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Molecular and Electrophysiological Characterization of Olfactory and Gustatory Perception in Honeybee and Cockroach for Application as Biosensor

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Molecular and Electrophysiological Characterization of Olfactory and Gustatory Perception in Honeybee and Cockroach for Application as Biosensor

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ABSTRACT

Chemoreception is an essential sensory modality for the survival and reproduction of many animals, especially insects. Food sources, mate and oviposition sites are located and evaluated through the use of the chemosensory apparatus. Despite growing knowledge of insect olfactory and gustatory detection and processing very little is known about the modulation and receptor function of the chemosensory system.

This objective of this thesis was to explore olfactory modulation of the peripheral olfactory system in the American cockroach, *Periplaneta americana* and to compare olfactory sensitivity between Asian honeybee, *Apis cerana* and European honeybee, *Apis mellifera*. In addition, this research concentrated on

determining the natural ligands such as sugar and amino acids of gustatory receptors of honeybee, *Apis mellifera*, in order to understand the gustatory world of insect. Finally, we developed a bioelectronics tongue using honeybee taste receptor for the detection of umami taste compounds with human like performance. Through the use of morphological, behavioral, electrophysiological, pharmacological, neuroanatomical, and biophysical techniques we have begun to elucidate the modulatory effects of internal factors, neuromodulators, on insect chemoreception. Also, we demonstrated functional role and characterization of gustatory receptors related to sweet and amino acids compounds in the honeybee, *Apis mellifera*. These studies provide a foundation for understanding the molecular and cellular basis of olfactory and gustatory coding and behavior.

Keywords: insect, olfaction, gustation, chemoreceptors, honeybee, cockroach.

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Introduction

Olfaction and taste are of critical importance to insects and other animals, since vital behaviors, including mate, food and host seeking, as well as predator and toxin avoidance, are guided by chemosensory cues (Hansson and Stensmyr 2011). Mate, food, and habitat choice are to a large extent determined by chemical signals, and chemoreceptors contribute accordingly to premating isolation barriers and speciation. Chemosensory neurons are present in specialized sensory hairs called sensilla (Keil 1999). In many insects olfactory sensilla are present on olfactory organs on the head, the antennae and the maxillary palps (L B Vosshall et al. 1999),(L B Vosshall, Wong, and Axel 2000), (Dobritsa et al. 2003) . Gustatory sensilla are present on several parts of the body including the internal and external organs such as mouth parts, legs, wing margins and brain, fat body, and ovipositor in females (Clyne 2000). In adult insects, each olfactory sensillum is innervated by up to four olfactory receptor neurons (ORNs) (A. Dahanukar, Hallem, and Carlson 2005). Pores in the cuticular walls of the sensilla enable passage of odorant molecules from the environment to the dendrites of ORNs (Leslie B Vosshall and Stocker 2007). Taste sensilla have a single pore at the tip and are innervated by one or more gustatory receptor neurons (GRNs), typically four, and a mechanosensory neuron (Isono and Morita 2010), (Liman, Zhang, and Montell 2014). Physiological and behavioral analysis has shown that GRNs are

tuned to four classes of chemicals: sugars, bitter substances, salts, and water (Moon et al. 2006), (Jiao et al. 2008), (A. Dahanukar et al. 2007).

Chemoreceptors, which bind to external chemical signals and then transform and send the sensory information to the brain, are at the core of the peripheral olfactory and gustatory system and have thus been the focus of recent research in functional characterization of chemoreceptors and modulation of neuronal and behavioral chemosensory responses to the environment. The molecular basis of insect olfaction and gustation became amenable to study through discovery of two large families of genes that encode candidate chemoreceptor proteins in *Drosophila melanogaster* (Clyne 2000), (Scott et al. 2001), (Robertson, Warr, and Carlson 2003). These proteins have at least seven transmembrane domains and although once thought to be similar to the known G-protein coupled receptor superfamilies, they share no sequence similarity with them (i.g. (Hill et al. 2002)) and appear to have the reverse membrane topology (Benton et al. 2006), (Wistrand, Käll, and Sonnhammer 2006), (Lundin et al. 2007). A super family of 60 odorant receptor (Or) and 60 gustatory receptor (Gr) genes encoding 62 and 68 different proteins was identified in Drosophila using bioinformatic and molecular approaches (Robertson, Warr, and Carlson 2003). Genes encoding related receptors have been recognized in the genomes of other insects, including the mosquito *Anopheles gambiae* (Hill et al. 2002), the moth

