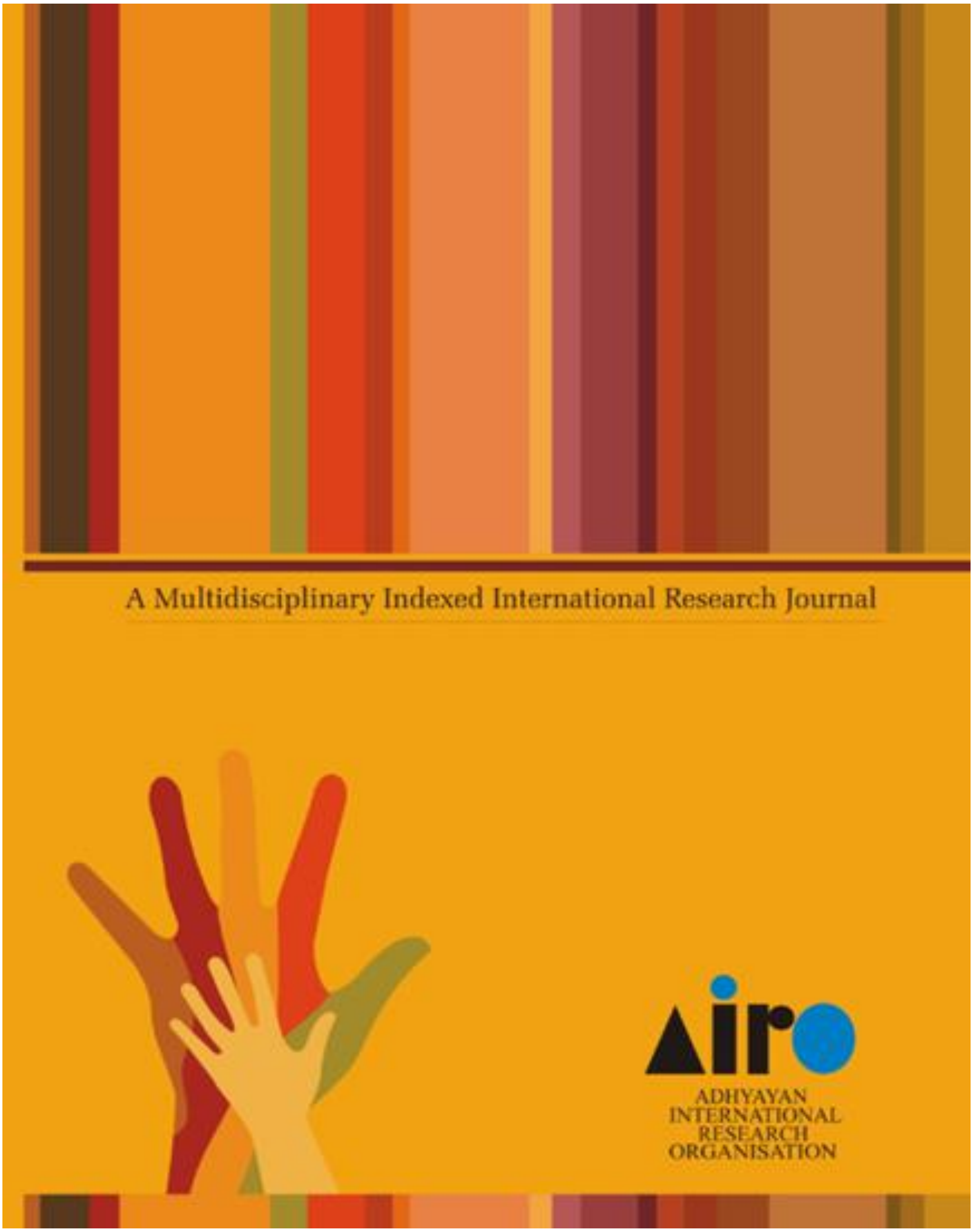
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ROLE OF CENTRAL OLFACTORY NEURONS IN HONEY BEE

