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Higher order visual input to the mushroom bodies in the bee, *Bombus impatiens*

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Abstract

To produce appropriate behaviors based on biologically relevant associations, sensory pathways conveying different modalities are integrated by higher-order central brain structures, such as insect mushroom bodies. To address this function of sensory integration, we characterized the structure and response of optic lobe neurons projecting to the calyces of the mushroom bodies in bees. Bees are well known for their visual learning and memory capabilities and their brains possess major direct visual input from the optic lobes to the mushroom bodies. To functionally characterize these visual inputs to the mushroom bodies, we recorded intracellularly from neurons in bumblebees (Apidae: *Bombus impatiens*) and a single neuron in a honeybee (Apidae: *Apis mellifera*) while presenting color and motion stimuli. All of the mushroom body input neurons were color sensitive while a subset was motion sensitive. Additionally, most of the mushroom body input neurons would respond to the first, but not to subsequent, presentations of repeated stimuli. In general, the medulla or lobula neurons projecting to the calyx signaled specific chromatic, temporal, and motion features of the visual world to the mushroom bodies, which included sensory information required for the biologically relevant associations bees form during foraging tasks.

Keywords

insect brain; color processing; novelty detection; honeybee; bumblebee

1. Introduction

The natural world offers a large array of challenges to the nervous system, which must process sensory cues and store information on the relevant stimuli to enable an animal to produce appropriate behavioral responses. A major question is: how does the nervous system process and filter the information to form associations between biologically relevant stimuli? This process is particularly pertinent to animals which navigate a complex environment, such as honeybees (*Apis mellifera* L.) and bumblebees (*Bombus* spp.). Bees have been studied for their extensive visual and olfactory learning and memory capabilities which they use to form

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associations while foraging for food (Giurfa, 2007; Menzel, 1999; Menzel and Giurfa, 2006; Ney-Nifle et al., 2001; Srinivasan, 1993; Srinivasan and Zhang, 1998). Therefore, bees provide an excellent opportunity to better understand how a relatively small nervous system can learn pertinent visual cues in a complex scene. The areas of the bee brain likely involved in the process of visual processing and formation of associations are the optic lobes (OLs), the principal visual centers of the insect brain, and the mushroom bodies (MBs), paired neuronal structures in the central insect brain.

The MBs have long been associated with learning and memory, although their necessary role in fly olfactory learning and memory has been the best-studied aspect of these structures (Review: Heisenberg, 1998, 2003). In the honeybee, electrophysiological experiments as well as local brain cooling and ablation experiments in combination with a number of behavioral tests have established the importance of the MBs to olfactory learning and memory (Erber et al., 1980; review: Menzel, 1999, 2001; Menzel and Giurfa, 2006). However, very little is known about the role of the MBs in processing other modalities, such as vision. Contextual visual learning and the detection of novel cues in the environment has been shown to require functional MBs in *Drosophila* (Liu et al., 1999). In addition, intact MB lobes also seem to be necessary for place memory in cockroaches (Mizunami et al., 1998). However, the functional role of the MBs in visual learning is likely species-specific, as the distribution of sensory input to the MBs differs among the insect groups. For instance, compared to most other insects, the MBs are not only relatively large in bees but they also receive different types of sensory input from the rest of the brain to key areas of the MBs, specifically the calyces.

The MB calyces, which are dorsal cup-shaped structures containing the dendrites of the parallel-projecting MB intrinsic neurons (Kenyon cells), receive numerous inputs from the rest of the brain (Gronenberg, 2001; Mobbs, 1982; Strausfeld, 2002). The MB calyces of most insect species receive input from the antennal lobes, the primary olfactory processing centers (Heisenberg, 1998; Menzel, 1999; Mobbs, 1982). In addition, in some insect taxa, the MB calyx receives assumed gustatory input (honeybee: Schröter and Menzel, 2003). However, while most insect taxa have little to no direct connections between the MB calyces and the OLs (cockroaches: Strausfeld and Li, 1999; Orthoptera: Honegger and Schürmann, 1975), there are substantial neuronal connections between the MBs and the OLs in ants, bees, wasps, and some butterflies (Hymenoptera: Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002; Lepidoptera: Kinoshita and Arikawa, 2007). This hymenopteran specialization of considerable input from the OLs to the MB calyces provides a unique opportunity to examine the role of vision in the integration of stimuli in bee MBs.

Another important aspect of bee MBs is that the sensory modalities are segregated within the MB calyces into three parts, namely the lip, collar, and basal ring (Mobbs, 1982; Fig. 1). While the lip receives olfactory input and the collar receives primarily optic lobe input, a mixture of modalities is represented in the basal ring (Mobbs, 1982; Gronenberg, 2001). Even among these divisions, Ehmer and Gronenberg (2002) found the input from the OLs to the MBs to be segregated, with separate areas of the OLs sending inputs into separate layers in the collar. In the only physiological examination of these visual MB inputs, Gronenberg (1986) found that the MB input neurons were selectively responsive to either flashes of light or to motion, which indicates that there are separate lines of visual information being sent to the MB calyces. Therefore, the anatomically segregated layering of visual inputs may represent a functional segregation of visual submodalities, such as color and motion.

This increase in the diversity of visual inputs may indicate that the function of MBs in bees has evolved to include a greater role in visual processing and may be the basis for the visual learning abilities of bees. The coincidence of sophisticated visually guided behaviors in advanced Hymenoptera could relate to the expansion of segregated color, pattern, and motion

information pathways to the MBs. As previous work has mainly been focused on the anatomical organization of visual MB input, we wanted to examine what kind of visual information is being sent to the MB calyces. To this end, we studied the responses of MB visual input neurons to determine how visual information is encoded, filtered, and relayed to the MB calyces.

To examine this segregation of visual information in the OL inputs to the MBs, we presented both color and motion stimuli to bees while recording intracellularly from OL neurons. While we recorded from both honeybees (*Apis mellifera* L.) and bumblebees (*Bombus impatiens*), we focused most of our research on bumblebees. It should be noted that both groups have demonstrated visual learning and memory capabilities (honeybees: Srinivasan and Zhang, 1998; bumblebees: Ney-Nifle et al., 2001), are closely related to one another (Roig-Alsina and Michener, 1993; Schultz et al., 2001), and have similar anatomical brain structures (Mares et al., 2005). In addition, both bumblebees and honeybees have trichromatic color vision with similar spectral sensitivities among the three photoreceptor types (Peitsch et al., 1992). In the course of our investigation, we were able to characterize 11 neurons which projected from the OLs to the MB calyces. In this study of the correlation of structure and function of the visual MB inputs on the single neural level, we found the neurons anatomically fit into the patterns shown in previous mass fill preparations (Ehmer and Gronenberg, 2002). In addition, the neural responses to color and motion stimuli indicate the visual MB input neurons are highly selective for novel cues or specific visual stimuli. Therefore, this study offers some insight into the nature of the visual information provided by the optic lobes to the mushroom body calyces.

2. Materials and methods

2.1. Animals and Preparation

Bumblebees (*Bombus impatiens*) were raised from commercially available colonies (Biobest Biological Services; International Technology Services, Michigan). Honeybees (*Apis mellifera* L.) were captured outdoors at the University of Arizona campus in Tucson, AZ. We used both species in the data collection but the bumblebees generally survived the procedure for a longer period of time relative to honeybees. The animals were cold anesthetized and immobilized in plastic tubes. The head capsules were opened frontally, salivary glands and part of the tracheal sheath were removed, and the brain was exposed for recording, with insect saline (Baumann and Hadjilazaro, 1972) applied at intervals.

2.2. Anatomy

To examine the connections between anatomical structures, we also performed mass fills of bee brain neurons as described by Ehmer and Gronenberg (2002). Again, the bees were cold anesthetized and immobilized in plastic tubes and the brain was exposed as described above. We filled neurons with Texas red[®] or fluorescein conjugated to 3000 MW dextran (Molecular Probes, Invitrogen) by penetrating optic lobes, including the medulla and the lobula, as well as the antennal lobes in the brain with the tips of sharp capillary tubes. The area was sealed with wax and cuticle and the bee was kept at 4°C overnight. The next day, the brain tissue was processed as described below.