Heliothis virescens (Krieger et al. 2004) and *Bombyx mori* (Sakurai et al. 2004), the red flower beetle *Tribolium castaneum* (Engsontia et al. 2008), and the honeybee *Apis mellifera* (Robertson and Wanner 2006a).

1. Peripheral olfactory system

Insect antennae come in a variety of shapes and size but can be subdivided by 3 segments; the scapus, the pedicellus and the flagellum (Keil 1999). The flagellum carries most of the olfactory sensilla yet other sensilla can be found on the labial or maxillary palps (Keil 1999). The number of antennal sensilla is dependent on the insects' life style, where males of some insect often have more sensilla compared to females reflecting sexsual dimorphic need to detect sex pheromone cues (Hansson and Stensmyr 2011). Irrespectively, adult insect antenna can carry tens of thousands of sensilla ensuing high functional fiedelity when "sieving" through the odorous environment (Keil 1999),(Jung et al. 2014). The structure, internal organization, and environment of the olfactory sensilla make them high fidelity detector of odors. Once through the pores in the sensillum cuticle, odor molecules bind to OBPs or PBPs making otherwise lipophilic molecules more hydrophilic (K. E. Kaissling 2009), (Stengl 2010). This complex of odor molecule and OBPs/PBPs then transverse the sensillum lymph to the olfactory receptors located on the ORN cilium. At the receptor site, the odor molecule binds to the receptor and induces signal transduction (Leslie B Vosshall

and Hansson 2011).

2. Modulation of the olfactory system

It is believe that each ORN only express one type of OR with the ubiquitous chapherone co-receptor, “Orco” (Couto, Alenius, and Dickson 2005), (Nef et al. 1992), (Leslie B Vosshall and Hansson 2011), (Nakagawa et al. 2005), (Larsson et al. 2004). Orco and expressed OR form a heterodimeric complex that an ionotropic (direct ion channel) and a metabotropic (through second messenger systems) mechanisms for signal transduction. Upon odor stimulation of the Orco/OR complex, induction of ionotropic and metabotropic currents results in a graded receptor potential that, at the spike initiation zone of the ORN, either increases the spike activity (excitation) or decreases spike activity (inhibition) (Leslie B Vosshall and Hansson 2011), (Wicher et al. 2008).

For the insect to be able to accommodate constant changes in both the external and the internal environment, the nervous system has to be able to change accordingly. A fast and reversible way of achieving such changes is by modulating the olfactory system through the use of neuroactive chemicals, i.e. neuromodulators. Alongside conventional neurotransmitters, such as acetylcholine and GABA, insect olfactory neurons are known to produce a large number of neuroactive substances (Nässel and Homberg 2006), (Nässel 2002). These neuromodulators, including biogenic amines and neuropeptides, are known to be

widely distributed within the olfactory system, especially the ALs and the MBs (Nässel and Winther 2010),(Heuer et al. 2012). However, direct physiological function within the olfactory system has so far only been attributed to a small subset of these neuromodulators (Root et al. 2011), (Ignell et al. 2009). Biogenic amines and neuropeptides are thought to be co-released with the conventional neurotransmitters and involved in synaptic transmission of information. Both biogenic amines and neuropeptides are believed to act through G-protein coupled receptors (GPCRs / metabotropic receptors) with a second messenger system that involves up- or down-regulation of cytosolic cyclic adenosine monophosphate (cAMP) (Schlenstedt et al. 2006), (Johnson, Becnel, and Nichols 2011)