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ABSTRACT

The antennae of honeybee (Apis mellifera) workers and drones differ in various aspects. One striking difference is the presence of Sensilla basiconica in (female) workers and their absence in (male) drones. We investigate the axonal projection patterns of olfactory receptor neurons (ORNs) housed in S. basiconica in honeybee workers by using selective anterograde labeling with fluorescent tracers and confocal microscopy analysis of axonal projections in antennal lobe glomeruli. Axons of S. basiconica-associated ORNs preferentially projected into a specific glomerular cluster in the antennal lobe, namely the sensory input-tract three (T3) cluster. T3- associated glomeruli had previously been shown to be innervated by uniglomerular projection (output) neurons of the medial antennal lobe tract (mALT). As the number of T3 glomeruli is reduced in drones, we wished to determine whether this was associated with the reduction of glomeruli innervated by medial-tract projection neurons. We retrogradely traced mALT projection neurons in drones and counted the innervated glomeruli. The number of mALT-associated glomeruli was strongly reduced in drones compared with workers. The preferential projections of S. basiconica-associated ORNs in T3 glomeruli together with the reduction of mALT-associated glomeruli support the presence of a female (worker)-specific olfactory subsystem that is partly innervated by ORNs from S. basiconica and is associated with the T3 cluster of glomeruli and mALT projection neurons. We propose that this olfactory subsystem supports parallel olfactory processing related to worker-specific olfactory tasks such as the coding of colony odors, colony pheromones and/or odorants associated with foraging on floral resources.

KEYWORDS: Insect, olfaction, parallel processing, projection neurons, specific lesion, olfactory conditioning.

INTRODUCTION

Olfaction is an ancient sensory modality and plays a crucial role in most animals for approaching or avoiding various odor sources and for judging their quality in a variety of behavioral contexts. Whereas odorant reception at the molecular level exhibits distinct differences between vertebrates and insects, the basic wiring pattern of receptor neurons with second-order neurons within the primary olfactory centers, the vertebrate olfactory bulb and the insect antennal lobe (AL) shows several striking similarities. These have been the subject of intense research over recent years. Insect antennae are covered with various types of sensory sensilla; most of them being specialized for chemoreception but also for hygro-, mechano- and thermo reception. Olfactory sensilla house the olfactory receptor neurons (ORNs) that extend axons into spheroidal structures termed glomeruli to form synaptic connections with local interneurons and projection neurons (PNs). Glomeruli represent the functional unitsof the AL. In the honeybee, Sensilla placodea, Sensilla trichoidea and Sensilla basiconica have been classified as olfactory sensilla, either according to their odor-response profiles in single-sensillum recordings (S. placodea) or based on specific anatomical features. ORN axons from olfactory sensilla project via four distinct AL sensory-input tracts to four clusters of glomeruli in the AL termed the T1-T4 cluster. Axons from ORNs in S. placodea project to all four clusters of glomeruli T1-T4 and single-sensillum recordings from S. placodea have revealed responses to a broad range of odorants. This might be either caused by the broad tuning of ORNs or attributable to the finding that S. placodea house many individual ORNs, each covering a certain spectrum of molecular receptive ranges.

In insects, PNs convey the olfactory information to the mushroom bodies (MBs), higher sensory association centers and sites associated with learning and memory. Uniglomerular PNs in the honeybee and other Hymenoptera have been shown to project to the MBs and lateral horn (LH) via two parallel tracts: the medial and the lateral AL tracts (mALT and lALT) forming a dual olfactory pathway. Comparative anatomical studies indicate that a dual olfactory pathway probably emerged in the basal Hymenoptera However; the selective pressure that promoted the evolution of a dual olfactory pathway within this group of insects remains to be further investigated. Female honeybee workers show complex social behavior that is largely influenced by pheromonal communication. This is different in male drones, which mainly perform reproductive tasks, do not forage actively for food and might not need to distinguish minor changes in colony pheromone concentrations. On the other hand, drones are highly sensitive to the queen sex-pheromone. Therefore, differences in the olfactory system reflecting these behavioral specializations are likely to exist between honeybee workers and drones. One striking sex-specific difference is the absence of S. basiconica on drone antennae. Furthermore, drone ALs contain a smaller number of glomeruli compared with both female castes (workers and queens) but comprise several enlarged macro glomeruli. The largest macro glomerulus has been shown to respond to the major component of the queen mandibular pheromone. The reduction of AL glomeruli is mostly associated with the T3 cluster, which has been demonstrated to be mainly innervated by medial tract PNs in honeybee workers. Comparative studies in other Hymenoptera indicate that the lack of S. basiconica in males is a characteristic trait across both social and solitary Hymenoptera. In the leaf-cutting ant Atta vollenweideri, S. basiconica have been found exclusively to innervate a specific (T6) cluster of AL glomeruli. The absence of S. basiconica, together with the reduction of glomeruli in the T3 cluster, in honeybee drones suggests that ORNs from S. basiconica preferentially innervate glomeruli in the T3 cluster and are associated with medial-tract PNs. To test this hypothesis, we investigated the axonal projections of ORNs in the hair-like olfactory antennal sensilla of female worker bees, with a special focus on S. basiconica and, in particular, their glomerular innervation patterns and their association with PN output tracts. Furthermore, we retrogradely labeled the mALT in drones to analyze whether glomeruli associated with this tract are reduced.