2.3. Histology

Briefly, a fixative solution (4% buffered paraformaldehyde solution in insect saline) was applied to the brain before dissection. The brains were dissected, rinsed, and dehydrated using increasing concentrations of ethanol and embedded in Spurr's low viscosity medium (EMS, Hartfield, PA). The resulting plastic blocks were sectioned at 10-20 µm using a sliding microtome and mounted on slides with Cytoseal (Apogent, Kalamazoo, MI) for imaging. Images of the brain sections were recorded using a Nikon PCM 2000 confocal laser microscope with argon and HeNe lasers (Nikon, Japan). Optical sections of each plastic section were taken

in steps of 2 μm . Neurons were graphically reconstructed from the image stacks and were adjusted for brightness and contrast for neuron visibility using Photoshop (Adobe, San Jose, CA). For each figure, unless otherwise noted, the neurons represented were two-dimensional reconstructions of projected image stacks across all the plastic sections where the neuron could be observed in the section.

In addition, to delineate the brain structures in reference brains we used osmium staining as described by Mares et al. (2005). Briefly, after formaldehyde fixation, brains were impregnated with 1% aqueous osmium tetroxide for 2-3 hours, rinsed and plastic-embedded and sectioned as described above.

Finally, the Bodian reduced silver staining method was used to label neurons along similar lines to the protocol described by Gregory (1980). Brains were removed from the head capsule and fixed overnight at 4°C in A.A.F. (64% Ethyl-Alcohol, 5% Acetic Acid, and 3.75% Formalin). After fixation the tissue was dehydrated and embedded in paraffin (Paraplast+, Oxford Labware, St. Louis, MO). Paraffin blocks were cut into 15 μm sections, and mounted on slides coated with albumin and incubated overnight at 37°C to ensure proper adhesion. The tissue was then deparaffinized and rehydrated before incubation in protargol solution: 2.5 g silver protein (Alfa AEsar) and 2 g metallic copper, in 250 ml distilled water. After 23 hours at 60°C slides were removed from the protargol solution and processed through 1% hydroquinone and 5% sodium sulphite for 5 min, 1% gold chloride under bright light for 7 min, (1%) oxalic acid for 5 min and 2.5% sodium thiosulfate for 5 min. Sections were again dehydrated and then cover-slipped using Entellan (Merck).

2.4. Intracellular recording procedure

Borosilicate thin-walled glass electrodes (tip resistances within 70-120 M Ω when measured with 1 M KCl) were pulled from capillary tubes (A-M Systems, Inc., Carlsborg, WA) on a laser based micropipette puller (model P-2000, Sutter Instruments, Inc., Novato, CA). The electrodes were backfilled with one of three different fluorescent dyes: Lucifer yellow (200 μM in 0.5 M LiCl) or one of two Alexa Fluor[®] hydrazide dyes (excitation spectra at 568 and 633 nm, 200 μM in 1 M KCl, Molecular Probes, Invitrogen). Signals were amplified (Neuroprobe 1600; A-M Systems, Inc.) viewed on an oscilloscope and recorded on a PC computer using a Power Lab data acquisition board (ADInstruments, Inc., Colorado Springs, CO). Data were captured at 20 kHz and saved for offline analysis.

A recording session lasted anywhere between 1 1/2 to 3 hours. During that time, several cells were recorded and filled sequentially with separate dyes to maximize the number of possible cells recorded and filled in any one brain. Penetration of a neuron was indicated by a drop in the membrane potential (between 20 and 60 mV) and the presence of an action potential. The bee was then subjected to an array of visual stimuli. After stimulus presentation, the neuron was labeled by injecting a current of -2 to -5 nA at 1 Hz for 1-3 minutes. The brain tissue was then histologically processed as described for mass fills above.

2.5. Visual Stimuli

Two main types of visual stimuli were applied to the bees' right eye, which was also the side of the recording (Fig. 1, 2). First, light flashes produced by a 60-light emitting diode (LED) array (J & E Electronic Co., LTD, Hong Kong) composed of three parallel rows each comprising 20 violet, blue, or green LEDs, respectively (peak wavelengths: violet: 404 nm; blue: 476 nm; green: 561 nm, average half-peak width: 22.13 ± 8.86 nm).

It should be noted that the violet, blue, and green LEDs did not match the short (UV), medium (blue), and long (green) wavelength photoreceptor types in either honeybees or bumblebees,

which have comparable photoreceptor sensitivities (Peitsch et al., 1992). Indeed, for both groups, the violet LEDs should produce the strongest response in the medium (blue) photoreceptor type (eliciting ~80% of the peak response; Peitsch et al., 1992). In addition, the blue LEDs should produce equal relative responses in the medium (blue) and long (green) photoreceptor types (Peitsch et al., 1992). However, the violet LEDs are the only LEDs which could stimulate the short wavelength-sensitive photoreceptors, since there is no overlap of the blue or green with the short wavelength-sensitive photoreceptor sensitivity curves in either honeybees or bumblebees (Peitsch et al., 1992). In other words, even though the LEDs are not spectrally matched to the photoreceptor types, the relative distribution of the LED colors did make it possible to discriminate the color sensitivities of the neurons.

All three LEDs were adjusted with resistors to have the same relative intensity level at 450 photons/count per LED, which were measured using a fixed grating spectrometer (Ocean Optics model S2000; Ocean Optics, Dunedin, FL). The approximate illuminance at the level of the ommatidia originating from the LED array was estimated using an illuminance-meter (Mastech LX1330; for measurement details, see below) to be about 1400 lux (an illuminance that bees might encounter under overcast skies).

The LED array was arranged in a half-circle such that the long axis of the array aligned with the long axis of the bee eye, spanning 2.9° by 123.3° of the visual field (Fig. 2), which could be moved out to test motion stimuli. In all the recordings, five 500-ms LED light flashes were presented at 1 Hz. The intervals between the series of five light flashes ranged between 20 seconds to 2 minutes. To test chromatic sensitivity, we lit the individual arrays of violet (v), blue (b), or green (g) LEDs at a time or in four different combinations (b-g, b-v, g-v, and b-g-v).

The second type of visual stimuli comprised a CRT monitor (vertical refresh rate of 160 Hz; Gateway 2000, EV900) which subtended 114.0° by 103.1° of the visual field of one eye (Fig. 2) controlled by a personal computer with a Radeon 9800 Pro video card (ATI Technologies, Inc., Ontario, Canada). Since, at no point in our recordings did we observe a cell phase-lock to a frequency of 160 Hz, we are confident that the neurons did not respond to the inherent high-frequency monitor flicker. The illuminance at the level of the ommatidia facing the monitor was estimated using an illuminance-meter (Mastech LX1330) directly at the monitor screen, which was estimated at 330 lux (white bars). This illuminance is lower than daylight conditions, but, in our experience, sufficient for bumblebees to fly in their flight cage (at 376 lux).

Using Vision Egg software (Straw et al., 2006), four main types of motion stimuli were computer-generated: a large field high contrast square-wave moving grating (filling the entire computer screen, spatial frequency at 12.89° , moving at a rate of $57.2^\circ/\text{sec}$), a small moving target (subtending 3.85° by 16.2° , moving at a rate of $73.57^\circ/\text{sec}$), a large moving bar (subtending 9.04° by 103.1° , moving at a rate of $54.25^\circ/\text{sec}$), and a moving black edge on a white background (filling the entire computer screen, moving at a rate of $87.69^\circ/\text{sec}$). The stimuli moved in eight different directions (up, down, left, right and 45° to these respective directions) and four different orientations (e.g. a grating was composed of either vertical lines, horizontal lines or lines oriented 45° to these orientations). Luminance changes of the CRT monitor resulting from the visual stimuli were recorded by a photodiode and stored with the neuronal signals.