3. Organization of gustatory system and functional analysis of gustatory receptors of insects

In general, gustatory receptor genes appear to be expressed in subsets of GRNs in the adults, in one or more gustatory tissues including mouth parts, antennae, legs, wing margins, and internal organs(Scott et al. 2001), (Chyb et al. 2003), (Wang et al. 2004), (Thorne et al. 2004). Similar to the *Or* genes, some *Gr* genes are expressed during larval stage (Scott et al. 2001). In addition, the expression of at least three Gr genes has been detected in the antenna, suggesting that some of them might have olfactory function (Scott et al. 2001). In fact, Gr21a is expressed in a neuron that is narrowly tuned to CO_2 (De Bruyne, Foster, and

Carlson 2001), but its role in this response is not clear. GRNs project to the subesophageal ganglion (SOG) and the tritocerebrum in the brain, which are the first order processing centers for gustatory information (Leslie B Vosshall and Stocker 2007). Although responses of GRNs in other insects have been examined in some detail (Glendinning, Davis, and Ramaswamy 2002), functional characterization of GRNs is limited in *Drosophila*. In adult flies, a minimum of three receptors are required for sensing all sugars tested, except for fructose: Gr5a, Gr64a, and Gr64f (a Dahanukar et al. 2001), (A. Dahanukar et al. 2007), (Jiao, Moon, and Montell 2007), (Jiao et al. 2008). These three receptors are co-expressed in the sugar-responsive GRNs in the labellum, along with five other related GRs that comprise the Gr-Sugar clade (A. Dahanukar et al. 2007), (Jiao, Moon, and Montell 2007). It seems likely that the other member of GR-Sugar clade contribute to sugar sensation. In addition, different sets of bitter-sensitive GRNs have distinct sensitivities. On the basis of their responsiveness of a panel of 16 bitter compounds, the L-, I-, and S-type sensilla that decorate the labella are classified into five groups, four of which are sensitive to bitter chemicals (Weiss et al. 2011). Unlike bitter compounds and most sugar, which are detected by a complex set of Grs, a single Gr, Gr43a, has been reported to detect fructose (Miyamoto et al. 2012). Genetic study demonstrated that Gr43a is required for responding specifically to fructose (Miyamoto et al. 2012).

4. Gustatory modulation of honeybee

Gustatory stimuli play a vital role in the life of honeybee which is our research model. In the foraging stage, foragers collect pollen and nectar, which provide protein and carbohydrate that are necessary for survival. Pollen contains protein as well as lipids and amino acids (Teixeira et al. 2005). Nectar consists of sugars such as sucrose, glucose, and fructose as well as lipids and minerals (Teixeira et al. 2005). Nurses within the hive may also use their gustatory senses for different purposes such as intracolony recognition and pheromone detection. In these circumstances, honeybees actively scan gustatory stimuli with their antennae. The tip of the antenna in the honeybee contains different types of chemo- and mechano-sensory sensilla that are known as taste hairs. The density of gustatory sensilla that take the form of hairs (sensilla chaetica) or pegs (sensilla basiconica) on the antenna is highest on the terminal antennomere (Esslen and Kaissling 1976), implying that the tip of the antenna is critical for antennal gustation. Honeybees use the region to evaluate the quality of food through antennal scanning behavior (Haupt 2004).

5. Bioelectronic tongue

Olfactory and gustatory bioelectronics sensor systems are very useful in the food industry and for environmental protection. Although people have a considerable amount of basic knowledge about smell and taste, the chemoreception is still in an

early stage of development. Only a few types of olfactory gustatory sensor systems are commercial use. For example, the electronic tongues usually utilize synthetic materials such as polymers, semiconductors or lipid membrane in the form of an array to detect known tastants. Even with technical advances, most previous artificial sensors could not mimic the natural features of human taste system. Some previous researches developed a bioelectronic tongue using heterodimeric human sweet taste receptors for the detection and discrimination of sweeteners with human like performance (Song et al. 2014). In addition, same research group developed a chemical pain sensor that could recognize chemical pain stimuli such as capsaicin and resiniferatoxin just like mammalian chemical pain sensor systems (Jin et al. 2013). Despite of the history of the research of the bioelectronics nose and tongue is not long, its progress has been extensively studied. Many materilas and methods have been applied in both the manufacture and signal processing of the biomimic sensor system, and many meaningful results have been obtained. However, less well known is developing electronic devices which mimic the gustatory system of insects.