REVIEW OF LITERATURE

In many sensory modalities, the nervous system uses parallel pathways to enable the separate processing of different stimulus features. The best described example of such parallel stimulus segregation is the case of visual processing in vertebrates and invertebrates which relies on the existence of one pathway involved in the processing of colors and shapes and of another pathway processing movement and spatial features. The study of such parallel processes usually follows a double approach:

(i) Functional recording (via electrophysiology or imaging, for instance) of individual responses from each of these pathways and

(ii) Selective pathway lesions allowing determining the capabilities affected by the injury and thus the functional role of both the lesioned and the intact pathways.

Combining both approaches is essential for understanding parallel processing in a given sensory system. In the olfactory modality, parallel processing is least known, although the anatomical organization of olfactory systems clearly suggests that such treatment exists. Both in vertebrates and insects, different subsystems are involved in the processing of pheromones and general odorants. Besides this segregation in terms of odorant classes, the general olfactory system needs to classify the chemical quality of odorants regardless of their concentration (“concentration invariance”) and also code the absolute concentration of an odor when an animal seeks its source. In addition, different chemical characteristics of odorant molecules (for instance their chain length or functional group) may need to be processed separately. Parallel processing in the olfactory system may constitute an adequate solution to these problems. However, how parallel olfactory systems encode and process chemical stimuli is still largely unknown.

The honey bee Apis mellifera is an influential model for the study of olfactory coding and processing. Olfaction is a key modality for honey bees, playing a major role in multiple aspects of their social life style and foraging behavior. The olfactory circuit of the bee exhibits two parallel olfactory pathways of almost equal size Dual olfactory pathway of the honey bee brain.

(A) Schematic overview of the dual olfactory pathway of the honey bee brain. Odorant molecules are detected by olfactory receptor neurons (ORN) on the antenna which project to the antennal lobe (AL). Then, projection neurons (PN) convey information to the mushroom bodies (MB) and the lateral horn (LH) via two main tracts, the medial antennal lobe tract (m-ALT, magenta) and the lateral antennal lobe tract (l-ALT, green). Lesion site of the m-ALT and of the optic lobe (OL) are indicated (lesion 1 and lesion 2 respectively).

(B) Mass staining in the AL, showing the course of l-ALT and m-ALT PNs from the AL to LH and the MB calyces. Abbreviations: m-ca, median calyx; l-ca, lateral calyx. 2013). Following odor detection by olfactory receptor neurons (ORNs) and subsequent primary processing in the antennal lobe (AL), two main neural tracts of projection neurons (PNs), the lateral and the medial antennal lobe tracts (l-ALT and m-ALT, respectively) convey the processed olfactory message to higherorder centers, the mushroom bodies (MBs) and the lateral horn (LH; Figure 1). The AL is composed of functional units, termed glomeruli, that each receives input from ORNs expressing the same olfactory receptor type. About half of the glomeruli located on the ventral surface of the AL (84 glomeruli) are innervated by the l-ALT while the other half located on the dorsal surface (77 glomeruli) are innervated by the m-ALT. The two tracts project to largely segregated areas within higher-order centers, with only limited overlap (Kirschner et al., 2006). Until now, only functional recordings have been used in the honey bee to study the role of these parallel pathways but no clear differences were found in their responses to general odorants, which are mostly redundant apart from small disparities in their spatiotemporal characteristics. The most apparent difference between both tracts was the fact that queen pheromone is processed by the l-ALT while brood pheromone is mainly processed by the m-ALT. Apart from these differences, the two pathways may also be differentially involved in olfactory learning, but this idea has not been explicitly tested. In this context, the use of selective tract lesions may help understand the functional role of l-ALT and m-ALT neurons.

HONEY BEE PREPARATION

Worker bees were collected in the morning from the entrance of outdoor hives. To facilitate handling and mounting, bees were anesthetized on crushed ice for 5 min. They were then placed into individual metal tubes, taking care to leave their antennae, mandibles, and proboscis free. Two adhesive strips were placed behind the head and the abdomen. The bees were then fed with 5µL sugar solution (50% w/w) to homogenize their satiety state. The lesions of the m-ALT being made unilaterally (to increase the success rate of this method), the appetitive olfactory conditioning also had to be performed unilaterally. For this reason, the antenna contralateral to the lessoned side was fixed with wax, and the flagellum was covered with 2-component silicone to prevent odor detection on this side. The efficiency of the silicone for blocking olfactory input was checked in a group of bees with both antennae covered (see Results). After attaching the bee's head with wax, an opening was made with a razor blade between the compound eyes, and the detached piece of cuticle was preserved, so that it could be placed back after the brain lesion. To allow access to the brain and to perform the m-ALT lesion, glands, and trachea covering the brain were removed.