2.6. Analysis of Responses to Visual Stimuli

Cell membrane potential data along with recordings of the visual stimuli were imported into Matlab (Mathworks, Natick, MA). Next, the data were down sampled to a resolution of 0.1 ms and were filtered to remove high frequency (2000 Hz) and low frequency (50 Hz) noise. In

addition, we digitally removed electrical relay switch noise produced by turning on and off the LEDs. The time points of the action potentials in the intracellular traces were detected by identifying above-threshold membrane potentials.

To determine if the neuron responded to visual stimuli, we defined a neuron as responsive when it changed its instantaneous spike rate 2 times the standard deviation above or below the mean of the background activity. In the second measure of responses, a response was defined as change in binned spike times 2 times the standard deviation above or below the mean activity across trials, which was calculated from the peristimulus time histogram (PSTH) of spike times. In all PSTH graphs, the histograms were smoothed with a Gaussian curve for visualization purposes only.

For motion stimuli, we measured the relative spike rate changes in response to the eight different motion directions. For the LED light flashes, we determined whether the cell responded differently to the different LED colors. We also examined if the cell habituated to the light flashes. A measure of habituation was calculated as the difference between the number of spikes during the first light flash and the mean number of spikes during the subsequent four light flashes. Data were normalized by dividing the difference by the sum of the number of spikes during the first light flash and the average number of spikes during the following light flashes. To determine if the spike rate differences between the first and the four subsequent light flashes were significantly different, we used the Mann-Whitney *U*-test, which was also used for the other statistical comparisons.

3. Results

We recorded intracellularly and morphologically reconstructed 11 spiking interneurons which project from the optic lobes (OLs) to the mushroom body calyces (MB input neurons; Fig. 3, 4). Ten of these neurons were from bumblebees (*Bombus impatiens*) while only one optic lobe MB input neuron was from a honeybee (*Apis mellifera* L.). We first describe the anatomical organization of these neurons and then focus on their physiological properties.

3.1. General morphological properties

The MB calyces receive input from the two main areas of the optic lobes, the medulla, the second optic lobe neuropil, and the lobula, the third optic lobe neuropil downstream of the medulla (Fig. 1B; Mobbs, 1982; Ehmer and Gronenberg, 2002). To determine whether the MB visual input regions are segregated from the olfactory inputs in the calyces of bumblebees, we anterogradely traced input neurons from the optic lobes (Fig. 1D, E) as well as from the olfactory processing centers, the antennal lobes (Fig. 1F). From such preparations, we observed that the visual inputs occupied different layers in the calyx' basal ring and collar depending on the OL input region (Fig. 1D, E). For example, the medulla inputs project to the outer region of the collar (Fig. 1D). When we filled a subset of both medulla and lobula neurons, we could trace the projections of the medullar inputs to the MB calyces on the outer collar (black arrows; Fig. 1E) while the lobula inputs projected into the inner areas (white arrows; Fig. 1E). In contrast, the olfactory inputs generally bypassed the collar region, sending inputs to the lip and the basal ring (Fig. 1F). The mass fills allowed us to correlate our individually labeled and recorded neurons with neuronal tracts and to establish general types or classes of neurons.

Based on specific anatomical cues, we assumed the direction of information flow within individual optic lobe MB input neurons was from the OLs (input) towards the MB calyx (output). We considered certain branching areas to be putative dendrites when we observed fine processes along their arborizations in the OL (Fig. 3A). The finer branching of the dendritic trees contrasted with the cells' projections into the MB calyces, where we observed relatively larger varicosities, which we labeled blebs, and considered putative pre-synaptic sites (Fig.

3B). Therefore, the MB input neurons project from the OLs to the MB calyces on the same side of the recording and visual stimulation.

Within the OLs, we observed a large array of branching patterns, but we were able to group the recorded MB input neurons into previously described cell types based on morphology. We recorded from visual MB input neurons which belonged to the following neuron types: large field lobula neurons projecting to the MB calyces (hereafter labeled large field lobula MB input neurons; $n=8$; Fig. 3C-F), small field lobula neurons projecting to the MB calyces (hereafter labeled small field lobula MB input neurons; $n=2$; Fig. 3G), and a medulla neuron projecting to the MB calyces (hereafter labeled medulla MB input neuron; $n=1$; Fig. 4F). The morphologies are described first followed by a description of their physiological responses.

3.2. Optic lobe branching patterns

3.2.1. Lobula dendritic branching patterns—The majority of the recorded optic lobe input neurons to the MB calyces had putative dendritic branching in the lobula ($n=10$). Eight of these lobula MB input neurons were large field lobula MB input neurons, where seven were from bumblebees (Fig. 3C-F) and one was from a honeybee (Fig. 4C). All large field lobula MB input neurons had large dendritic branching in the lobula, spanning an average of 100-200 μm , though they varied widely in their dendritic branching patterns (Fig. 3C-F). However, the cells' neurites were restricted to the inner or anterior lobula and never branched in the outer or posterior rim of the lobula (see next section). In addition, the types of dendritic branching varied from fine neurites (Fig. 3A) to trees containing both spines and varicosities (Fig. 4D). The large field lobula MB input cell bodies, when filled, were situated ventral and lateral to the lateral MB calyx and ipsilateral to the cell's dendritic projections. The cells exited the lobula via the lobula optic tract (LOT; Mobbs, 1984).

As opposed to the large field lobula MB input neurons, the small field lobula MB input neurons had dendritic branching spanning 50 μm or less ($n=2$; Fig. 3G). As observed in the large field lobula MB input neurons, small field lobula MB input neurons did not branch in the outer or posterior rim of the lobula, which correlated with the layered structure of the lobula (Ribi and Scheel, 1981).

3.2.2. Correlation of the lobula structure and dendritic branching patterns—Ribi and Scheel (1981) described six layers within the bee lobula, progressing from one (posterior or outer-most) to six (anterior or inner-most), best illustrated in osmium-stained horizontal sections of the bee brain (Fig. 4A, B). We observed that all the lobula MB input neurons sent branches into layers 5 and 6 of the lobula, but not to layers 1 through 4. We also recorded and morphologically identified other lobula neurons that projected to layers 1 through 4 (not subject of the current account). This is illustrated in Fig. 4C, where the MB input neuron had dendritic branching in the anterior region of the lobula (white) and the other lobula neurons which did not input to the MBs (magenta) sent their branching into the posterior edge of the lobula. Interestingly, neurons with dendritic branching in layers 1-4 never projected to the MB calyces and often projected to more posterior regions of the protocerebrum (magenta neurons in Fig. 4C).

3.2.3. Medulla neuron—We also recorded a single medulla neuron with projections into the MB calyces (Fig. 4E,F). The dendritic arborizations of this medulla MB input neuron (Fig. 4E,F) extended into the medial medulla along its posterior edge within the medulla into an area labeled the serpentine layer. Composed of axons and tangential neurons, the serpentine layer separates the inner from the outer medulla (Ehmer and Gronenberg, 2002). The medulla MB input neuron branched around the medulla within the serpentine layer with its cell body near

the base of the lateral calyx. The cell's axon entered the anterior inferior optic tract and projected toward the MB calyces (Mobbs, 1982, 1984; Ehmer and Gronenberg, 2002).

3.3 Projections: output regions

3.3.1. Calycal projections—The optic lobe neurons projecting to the MB calyces sent blebbed inputs into three main regions of the collar and basal ring of the MB calyx. First, the large field lobula MB input neurons all projected into the basal ring and the inner rim of the collar of the lateral and medial MB calyces (Fig. 5A, B). While the small field lobula MB input neurons also projected into the basal ring and inner rim of the collar of the lateral and medial MB calyces, the projections were lateral, or closer to the outer collar, compared to the large field lobula MB input projections (Fig. 5C). Finally, the medullar MB input neuron projected into the outer basal ring and the outer portion of the MB collar in both the medial and lateral ipsilateral calyces (Fig. 1D, E, 5D).