6. Specific Aims

The aim of this research topic is to give an update on the breadth and depth of research currently in progress related to understanding the molecular and physiological mechanisms of insect chemoreception, with specific emphasis on

the olfactory receptor and gustatory receptor. This present research demonstrated that the characterizing distribution patterns of olfactory sensilla, olfactory responses to floral volatile compounds, and the expression level of Orco in antennae by employing electron-microscopic observation, electrophysiological approaches, and western blot analysis. In addition we investigated the anatomical organization of neuronal circuits governing the alteration of olfactory sensitivity by neuromodulators and explored the observation on the physiological effects of neuromodulators on olfactory sensitivity in the antenna. The cellular function of honeybee gustatory receptors was tested by two heterologous expression in HEK293T cells and *Xenopus* oocytes. Cells and *Xenopus* oocytes expressing AmGr1 are responsive to sugar substances such as sucrose, glucose, maltose, and trehalose in a dose-dependent manner. Also, HEK293T cell expressing AmGr3 selectively responded to fructose with an influx of extracellular Ca^{2+} ions. Amino acids are ligands for an AmGr10 and this receptor responses to nearly all L-amino acids were dramatically enhanced by low doses of inosine-ribonucleotide (IMP), which can be umami taste receptor in honeybee. Finally, we first prepared nanovesicles containing honeybee umami taste receptor, which is activated by MSG. This sensor platform can be utilized for various practical applications such as food screening tools.

In addition to fundamental physiological, ecological and evolutionary

consideration, the knowledge of insect taste and olfaction is also of great importance to human economies, since it facilitates a more informed approach to the management of insect pests of agricultural crops and forests, and insect vectors of diseases.

CHAPTER I

Neuromodulation of olfactory sensitivity in the peripheral olfactory organs of the American cockroach, *Periplaneta americana*

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Abstract

Olfactory sensitivity exhibits daily fluctuations that are regulated by the circadian system. A line of studies suggested that the olfactory system in insects is modulated by both biogenic amines and neuropeptides. However, molecular and neural mechanisms underlying olfactory modulation in the periphery remain unclear since neuronal circuits regulating olfactory sensitivity have not been identified. Here, we investigated the structure and function of these signaling in the peripheral olfactory system of the American cockroach, *Periplaneta americana*, utilizing *in situ* hybridization, qRT-PCR, and electrophysiological approaches. We showed that tachykinin was co-localized with octopamine receptor in neurons present near antennal nerves of the antennae. In addition, tachykinin receptor was expressed in most olfactory receptor neurons in antennae. Functionally, the direct injection of tachykinin peptides and dsRNA of tachykinin and tachykinin receptors as well as octopamine receptors into the antenna area added the evidence to modulate the olfactory sensitivity. Taken together, these findings demonstrated that octopamine and tachykinin in antennal neurons were potential olfactory regulators in the periphery, of which the expression levels were also affected by circadian control. We propose here the hypothesis that octopamine released from neurons in the brain regulates the release of tachykinin

from the octopamine receptor neurons in antennae, which in turn modulates the olfactory sensitivity of olfactory receptor neurons, which house tachykinin receptors.