OLFACTORY CONDITIONING OF THE PER PER

conditioning was performed in standard conditions. A conditioning session consisted in five conditioning trials, in which an odor was associated with sucrose, separated by 10 min inter-trial intervals. The conditioned stimulus (CS) was the odorant 1-nonanol (C9- ol, Sigma, Deisenhofen, Germany). The presentation of the odor was performed manually at the bee's antennae, using a 20 mL syringe containing a 1 cm2 filter paper strip soaked with 5µL of pure odor solution. The unconditioned stimulus (US) was a sugar solution (50% w/w) applied with a toothpick to the bees' uncovered antenna, and then to the proboscis. In the experiment with both antennae covered, both covered and uncovered bees received the US on the proboscis only. A conditioning trial lasted 30 s. One bee at a time was placed in the stimulation site in front of an air extractor and left for 15 s to accommodate to the experimental situation. Then, the CS (odor) was presented for 5 s and the US (sugar solution) was applied for the last 2 s of CS presentation. The interval between CS and US onsets was thus 3 s. The bee was left in the set up for 10 more seconds until the end of the trial. During conditioning, the responses (complete proboscis extension) to CS and US were recorded. Bees that did not respond to the US at any time during the experiment were excluded from the analysis as they were not considered motivated enough for the experiments.

ROLE FOR PNS IN OLFACTORY ACQUISITION

Previous neurophysiological studies showed that l-ALT and mALT neurons respond to a mostly redundant array of general odorants (i.e., non-pheromonal odorants), albeit with somewhat different spatiotemporal characteristics. This observation suggested that both neural tracts could be functionally redundant for the learning of these odorants. In particular, optical imaging recordings showed clearly that both l-ALT and m-ALT subsystems respond to the odorant 1-nonanol, the CS we used to train the bees. Therefore, if both subsystems were totally redundant, bees should be perfectly able to learn to associate 1-nonanol with sucrose even in the absence of a functional m-ALT tract. This was not the case as bees with an m-ALT lesion showed a strong decrement of acquisition and retrieval performances compared to bees with an OL lesion. This result contradicts the common idea that normal olfactory function within the antennal lobe alone is sufficient for olfactory acquisition. This idea stems from the results of several studies. Erber et al. (1980) were the first to suggest a role for the AL in appetitive olfactory learning by showing that local cooling of the AL in the first 3 min after a single-trial conditioning strongly reduces bees' conditioned responses. Later, this role was confirmed by Hammer and Menzel (1998), who showed that injection in the AL of octopamine, the neurotransmitter mediating the reinforcing properties of the sucrose US, is sufficient for inducing significant acquisition if it follows immediately an odor presentation. In the same line, Farooqui et al. (2003) confirmed that blocking OA neurotransmission in the AL also blocks acquisition. Together, these results indicate that an olfactory memory supporting normal acquisition performance is established through association of the odor CS and OA-mediated US information in the AL. Other studies repeatedly showed appetitive learning-induced plasticity both in the structure and activity of AL networks. However, these data do not give any insights into the role of connecting processes between AL and MBs. Our results suggest that after the formation of a CS-US association in the AL, associative plasticity would be transmitted via PNs to the MB calyx for further acquisition and memory consolidation. Such transfer processes are also found in other memory systems, for instance between the hippocampus and the cortex or between the cerebellum and the vestibular nuclei. The drop in acquisition after an m-ALT lesion could either indicate that the m-ALT tract alone is involved in such transfer, or that concomitant activity from both m-ALT and l-ALT neurons is necessary for this task. At this time, it is difficult to decide between these two hypotheses, because up to now no study could perform a perfectly specific l-ALT lesion. However, one previous study has provided interesting clues. As a control for an optical imaging experiment, Peele et al. (2006) applied an l-ALT lesion between the LH and the MB calyces in one hemisphere of the bee brain. The authors observed a similar effect as in the present study: unilaterally lesioned bees conditioned with a bilateral CS did not respond to this CS when it was presented on the lesioned side. If the observed effect was due to thel-ALT lesion, this data suggest that the l-ALT is also necessary for normal olfactory learning. Yet, in this study it is unclear if the applied lesion also severed the m-ALT, which is also found at this location. Therefore, if the observed effect was rather due to the m-ALT lesion, it would suggest that the LH would be the target of the plastic message carried by m-ALT neurons. Only further work with specific lesions of the l-ALT or m-ALT at different locations in the brain may help clarify this point. In any case, our results together with the study by Peele et al. (2006) identify a prominent role of PNs in olfactory learning performance.