Interestingly, five large field lobula MB input neurons sent blebbed projections into the outer rim of the MB calycal lip region (Fig. 5E), which has been shown previously to have predominantly olfactory input (Fig. 1F; Gronenberg, 2001). In sum, we confirmed that MB input from the medulla and the lobula, respectively, is segregated in different calycal layers, and we found that some large field lobula MB input neurons sent projections into calycal areas previously thought to receive exclusive olfactory input.

3.3.2. Projections across the brain—Another feature we observed among the recorded and filled MB input neurons was that some neurons appeared to project unilaterally while others appeared to project bilaterally to both MB calyces. While we were not able to completely fill the long collaterals across the brain, we found that the medullar MB input neuron as well as the small field lobula MB input neurons had faded projections past the midline of the brain (white arrow, Fig. 3G), which could indicate bilateral projections to the calyces of the MB. Conversely, in all the large field lobula MB input neurons, there were no obvious projections, faded or otherwise, toward the midline or toward the contralateral MB calyces (white arrow, Fig. 3C) while arborizations in the ipsilateral calyces were brightly stained. This suggests the large field lobula MB input neurons send unilateral input to the MB calyces.

3.3.3. Additional projections into the dorsal protocerebrum—One distinctive feature for many of the lobula neurons with projections into the MB calyces was the fact that they had additional projections into the protocerebrum before the axons entered the MB calyces (Fig. 5F; Table 1). This is noteworthy because such additional projections have not been observed previously and were absent in medulla visual MB input neurons (Ehmer and Gronenberg, 2002; Gronenberg, 1986). These protocerebral projections often resided in the dorsal region of the protocerebrum immediately ventral to the MB calyces, called the superior lateral protocerebrum (s1 pr; Strausfeld, 2002; also called the lateral protocerebral lobe: Kirschner et al., 2006; Rybak and Menzel, 1993), but the diffuse structure of the region has made it difficult to localize the projections to any one region of the superior lateral protocerebrum. Not only that, but the lobula MB input projections into the protocerebrum were varied, where some projected into anterior superior lateral protocerebrum, others projected into the posterior superior lateral protocerebrum, and still others projected in the peri-peduncular protocerebrum. Nevertheless, in every case, the protocerebral projections were blebbed as observed in the MB calyces (Fig. 5F), possibly indicating that select areas of the protocerebrum receive outputs from these large field lobula MB input neurons.

3.4. Physiological properties

Even among this small group of sampled neurons, we found a diversity of physiological properties and responses to visual stimuli. In fact, we could not find significant differences in

the visual responses or the spontaneous activity that correlated with the anatomical types, indicating that, even among the anatomical classes, the visual MB input neurons varied considerably in their physiological properties. For example, in terms of the baseline spiking activity, the spontaneous firing rates of the neurons varied largely, ranging from 0.1 to 39 Hz (mean = 13.06 ± 9.988 Hz, excluding the 43 Hz firing cell) which did not correlate with cell type. For this reason, we pooled the cell responses to examine how all the optic lobe input neurons to the MB calyces respond to chromatic cues, to motion cues, and then we examined the temporal aspects of their responses to visual cues (Table 1).

All but one of the optic lobe neurons with projections to the MB calyces responded to visual stimuli. For the single cell that did not respond (in a *B. impatiens* preparation, Fig. 3C), the cell's activity was high (firing frequency of 43 Hz; probably injured) and the period of stimulus presentation and recording was not long (2-3 minutes), which may be the reason no responses were observed. The honeybee lobula MB input neuron possessed similar morphological and response properties to the bumblebee large field lobula MB input cells and was therefore combined with the rest of the neurons for analysis (Table 1).

3.4.1. Receptive fields—Only two recordings lasted long enough to fully test receptive fields (a large field lobula MB input neuron and the medulla MB input neuron). In the case of a large field lobula MB input neuron, the receptive field spanned the entire LED array, matching with the cell's large dendritic branching pattern in the lobula (Fig. 3F). When we tested the receptive field of the medulla MB input neuron, it had a localized receptive field in the upper area of the visual field, again matching the cell's narrow dendritic branching pattern in the dorsal medulla (Fig. 4F).

3.5. Chromatic responses

Both the honeybee and bumblebee neurons demonstrated chromatic selectivity, which could include both excitation and inhibition in response to the light flashes (Table 1). Most of the optic lobe neurons projecting to the MB calyces preferentially responded to one or two types out of three LEDs ($n=10$). As pointed out previously, there is not a simplistic one-to-one correspondence between the types of LEDs and the types of color receptors stimulated. However, we were able to deduce the types of color receptors which input to the individual neurons by examining the patterns of responses to the LEDs (Table 1). For example, we could deduce the type of color receptor sending inputs to the medulla MB input neuron by comparing its response to blue, green, and violet LEDs (Fig. 6A). The medulla neuron responded to the violet LEDs almost exclusively with a phasic burst of activity across trials (Fig. 6A), with little to no response to green or blue light flash stimuli (Fig. 6B). Therefore, this cell seemed to mainly receive input from the ultraviolet photoreceptors. In another type of chromatic selectivity, a large field lobula MB input neuron was excited by blue light, inhibited by green light, but had no response to violet light, indicating it had blue and green, but not ultraviolet, photoreceptor input (Fig. 6C).

In four MB input neurons, we also observed chromatic opponency, where the presence of one color changed the cells' responses to another color. In the first example of chromatic opponency, the burst of excitatory spikes at the onset of the green light (Fig. 6D) was suppressed by the simultaneous presentation of violet light (Fig. 6D). In the second example of chromatic opponency (Fig. 6E), the neuron showed excitatory responses to green and blue stimuli while the simultaneous presentation of violet light inhibited the blue and green responses. Incidentally, this inhibitory effect was evident only during subsequent stimulations after the first light flash (arrows in Fig. 6E), corresponding to the change in response to the violet stimulus alone (habituation; see below). We took the interactions of the different color channels into account when we inferred what photoreceptor types input to each neuron (Table 1).

Based on our inferences of what photoreceptor types eventually input to these neurons, we found 7 of the MB input neurons likely receive input from the blue (medium wavelength) photoreceptor type, while 5 receive input from the green (long wavelength) photoreceptor type, and 4 neurons have UV (short wavelength) photoreceptor input (Table 1). In other words, the different photoreceptor types appear to all play a role in the responses observed in the recorded optic lobe neurons with projections in the MB calyces.

3.6. Motion responses

Only four of the eight tested MB input neurons had a response to motion stimuli (Fig. 7). None of the neurons responded to all types of motion stimuli presented, indicating that the cells were selectively responsive to certain types of motion. Motion selectivity included responses to either directional, non directional, or bidirectional motion (where the neuron responded to two opposing directions, but not to all directions, of motion; Table 1). One wide field lobula MB input neuron showed clear directional preferences for a moving black and white edge (Fig. 7A). A small field lobula MB input neuron also responded to the black and white moving edge, although it did not exhibit clear directional preferences (Fig. 7B). Finally, one large field lobula MB input neuron was temporarily inhibited by the directional motion of a moving grating, with inhibition highest for certain directions (Fig. 7Ci, Cii) and lower for others (Fig. 7Ciii, Civ). Overall, though, the neurons did not preferentially respond to directional motion (Table 1).

3.7. Temporal response properties

3.7.1. Temporal response properties of individual neurons—In addition to chromatic selectivity and some motion sensitivity, the MB input neurons all exhibited some distinctive temporal response properties when stimulated by the LED light flashes. For instance, one temporal characteristic of the neurons with low firing rates was the fact that, when they did respond, they displayed temporally precise spikes which were time-locked to the stimulus ($n=4$; Fig. 8A, B), which is illustrated in the peristimulus time histogram (PSTH) with bins at 5 ms (Fig. 8A, B, bottom plot). This behavior was observed in two large field lobula MB input neurons, one small field lobula MB input neuron, and in the medulla MB input neuron.