Keywords: olfaction, olfactory sensitivity, tachykinin, biogenic amine, olfactory modulation

Introduction

Olfaction is an important sensory modality for social interactions, perception, and efficient orientation to food sources in most animals (Kaupp 2010). While the circadian regulation of a large number of physiological and behavioral processes has been studied extensively, circadian rhythms in olfaction are some of the more recently discovered and the least well-characterized in mammals and insects (Granados-Fuentes, Tseng, and Herzog 2006), (Merlin et al. 2007). In both *Drosophila melanogaster* and *Leucophaea maderae*, it has been demonstrated that olfactory sensitivity in the antennae is regulated by a circadian rhythm, where the minimum olfactory sensitivity are present at the time of maximum food searching behaviors in both animals (Krishnan, Dryer, and Hardin 1999), (Page and Koelling 2003). In addition, several olfactory-driven insect behaviors exhibit circadian rhythms, suggesting that the circadian oscillation of olfactory systems at the level of olfactory sensitivity in the antennae may have an effect on the regulation of olfactory behavior (Marianna I. Zhukovskaya 1995), (Kawada and Takagi 2004), (Rymer et al. 2007). However, the molecular and neural mechanism underlying the regulation of peripheral olfactory sensitivity by the circadian system is not yet understood.

Available evidence suggests that modulation of olfactory sensitivity is controlled by neuropeptides and biogenic amines (Leinwand and Chalasani 2011),

(Carlsson et al. 2010), (Root et al. 2011). Among them, octopamine (OA) is known as a neurotransmitter, a neuromodulator, and a neurohormone in insects (Roeder 1999). In particular, OA is released into the antennal heart of the cockroach (P and H 1992), and the injection of OA can either decrease (Marianna I. Zhukovskaya 2008) or enhance (Vander Meer, Preston, and Hefetz 2008) olfactory responses depending on the insect species. While the molecular mechanisms underlying the OA in olfactory modulation are not known, it seems to have several roles on the regulation. In addition to OA, it is likely that certain neuropeptides are also employed as neuromodulators in insect olfactory systems. One neuropeptide of particular interest is tachykinin (TK) (Pennefather et al. 2004). Several isoforms of TK peptides have been shown to be expressed in the central and peripheral nervous systems (Predel et al. 2005),(Takeuchi et al. 2004) (Meola et al. 1998). A recent study found that flies deficient with tachykinin-related peptides exhibited the decrease of sensitivity for odor perception, implicating their significant role of neural modulation in olfactory systems (Winther, Acebes, and Ferrús 2006). In addition, it has been reported that *Drosophila* tachykinin receptor (DTKR) expressed in axon terminals of olfactory receptor neurons (ORNs) modulated by TK in local interneurons (LNs) of antennal lobes (ALs) inhibits the olfactory responses of ORNs (Ignell et al. 2009). However, most research has focused on the central nervous system (CNS), and

thus information on how the function of peripheral olfactory systems can be regulated is largely unknown.

In this regard, we have here investigated the functional organization of neuronal circuits governing the alternation of olfactory sensitivity by both OA and TK signaling in the peripheral olfactory organ employing *in situ* hybridization, immunostaining, and quantitative real-time polymease chain reaction (qRT-PCR) methods. We also report that the expression of both octopamine receptor and tachykinin genes is strongly dependent on the time of day. In addition, we report the first findings of the functional characteristics of tachykinin, octopamine, and octopamine receptors on the modulation of olfactory sensitivity in the antennae of *Periplaneta americana*. Our current data demonstrate that molecular machineries and olfactory systems enabling to modulate olfactory sensitivity exist in the peripheral olfactory organ and they are likely to be affected by circadian rhythms and the status of hunger.

Materials and Methods

Insect and tissue preparation

P. americana were raised under a light cycle of 12:12 hours light and a dark (L/D) cycle at 26±1ºC as described (Saifullah and Page 2009). Intact adult male cockroaches were used for qRT-PCR and electrophysiological recordings. Animals with external damages such as missing antennal segments were discarded for tissue preparation. Male cockroaches were anesthetized by chilling on ice before dissection after which antennae were immediately removed on dry ice. Collected antennal tissues were further treated depending on different experimental procedure described below.