FEMALE-SPECIFIC OLFACTORY SUBSYSTEMS AND THEIR POSSIBLE FUNCTION

Demonstrate that the reduction of glomeruli in the AL of honeybee drones is higher in the mALTcompared to the lALT-innervated hemilobe of the AL. ORN axons in the glomeruli of the T3 cluster are innervated by mALT PNs in females. This cluster is reduced in drones, whereas the T1 cluster is less reduced compared with that in the female AL. As drones lack S. basiconica, the reduction of the mALT-associated parts of the T3 glomeruli is likely to be related to the absence of ORNs from the S. basiconica. As three enlarged glomeruli are present in the T1 cluster in drones, the slight reduction in the number of T1 glomeruli in drones might favor the macro glomeruli. Several physiological studies, so far, have shown that no part of the honeybee AL and therefore neither the mALT nor the lALT, is selectively specialized for either only social or floral odorants. However, as honeybees are exposed to an enormous odor space in their natural environments, more odorants, inparticular social (colony) cues and pheromones, remain to be tested in more detail and might give further indications concerning selective physiological properties and the molecular receptive range of S. basiconica ORNs. The ants Camponotus floridanus, Camponotus japonicus and A. vollenweideri have similarly been demonstrated to have a reduced number of glomeruli in males compared with females. Kelber et al. (2010) investigated projection patterns of S. trichoidea and S. basiconica ORNs in A. vollenweideri. Here, the ants possess six AL glomerular clusters (T1-T6) and S. trichoidea ORNs have been shown to project to all of them. Up to five different glomerular clusters have been found to be innervated by ORNs from a single S. trichoideum. In contrast, S. basiconica ORNs only projected to the T6 cluster. In honeybee workers, we were able to reveal that S. basiconica preferentially project into the T3 cluster. C. japonicus ALs comprise seven glomerular clusters (T1-T7) and the glomerular cluster T6 is also worker-specific, similar to the T6 cluster in A. vollenweideri. Furthermore, here the T6 output neurons were demonstrated to project to specific sub regions within the MBs and the LH and the authors speculate that the input to these glomeruli origins from S. basiconica-associated ORNs. Nishikawa et al. (2012) argued that these brain regions are likely to be involved in social tasks in C. japonicus, as drones do not need to fulfill extensive social duties within the colony.

Another important aspect is that in several solitary bee species, the males also lack S. basiconica. In parasitoid wasps, cuticular hydrocarbon profiles have been shown to vary between closely related species, between sexes and according to the developmental environment. Better detection abilities for cuticular hydrocarbons could serve females in kin detection and thus help them to avoid insemination by related males or even by males from other species. Another major distinction between drones and workers in both ants and social bees is that drones do not forage. This implies the involvement of S. basiconica in the detection of floral odorants. These studies show that various hymenopteran species possess female-specific olfactory subsystems consisting in specific sensilla, their respective ORNs and downstream odor-processing brain structures. The separation of these subsystems from the sexual isomorphic structures can be more (A. vollenweideri, C. japonicus) or less (A. mellifera) pronounced. Thus, variations in olfactory tasks and structures between males and females might differ across species.

CONCLUSION

Honeybee workers possess a specific olfactory subsystem comprising S. basiconica; parts of the T3 cluster of glomeruli and a significant proportion of mALT PNs. Drones completely lack this olfactory subsystem and, additionally, have far fewer S. trichoidea, all features that might favor a more elaborated queen-pheromone-processing system and the associated higher numbers of S. placodea present in drones. At the behavioral level, drones, therefore, are likely to have more limited odor discrimination and recognition abilities compared with females. This limitation is likely to be associated with social (colony) odors and/or floral odors. The adaptation in drones for improved queen-pheromone detection including high numbers of S. placodea and pheromone-processing macro glomeruli is likely to increase mating probabilities. Honeybee workers, in contrast, are exposed to high selective pressure to identify and locate correctly a wide variety of odorants, including floral odorant mixtures, a large variety of pheromones and colony (social) cues. The different types of sensilla and the associated olfactory subsystems of glomeruli and output tracts in the AL appear to be well adapted for these tasks.

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