Another temporal response property was found in a single small field lobula MB input neuron (Fig. 8C, D). This neuron responded weakly to the first blue light flash, but progressively increased its firing rate with consecutive stimulations, a response which we call a ‘summation effect’ (Fig. 8C). This summation effect only occurred when blue or green were present in the stimuli, but was not present when stimulated exclusively with violet flashes, even though it did have a weak response to violet (Fig. 8C). We quantified this change by summing the number of spikes during and 500 ms after the light flash for each of the five light flashes and for 1 second after the last light flash across single trials of each color or color combination. We found that the number of spikes did increase with the presentation of sequential light flashes for green, blue, and blue-green-violet light but not for violet light (Fig. 8D).

3.7.2. Stimulus entrainment—In addition to precise spike times and summation over sequential stimulations, a striking response pattern was observed in one particular large field lobula MB input neuron (Fig. 3F). This neuron shifted its spiking ahead of the light flashes, in a stimulus entrainment response (black arrows, Fig. 9A). This property was more obvious at the end of a stimulus train when the neuron produced a burst of activity even though no stimulus was present (white arrow, Fig. 9A). This stimulus entrainment response occurred reliably after violet light stimulus trains ($n=8$). The neuron also responded to other colors (blue, Fig. 9A), although in that case, the response latency was constant. To illustrate this difference, we computed PSTHs across 11 trials of five sequential LED light flash stimuli where the light flash color combinations included violet light (white) and 11 trials where the light flashes

included only blue or green light (black) (Fig. 9B). Notably, the neuron's responses to stimuli with violet light shifted ahead of the light flash, with a large burst occurring a period of time after the final light flash. The responses to only blue or green light combinations exhibited a repeatable phasic-tonic burst of activity (Fig. 9B).

To determine if this stimulus entrainment involved a phase progression ahead of the light flashes, we measured the mean spiking activity relative to the beginning of each consecutive light flash (Fig. 9C, D). In this case, the repeated LED light flash stimuli were treated as a sinusoidal wave of 1 Hz, where the positive phase of the wave is the period of the light flash and the negative phase is when the light flash is off (i.e. 0 is the beginning of the light flash and 180 is the end). When the averaged binned spike times are plotted along the polar plot, the stimuli without violet light generally responded after the onset of the light flash (black arrow). When violet light was included in the stimuli (grey arrow), the mean response significantly advanced relative to the non-violet stimuli responses (Fig. 9C; Mann-Whitney *U*-test; rank sum statistic = 316480; *z*-statistic = 9.5093; *p* < 0.00001). This difference was even more obvious when the data was analyzed for the responses to the final four consecutive light flashes, excluding the first of five stimulus presentations. In this case, the mean phase of the response was significantly shifted ahead of the light flash (Fig. 9D; Mann-Whitney *U*-test; rank sum statistic = 138539; *z*-statistic = 8.0390; *p* < 0.00001).

3.7.3. Habituation—A property observed in most of the MB input neurons was some form of habituation, where MB input neurons either changed, reduced, or stopped their responses after the first light flash when stimulated by a series of five light flashes (*n*=10 neurons, including the medulla and the honeybee neurons). Habituation depended on color: some neurons showed the strongest habituation response to blue stimuli (Fig. 10A) whereas others showed habituation for green or violet (Fig. 6E). Notably, if the stimulus was changed to another color or if the stimulus was presented 4-5 seconds later, the neurons reproduced their original responses.

We noted that the habituation response contrasted sharply with the responses of lobula neurons with inputs in lobula layers 1-4 that projected into the central brain outside of the MBs (hereafter referred to as 'non-MB neurons'; magenta neurons, Fig. 5E, F; otherwise not covered in the current account). All these non-MB neurons had strong responses to most of the directional motion stimuli and were not color sensitive in that they responded to all three LED colors equally across repeated stimuli (Fig. 10B). In order to see if the non-MB neurons demonstrated habituation similar to the MB neurons, we selected twenty-one lobula non-MB neurons for comparison. Four of the non-MB neurons were recorded in the same brains as four MB visual input neurons. The MB input neurons had significantly higher normalized spike rate differences between the first and subsequent light flashes than the non-MB neurons (Fig. 10C; Mann-Whitney *U*-test; rank sum statistic = 4529; *z*-statistic = -9.2801; *p* < 0.00001).

4. Discussion

The mushroom bodies receive multimodal information from many parts of the brain (Fig. 11A; Gronenberg, 2001; Mobbs, 1982, 1984; Strausfeld and Li, 1999) and are central integration centers assumed to be the place where associations are formed (Heisenberg, 1998, 2003; Menzel, 1999, 2001). While the MBs have been demonstrated to have a role in olfactory associations, the presence in bees, wasps, and ants of visual interneurons which directly project to the MBs from the visual processing centers, the optic lobes (OLs), brings up a major possibility of the role of the MBs in visual learning and memory.

4.1. Anatomy

The morphological patterns we observed for the large field lobula MB input neurons illustrated two points that, until now, were not known with regard to visual MB inputs. First, we report that the large field MB input neurons project into the superior lateral protocerebrum in addition to the MBs. This side projection is remarkable considering the similarity of this type of projection to the olfactory input neurons to the MB calyces (Homberg, 1984; Kirschner et al., 2006). In the case of the olfactory input to the MB calyces, many projection neurons from the antennal lobe send fine output projections into the lateral protocerebrum (lateral horn) as well as projecting into the lip of the MB calyces (Homberg 1984; Kirschner et al., 2006). By having two output areas to the central brain, the MB input cells can send their outputs to additional circuits within the bee brain. However, the large field lobula MB input projections to the superior lateral protocerebrum likely do not overlap with olfactory inputs (Kirschner et al., 2006). Instead, the large field lobula MB input neurons could be interfacing with feedback circuits from the superior lateral protocerebrum back into the OLs. The presence of feedback cells could explain some aspect of the habituation or the stimulus entrainment response, but further recordings are necessary to determine if this is the case.

Second, we note that the large field lobula MB input neurons may not actually project bilaterally, as has been suggested for the optic lobe input neurons to the MB calyces (Ehmer and Gronenberg, 2002; Gronenberg, 2001; Mobbs, 1982, 1984). However, in past studies, most of these neurons were identified using mass filling or Golgi techniques, which makes it hard to follow individual neurons. By filling and recording from individual neurons, we found that some of the large field lobula MB input neurons, particularly the neurons with the most extensive arborizations on the lobula, projected unilaterally. In this respect, large field lobula MB input neurons resemble the unilateral olfactory inputs to the MBs (Fig. 11A; Homberg, 1984, Gronenberg, 2001; Kirschner et al., 2006). Therefore, three different populations of visual inputs to MB calyces seem to exist: bilaterally projecting medulla inputs, bilaterally projecting lobula inputs, and unilaterally projecting lobula inputs (Fig. 11A). As information about all visual stimulus parameters appears to be distributed to the ipsilateral as well as to the contralateral MBs we cannot draw any functional conclusions about the differences in side-specificity.

Thirdly, we found that the lobula inputs to the mushroom bodies could also be segregated among layers, just as the MB calycal medulla inputs are segregated within layers (Fig. 11B; Ehmer and Gronenberg, 2002). The small field lobula MB input neurons projected in an area of the collar outside the inner projections of the large field lobula MB input neurons (Fig. 11B). This result could mean that the medulla forms the outer ring of the collar, forming a shell around the projections of the small field lobula neurons, which then could form a ring around the innermost optic lobe input projections of the large field lobula input neurons in the MB calyces.

Finally, a few of the large-field lobula MB input neurons sent fine projections into the base of the lip (Fig. 11B). Even though, overall, the MB calyces are divided in terms of their sensory inputs, with the lip receiving olfactory input and the collar receiving visual input, we did observe some crossover between the sensory input pathways (Fig. 11B). Particular MB Kenyon cells that have their dendritic domains in the inner lip area, such as shown by Strausfeld (2002), would be able to integrate the overlapping visual and olfactory inputs. This fact speaks further to the possibility of integrating visual and odor cues in the MBs at the level of individual Kenyon cells.

