biol. Chem.* **267**, 18589–18597.

Christensen, T. A. and Hildebrand, J. G. (1987). Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta*. *J. Comp. Physiol.* A **160**, 553–569.

Davis, N. T., Veenstra, J. A., Feyereisen, R. and Hildebrand, J. G. (1997). Allatostatin-like-immunoreactive neurons of the tobacco hornworm, *Manduca sexta* and isolation and identification of a new neuropeptide related to cockroach allatostatins. *J. Comp. Neurol.* **385**, 265–284.

Davis, R. L. (1993). Mushroom bodies and *Drosophila* learning. *Neuron* **11**, 1–14.

Denninger, J. W. and Marletta, M. A. (1999). Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim. Biophys. Acta* **1411**, 334–350.

Ewer, J. and Truman, J. W. (1996). Increases in cyclic 3′,5′-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. *J. Comp. Neurol.* **370**, 330–341.

Foster, D. C., Wedel, B. J., Robinson, S. W. and Garbers, D. L. (1999). Mechanisms of regulation and functions of guanylyl cyclases. *Rev. Physiol. Biochem. Pharmac.* **135**, 1–39.

Gelperin, A. (1999). Oscillatory dynamics and information processing in olfactory systems. *J. Exp. Biol.* **202**, 1855–1864.

Gronenberg, W. (1999). Modality-specific segregation of input to ant mushroom bodies. *Brain Behav. Evol.* **54**, 85–95.

Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learning Memory* **5**, 146–156.

Han, P. L., Levin, L. R., Reed, R. R. and Davis, R. L. (1992). Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. *Neuron* **9**, 619–627.

Heinbockel, T., Christensen, T. A. and Hildebrand, J. G. (1999). Temporal tuning of odor responses in pheromone-responsive projection neurons in the brain of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* **409**, 1–12.

Hildebrand, J. G. and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* **20**, 595–631.

Homberg, U., Montague, R. A. and Hildebrand, J. G. (1988). Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta*. *Cell Tissue Res.* **254**, 255–281.

Homberg, U., Vitzthum, H., Muller, M. and Binkle, U. (1999). Immunocytochemistry of GABA in the central complex of the locust *Schistocerca gregaria*: identification of immunoreactive neurons and colocalization with neuropeptides. *J. Comp. Neurol.* **409**, 495–507.

Ilius, M., Wolf, R. and Heisenberg, M. (1994). The central complex of *Drosophila melanogaster* is involved in flight control: studies on mutants and mosaics of the gene *ellipsoid body open*. *J. Neurogenet.* **9**, 189–206.

Lewin, M. R. and Walters, E. T. (1999). Cyclic GMP pathway is critical for inducing long-term sensitization of nociceptive sensory neurons. *Nature Neuroscience* **2**, 18–23.

Lowe, D. G. (1992). Human natriuretic peptide receptor-A guanylyl cyclase is self- associated prior to hormone binding. *Biochemistry* **31**, 10421–10425.

McBride, S. M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G. and Siwicki, K. K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster*. *Neuron* **24**, 967–977.

Mizunami, M., Weibrecht, J. M. and Strausfeld, N. J. (1998). Mushroom bodies of the cockroach: their participation in place memory. *J. Comp. Neurol.* **402**, 520–537.

Muller, U. and Hildebrandt, H. (1995). The nitric oxide/cGMP system in the antennal lobe of *Apis mellifera* is implicated in integrative processing of chemosensory stimuli. *Eur. J. Neurosci.* **7**, 2240–2248.

Nighorn, A., Gibson, N. J., Morton, D. B. and Hildebrand, J. G. (1998). The NO/cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. *J. Neurosci.* **8**, 7244–7255.

Nighorn, A., Healy, M. J. and Davis, R. L. (1991). The cyclic AMP phosphodiesterase encoded by the *Drosophila dunce* gene is concentrated in the mushroom body neuropil. *Neuron* **6**, 455–467.

O'Shea, M., Colbert, R., Williams, L. and Dunn, S. (1998). Nitric oxide compartments in the mushroom bodies of the locust brain. *Neuroreport* **9**, 333–336.

Olshevskaya, E. V., Ermilov, A. N. and Dizhoor, A. M. (1999). Dimerization of guanylyl cyclase-activating protein and a mechanism of photoreceptor guanylyl cyclase activation. *J. Biol. Chem.* **274**, 25583–25587.

Prescott, D. J., Hildebrand, J. G., Sanes, J. R. and Jewett, S. (1977). Biochemical and developmental studies of acetylcholine metabolism in the central nervous system of the moth *Manduca sexta*. *Comp. Biochem. Physiol*. **56**C, 77–84.

Sanes, J. R. and Hildebrand, J. G. (1976). Structure and development of antennae in a moth, *Manduca sexta*. *Devl. Biol*. **51**, 280–299.

Simpson, P. J., Nighorn, A. and Morton, D. B. (1999). Identification of a novel guanylyl cyclase that is related to receptor guanylyl cyclases, but lacks extracellular and transmembrane domains. *J. Biol. Chem.* **274**, 4440–4446.

Skoulakis, E. M., Kalderon, D. and Davis, R. L. (1993). Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. *Neuron* **11**, 197–208.

Stengl, M., Zufall, F., Hatt, H. and Hildebrand, J. G. (1992). Olfactory receptor neurons from antennae of developing male *Manduca sexta* respond to components of the species-specific sex pheromone *in vitro*. *J. Neurosci.* **12**, 2523–2531.

Stopfer, M. and Laurent, G. (1999). Short-term memory in olfactory network dynamics. *Nature* **402**, 664–668.

Strausfeld, N. J. (1976). *Atlas of an Insect Brain*. New York: Springer-Verlag.

Strausfeld, N. J. and Li, Y. (1999). Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. *J. Comp. Neurol.* **409**, 603–625.

Sun, X. J., Tolbert, L. P. and Hildebrand, J. G. (1997). Synaptic organization of the uniglomerular projection neurons of the

antennal lobe of the moth *Manduca sexta*: a laser scanning confocal and electron microscopic study. *J. Comp. Neurol.* **379**, 2–20.

Tolbert, L. P., Matsumoto, S. G. and Hildebrand, J. G. (1983). Development of synapses in the antennal lobes of the moth *Manduca sexta* during metamorphosis. *J. Neurosci.* **3**, 1158–1175.

Wilson, E. M. and Chinkers, M. (1995). Identification of sequences mediating guanylyl cyclase dimerization. *Biochemistry* **34**, 4696–4701.

Zars, T., Wolf, R., Davis, R. and Heisenberg, M. (2000). Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: In search of the engram. *Learning Memory* **7**, 18–31.

Zhao, Y. and Marletta, M. A. (1997). Localization of the heme binding region in soluble guanylate cyclase. *Biochemistry* **36**, 15959–15964.

Ziegelberger, G., van den Berg, M. J., Kaissling, K. E., Klumpp, S. and Schultz, J. E. (1990). Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *J. Neurosci*. **10**, 1217–1225.

Zufall, F. and Leinders-Zufall, T. (1997). Identification of a long-lasting form of odor adaptation that depends on the carbon monoxide/cGMP second-messenger system. *J. Neurosci*. **17**, 2703–2712.