Other than these differences, we also saw a number of similarities with previous work. For example, the input neuron from the medulla matches closely what Ehmer and Gronenberg (2002) described in a population of nearly 300 medullar neurons, all of which assumedly have bilateral projections restricted to the outer rim of the MB calyx. Small field lobula MB input

neurons have been recorded (yet less well characterized) by Gronenberg (1986), who also demonstrated that these neurons have some motion sensitivity and responses to light flashes. We were able to record and fill two of these types of cells, out of a population assumed to comprise nearly 100 neurons (Ehmer and Gronenberg, 2002).

Interestingly, the cell population we found more often, the large field lobula MB input neurons, has only been described once before. A single widely-branching lobula MB input neuron that showed some motion and light flicker responses has been previously described (Gronenberg, 1986). As to why this neural type has not been examined, we hypothesize that it has to do with the histological techniques used. Most of the mass filling techniques used in describing the anatomy of these neurons involved injecting dye into the brain dorsally or anteriorly (Ehmer and Gronenberg, 2002; Gronenberg, 2001; Mobbs, 1982, 1984), while most of the large field lobula MB input neurons have their cell bodies and major processes relatively posterior in the tissue. In fact, the best approach is probably to examine these neurons by using Golgi techniques, by pressure injection, or by filling individual neurons, as we have done here.

4.2. Response properties

Most of the MB input neurons were color-sensitive and a subset was motion sensitive, but the separate visual sub-modalities of color and motion were not explicitly segregated (Table 1). Instead of receiving visual information about generalized large-field movement (such as is required for gaze stabilization and flight control) or general luminance information (i.e. the presence of light), the MBs receive complex color information, specific motion information, and information on the novelty of the stimulus (habituation) or its repetitive nature (entrainment or summation). Therefore, MB Kenyon cells “see” only selected chromatic, temporal, and motion features of the visual world provided by the optic lobe MB input neurons. These features of the environment could be important to forming associations between visual cues and other sensory stimuli, such as odors, in navigating the environment. MBs therefore receive higher-order information in general, which corresponds with the concept of the mushroom bodies being involved in contextual integration and in forming associations (Heisenberg, 1998; Giurfa, 2007).

4.3. Processing of color information in bees

We tested monochromatic light stimuli as bees are well-known for using color cues to navigate to a reward and to exhibit color constancy (Backhaus et al., 1987; Giurfa, 2007; Ney-Nifle et al., 2001; Srinivasan and Zhang, 1998). A number of studies in honeybees through the years have also been done on the color processing mechanisms using similar intracellular techniques (Kien and Menzel, 1977a, 1977b; Hertel, 1980; Hertel and Maronde, 1987; Hertel et al., 1987; Yang et al., 2004). A common feature in all these studies was that the visual neurons in the bee OLs were divided into broad-band (color insensitive), narrow-band, and color opponent neurons. We found that the MB input neurons in the bumblebee also could be generally classified as either narrow band or color opponent neurons. Therefore, the MB visual inputs are part of a distinct class of bee visual interneurons that signal color cues as well as select motion cues.

4.4. Complex stimulus responses

Surprisingly, the least complex visual stimulus tested in this study, light flashes, elicited some of the most remarkable responses. While flicker stimuli (usually white light of higher frequencies) are commonly used to analyze temporal properties in visual research (Reinagel and Reid, 2000) and to characterize motion processing in insect OLs (Gilbert et al. 1991, Douglass and Strausfeld 1996), regular, repeated monochromatic stimuli are rarely tested, particularly at the relatively low frequency and long duration used in these experiments. The

responses of the neurons to the light flashes suggest complex underlying processing within the OLs of the bee brain, which included color opponency, habituation, and stimulus entrainment.

The habituation seen in MB input neurons could be a type of novelty signaling process. A similar phenomenon has been shown in the locust lobula, where the LGMD neuron is involved in triggering the escape jump and signals novel approaching objects. Its activity declines if the same stimulus is presented repeatedly (review: Rowell, 1971). Recently, novelty has been correlated with overall brain activity in insects, where local field potentials of populations of neurons in the *Drosophila* brain signaled novelty in an attention-like process (van Swinderen, 2007). We suggest that, through habituation, individual MB input neurons could be participating in increasing the attention of the insect to novel cues, as would be important for bees approaching landmarks or adapting to a new environment.

In addition to habituation, we observed a single neuron entrain and anticipate the timing of a stimulus (Fig. 9). This type of stimulus entrainment has been observed in crayfish jittery movement fibers and may represent a mechanism for detecting the irregularity of a stimulus (Shimozawa, 1975). Anticipatory, or entrainment, responses to visual stimuli have been shown in honeybees and ants by extracellularly recording visually evoked potentials from larger populations of neurons (Ramón and Gronenberg, 2005). Interestingly, the neuron showing anticipatory responses in the bee lobula is probably only 3-5 synapses away from the photoreceptors (Ribi, 1975; Ribi and Scheel, 1981). This means that the complex processing involved in stimulus entrainment is largely contained in the OLs and possibly in the dendritic tree of single cells. This remarkable complexity in single cells has been observed in other individual insect neurons (Hammer, 1997; Mauelshagen, 1993), but this is the first reported case of an insect neuron in the OLs anticipating an unrewarded stimulus based on periodicity and color.

4.5. Postsynaptic cells: mushroom body neurons

These results indicate that MB visual input neurons filter the visual information by signaling temporal features (i.e. novelty and periodicity) or select chromatic and motion cues. The filtering is likely important for the postsynaptic neurons directly receiving the visual input, the Kenyon cells (Howse and Williams, 1969), the substrates for MB associative memory formation (Heisenberg, 1998; Menzel, 1999). Each MB input neuron contacts only a limited range of calycal territory and provides relatively largely spaced 'blebs' (presumable output sites; Fig. 5, 11B) compared to the localized and compact dendritic trees of Kenyon cells (Mobbs, 1982, Strausfeld, 2002). This distribution suggests that each MB input neuron makes only a few synapses with any given Kenyon cell. In turn, each Kenyon cell probably receives input from many of these visual MB input neurons. One would therefore assume that each Kenyon cell should have a highly selective configuration of complex stimulus combinations that excites it maximally. This would support the idea of Kenyon cells as coincidence detectors (Perez-Orive et al. 2004) and the 'sparse' coding properties demonstrated for the responses of Kenyon cells to olfactory stimuli in locusts (Perez-Orive et al., 2002, Cassenaer and Laurent, 2007) and honeybees (Szyszka et al., 2005).

In addition, Kenyon cells in locusts demonstrate high rates of adaptation and high spike initiation thresholds (Laurent and Naraghi, 1994). This conclusion is supported in a study using calcium imaging in honeybees, where KCs only respond during the first 100 ms of an odor stimulus presentation (Szyszka et al., 2005), indicating that the KCs code the initial presentation of the stimulus, but not the stimulus duration, similar to many responses found in the visual MB input cells (e.g. Fig. 10). We hypothesize that the MB visual input neurons in bees adapt quickly, or habituate, to ensure a response in the KCs. Additionally, the selectivity and precise spike times observed in the OL neurons projecting to the MB calyces likely ensure that only temporally correlated associations involving color or specific features of the environment are

formed by the KCs and their outputs, the MB extrinsic neurons, which have demonstrated a large range of spike timing-dependent plasticity (Cassenaer and Laurent, 2007; Li and Strausfeld, 1997; Mauelshagen, 1993; Menzel and Manz, 2005).

The MB visual input neurons not only signal color and motion information to the mushroom bodies, but signal temporal aspects of the stimulus which expands the functional repertoire of the mushroom bodies themselves. If some amount of higher-order visual processing occurs at the level of the optic lobes, then the mushroom bodies could be receiving information on the features of the environment, not just the presence of one color, but the presence of specific color combinations, specific types of movement, whether the objects are novel or not, or, possibly, pattern information. The next step would be to test the MB visual input neurons with a larger array of visual cues to determine what specific features these neurons detect. In addition, a key step in our understanding of the mushroom bodies is to examine the convergence of multimodal information on the Kenyon cells themselves to better determine how associations are formed in the bee brain.

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Fig. 1. Anatomy of the bumblebee brain. (A) The bumblebee brain structures which are visible superficially are the retina (ret) which sends visual inputs to the optic lobes (ol), the antennal lobes (al), which are the primary olfactory processing centers, and the central brain, which includes the mushroom bodies, the calyces of which (mbc) are visible superficially. (B) frontal brain section (osmium stained) showing the second (medulla, me) and the third optic lobe neuropil (lobula, lo), the central protocerebrum outside the mushroom bodies (pr) and the mushroom bodies (mb) composed of a lateral and medial calyx (mb-lc and mb-mc, respectively) and mushroom body lobes (mb-l). (C) The box in (B) highlights a section of the lateral calyx, which is comprised of the lip (lip), collar (col), and basal ring (br) and the Kenyon cell bodies (KCs) enlarged in this Bodian preparation. (D-F) Fluorescent fills showing visual inputs from the medulla (D), the medulla (black arrowheads) and the lobula (white arrowheads)

(E) and olfactory inputs from the antennal lobe (F) to the calyx. Unless otherwise noted, scale bar = 100 μm . All images from bumblebee preparations.

Fig. 2.

Visual stimuli. The layout of the two types of visual stimuli relative to the bee is represented by a sphere around the bees' head. Both the light emitting diodes (LEDs) and motion stimuli using the computer monitor (CRT) were presented on the right side of the bees, but, for the sake of clarity, are placed on either side of the bee in this figure. The array of sixty violet, blue, and green LEDs are oriented to the long axis of the eye subtending 2.9° by 123.3° of the visual field, the computer monitor subtended 114.0° by 103.1° .

Fig. 3.

Mushroom body (MB) visual input neuron projections. (A) Input regions of the MB visual input neurons with characteristic fine processes in the lobula. (B) Outputs in the mushroom body calyx with characteristic blebbed varicosities. (C-G) lobula MB input neurons, showing the large range of differences in the dendritic branching patterns projecting in the lobula (lo) to the MB lateral and medial calyx (mb-lc and mb-mc), and to the superior lateral protocerebrum (s l pr). (C-F) Large field lobula MB input neurons have dendrites that span much of the lobula. However, these neurons do not have processes extending to the other side of the brain (white arrowhead in C). (G) Conversely, this small field lobula MB input neuron with fine dendrites in a small region in the lobula has a projection leaving the medial MB calyx toward the midline (white arrowhead). Boxes in (D) represent areas enlarged in Fig. 4 E and

5E and F, respectively. Boxes in (F) and (G) represent areas enlarged in Fig. 5 A and c, respectively. In (F), two large field lobula MB input neurons are filled and reconstructed in the same brain. For (A) and (B), they are two-dimensionally projected images single 15 μm plastic sections. For (C-G), the entire dendritic projection of each neuron in the lobula and the axons in the protocerebrum outside of the MB calyces are represented here as two-dimensional projections across plastic sections. Scale bar = 100 μm

Fig. 4.

Distinctive branching patterns of visual mushroom body input neurons. (A) Frontal view of an unstained bumblebee brain (top; broken line indicates position of section shown below) and horizontal section (bottom) of an osmium-stained preparation; inset enlarged in (B) to show the six layers in the lobula (Ribi and Scheel, 1981). (C) Dendritic arborizations of recorded and labeled lobula MB input neurons (white) and lobula non-MB input neurons (magenta) can be assigned to the different lobula layers shown in (B) in this horizontal section. Notably, the MB input neurons branch in layers 5 and 6 while the non-MB input neurons send their branches into layers 1-4. (any overlap of the magenta branching with layer 5 is due to the three-dimensional structure of the lobula layers and the neurons, which is difficult to depict two-dimensionally) (D) Lobula specializations of the neuron shown in Fig. 3D two-dimensionally projected within a single 15 μm plastic section. Note the close vicinity of spines (blue arrows) and bleb-like structures (red arrows) in the lobula (D). (E) The medulla neuron with input to the MBs projects through the medulla (me), around the lobula, to the MB lateral and medial calyx; cell body located below the lateral calyx. (F) A closer view of the medulla dendritic arborizations. Species represented: *Bombus impatiens*: A,B,D-F; *Apis mellifera*: C. Boxes in (C) and (E) represent areas enlarged in Fig. 5 B and D, respectively. Unless otherwise noted, scale bar = 100 μm.

Fig. 5.

Terminal fibers of visual input neurons in the calyces. (A-C) The lobula MB input neurons feature blebbed terminals in the inner rim of the MB calyces (arrows). The large field lobula MB input neuron projected into the innermost collar region (A, B) while the small-field lobula MB input neuron projected closer to the outer collar region (C). (A, C) frontal sections; the dorsal view (horizontal section) in (B) reveals that the lobula MB input neurons input branches project all around the inner rim of the calyx. (D) Medulla MB input neuron sends blebbed varicosities into the outer rim of the collar in the calyces (arrows). (E, F) Specializations of the neuron shown in Fig. 3D. Bleb-like terminals (arrows) near the lip of the calyx (E) and in superior lateral protocerebrum (s l pr) below the lateral MB calyx (F). MB pedunculus (ped); In all cases, the images are two-dimensionally projected within a single 15 μ m plastic section. Species represented: *Bombus impatiens*: A, C, D-F; *Apis mellifera*: B. A-D: Scale bar = 100 μ m. E, F: Scale bar = 10 μ m.

Fig. 6.

Chromatic response characteristics of MB input neurons. (A) The medulla MB input neuron responded phasically to a flash of violet light. (B) The medulla neuron preferentially responded to the presence of violet light over green or blue light as seen in a raster plot and peristimulus time histogram (PSTH; bin-width 5 ms) of the cell's responses to five consecutive light flashes to stimuli including violet (black bars) or not including violet light (grey bars). Abbreviations: blue (b), green (g), blue-green (b-g), violet (v), and violet-green (v-g) light. (C) A large field lobula MB input neuron is phasically inhibited by green, excited by blue, and has no obvious response for violet LED light. (D) Another large field lobula MB input neuron had no response to violet light, had a phasic response for green light which was suppressed by the simultaneous presence of violet light. The response presented here is to the first of five light flashes in all three cases. (E) The response of a large field lobula MB input neuron to blue and green light was inhibited by the concurrent presence of violet light (arrows). The black bars signify the duration of the light flash stimulus, which is 500 ms in all cases.

Fig. 7.

Motion response characteristics of MB input neurons. (A) A large field lobula MB input neuron responded to directional motion of a moving edge. Traces (Ai) and (Aii) show different trials. (B) A small field lobula MB input neuron responded differently to a leftward moving black edge and a rightward moving white edge (Bi) compared to a rightward moving black edge and a leftward moving white edge (Bii), but was not sensitive to directional motion. (C) A large field lobula MB input neuron responded to a directionally moving grating with inhibition of different strength in response to different directions (Ci- Civ). Arrows indicate direction of movement. Black bars signify stimulus duration in all cases.

Fig. 8.

Temporal response characteristics of individual MB input neurons. (A) One small field lobula MB input neuron responded with a phasic, highly precise spike at the beginning of the light flash to simultaneously presented blue-green-violet stimuli over fifteen trials, as illustrated in the raster plot and peristimulus time histogram (PSTH). Highly precise spikes responses is also observed in two large field lobula MB input neurons, with an example in (B) and in the medulla MB input neuron (Fig. 6A,B). (C) A small field lobula neuron increases its spike rate over five sequential light flashes for blue and somewhat for green light, but not for violet light. (D) In addition, the normalized spike rate for each light flash increases with the progress of light flashes for blue-green-violet, blue, and green stimuli (black) but not for violet (grey) (one trial

for each light flash color). A-C: Black bars signify the duration of the light flash stimulus, which is 500 ms in all cases.

Fig. 9.

Stimulus entrainment. (A) After two to three presentations of violet light, the spiking 'response' of the large field lobula MB input neuron actually precedes the light flashes (black arrows); this effect is much less pronounced or absent in responses to the blue light stimulus. Only after the end of violet stimulus series, the neuron produced a burst of activity (white arrow) which coincided with the 'expected' timing of a sixth light flash which was not present (colored in gray). (B) The presence of violet light with or without blue or green (white) resulted in a shift in the activity ahead of the light flashes and an additional burst after the last light flash, which was not observed in presentation of blue or green light only (black), as seen in smoothed PSTHs of spike times binned over 11 trials. Grey shading indicates the black curve 'hidden' behind (= smaller than) the white curve. (C, D) The binned spike times were averaged to get the phase of the spike times relative to the light flashes, where half of the plot is when the light flashes are on (white bar), and the second half is when the light flashes are off (black bar). (C) Phase plots of the responses to all five consecutive light flashes. (D) Phase plots of the responses to the last four consecutive light flashes. The responses to the blue and green LED light flashes without violet light (black arrows) followed the onset of the light flash. When violet light was present in the stimulus (grey arrows), the mean phase of the response was shifted ahead of the light flash. The phase of the responses was significantly different between the non-violet and violet responses. (For all five sequential light flashes across trials: Mann-Whitney U -test; rank sum statistic = 316480; z -statistic = 9.5093; $p < 0.00001$; for the last four light flashes: Mann-

Whitney *U*-test; rank sum statistic = 138539; z-statistic = 8.0390; $p < 0.00001$). A-B: Black bars signify the duration of the light flash stimulus.

Fig. 10.

Habituation responses. (A) The response strength to the first light flash of the optic lobe neurons projecting to the MB calyces decreased considerably to the subsequent light flashes in terms of both excitatory (Ai) and inhibitory (Aii) responses to blue and blue-green light, respectively. (B) In contrast, a lobula neuron not projecting to the mushroom bodies (non-MB neuron) responds similarly to blue (b), green (g), violet (v), and to combinations of the colors across the five subsequent light flashes. (C) Measurement of the habituation responses between the MB input neurons and the non-MB input neurons showed the MB input neurons had significantly higher habituation response values compared to 21 non-MB input neurons (Mann-Whitney *U*-test; rank sum statistic = -9.2801; *z*-statistic = 3.5199; $p < 0.00001$). A, B: Black bars signify the duration of the light flash stimulus.

Fig. 11.

Summary diagram of the sensory inputs to MB calyces examined in this study. (A) Bumblebee brain with different input pathways color coded. Dark gray: Olfactory input from the antennal lobe (two separate pathways; unilateral projections); Green: an input pathways from the medulla (bilateral projections); Magenta: small field lobula MB input neurons (bilateral projections with additional projections into the superior lateral protocerebrum (s.l.pr; arrow)); Blue: large field lobula MB input neurons (unilateral with additional projections into the superior lateral protocerebrum (arrow)). The large field and small field lobula MB input neuron schematics are presented on either side for visualization purposes only. Abbreviations: mushroom body (B) A closer view of half of a calyx shows general sensory inputs segregate in the calyx with the olfactory inputs in the inner basal ring and lip (dark gray shading) and visual inputs in the collar and outer basal ring (light gray and white shading). In addition to schematic drawings of the blebbed optic lobe visual inputs to the MB calyces, the images of the optic lobe projections into the MB calyces have been combined across preparations and false-colored to indicate the relative depths of the visual MB input within the lip (top), collar (col; middle), and basal ring (br; bottom). Visual input from the medulla occupies the outer collar and basal ring (area: light gray; individual projections: green in the schematic and in the insets). The small field lobula MB inputs generally terminate on the inner collar and basal ring relative to the medulla inputs (area: white; individual projections: magenta). The large field lobula MB inputs generally terminate on the inner-most collar and basal ring, and outer lip

(area: white; individual projections: blue). Additional abbreviations: antennal lobe, al; medulla, me; mushroom body lateral calyx, mb-lc; mushroom body medial calyx, mb-mc; mushroom body vertical lobe, mbvl; lobula, lo.

TABLE 1
Visual sensitivity and anatomy of mushroom body input neurons and non-mushroom body input neurons.¹

Dendrite type	MB calyx output	Projections Outside MB calyx	Cell body	Resp. to LEDs	Photorec. input	Chrom. opponent	Directional selectivity	Figure num.
Small field lobula	i br, i col	Med. s l pr	unknown	b g +	UV 0	none	Prog./regr.: bidir.	7B, 8A
Small field lobula	i br, i co	Ant. s l pr	Post. dors.	+ +	0 0	none	Not tested	3G, 5C, 8C,D
Large field lobula	i br, i co, o li	Med. s l pr	Post. dors.	+ +	0 0	B+G+ UV-	0	3A,B,F, 5A, 6D, 10Ai
Large field lobula	i br, i co, o li	Med. s l pr	Post. dors.	+ -	0 0	B+G- UV0	0	3A,B, F, 5A, 6C
Large field lobula (Apis)	i br, i co, o li	Med. s l pr	Post. dors.	+ +	0 0	none	0	4C, 5B
Large field lobula	i br, i co	Post. s l pr	unknown	+ +	0 0	B+G+ UV-	0	none
Large field lobula	i br, i co, o li	Post. s l pr	Post. dors.	+ +	- -	B+G+ UV-	Prog.	3D, 4D, 5E,F, 6E, 7A, 9
Large field lobula	i br, i co	Ant. s l pr	unknown	- -	0 0	none	Up/down: bidir..	3E, 7C, 10Ai
Large field lobula	i br, i co	none	unknown	+ +	0 0	none	Non-Dir.	8B
Large field lobula	i br, i co, o li	Ant. s l pr	Post. dors.	0 0	0 0	none	Not tested	3C
Medulla	o br, o co	none	Med. dors.	0 0	+ +	none	Not tested	4E,F, 5D, 6A,B
Lobula layers 1-4 (n=21)	none	Post. prot.	variable	+ +	+ +	none	variable	4C, 10C

¹The MB input neurons could have different types of dendrites (Dendrite type; lobula: lo.), have output projections in the inner or outer basal ring (i or o br), inner or outer collar (i or o co), or the outer lip (o li). The projections outside of the MB calyx can be in the anterior (Ant.), medial (along anterior-posterior axis, Med.), and posterior (Post.) superior lateral protocerebrum (s l pr). The locations of all the filled cell bodies were all lateral to the lateral MB calyx, but they could be posterior (Post.) or medial (Med.). A cell could either respond to the blue (b), violet (v), or green (g) light flashes with inhibition (-), excitation (+), or have no response (0) to the visual stimuli presented. Based on their responses to the LEDs, we deduced what photoreceptor types (Photorec. Input; ultraviolet, UV; blue, B; green, G) input to the neurons and how each input interacted, such as whether the UV input inhibited the excitatory B input (i.e. B+UV-) in the chromatically opponent neurons (Chrom. opponent). Motion sensitivity (Directional selectivity: Dir. select.) was categorized as such when the neuron had a significant response to progressive (prog), regressive (regr), or up/down moving stimuli on the computer monitor, if they preferentially responded to both directions of movement (bidir.), to all directions (Non-Dir.), or had no response (0). Motion stimuli were not tested for all cells (not tested). Finally, twenty-one non-MB input neurons with dendritic branching in Lobula layers 1-4 are included in the table to illustrate their response properties, which include responsiveness to all three colors and motion sensitivity. The figures where the anatomy of the neurons is pictured are in bold while the images of the physiological responses of the neurons are italicized (Figure num.).