Gene Cloning of octopamine receptor, tachykinin, and tachykinin receptor

Total RNA was extracted from three male *P. americana* antennae and brains using the Qiagen RNasy kit by manufacturer's instruction (Qiagen, Valencia, CA, USA), after which RQ1 RNase-free DNase I (Promega, Madison, WI, USA) was treated by manufacturer's instruction. Reverse transcription procedures were carried out as described previously (H.-W. Kwon et al. 2006). Information on each gene and and primer sets is provided in Table S2. TKR genes of *L. maderae* (Johard et al.

2001) (CAC36957.1), *Tribolium castaneum* (XP_970102.1), *Anopheles gambiae* (XP_312088.3), *Apis mellifera* (XP_395081), and *D. melanogaster* (AAA28722.1) were used for multiple alignment for conserved domain of the transmembrane protein regions (Fig. S1).

Quantitative real-time PCR (qRT-PCR)

A male cockroach after emergence from the last nymphal stage was isolated from a colony and kept in a plastic cage in which each animal was provided with food and water *ad lib*. Seven days after isolation, two groups of three male cockroaches were collected on 1 h before light-on (CT23) and 1 h before light-off (CT11). cDNA synthesis was conducted as described above. qRT-PCR was carried out with the StepOnePlus (Applied Biosystems, Foster City, CA, USA) using SYBR green qPCR Master Mix (Fermentas, Ontario, Canada). Primer information for qPT-PCR was described in Table S1. Quantitative analysis was employed by StepOne plus Software V. 2.0 (Applied Biosystems). Results were normalized to a validated control gene, *actin*, using the ΔΔCt method (Pfaffl 2001). pQE30 vector sequence were used as a control.

In situ Hybridization, immunostaining, and imaging

RNA probes of *Pa*OA1 and *Pa*TKR for *in situ* hybridization were prepared with DNA clones obtained described in Table S1 by the procedures as described previously (H.-W. Kwon et al. 2006). Briefly, signals were visualized by peroxidase (POD) coupled to anti-DIG antibodies (Roche, Indianapolis, IN), followed by the visualization by a tyramid signal amplification (TSA) kit by manufacturer's instruction (PerkinElmer, Waltham, MA, USA). Alternatively, anti-tachykinin antibody against *Locusta migratoria* tachykinin (Schoofs, Broeck, and Loof 1993) (gifted from Dr. Yoon-Seong Park at Kansas State University), were diluted 1:2000 in PBS with 0.1% Tween 20 and incubated at 4^{o}C overnight. Additionally, anti-DmOrco antibody (gifted from Dr. Leslie Vosshall at Rockefeller University) was used to localize Orco of *P. americana*. Cell and neuronal staining was conducted by TOTO-3 (Invitrogen) and anti-horseradish peroxidase (HRP) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. Stained preparations were embedded in Spurr's epoxy resin (H.-W. Kwon et al. 2006) and images were captured by a LSM 510 confocal microscope (Zeiss, Thornwood, NY).

Injection of tachykinins, dsRNAs, and octopamine agonist and antagonist

Fifteen tachykinin peptides identified in the cockroach, *P. americana* (*Pa*TKs) (Predel et al. 2005) were synthesized with 95% purity (Anygen, Gwang-Ju, Korea) (Table S2). dsRNAs for *Pa*OA1, *Pa*TK, *Pa*TKR genes as well as pQE30 as a control gene were generated using MEGAscript RNAi kit (Ambion) with specific primer sets (Table S1). For the injection of tachykinins and dsRNAs, 3 μl of each active tachykinin with 60 nmol and 0.5 μl of synthesized dsRNA (5 μg/μl) were injected into the antennal base of intact adult male cockroaches caught at CT11 using a Hamilton micro syringe, needle G30 (Becton Dickinson). Injected cockroaches were kept individually. Control cockroaches were injected with saline (PBS. pH=7.4). 2 μl of octopamine agonist (clonidine) and antagonist (yohimbine) (Sigma-Aldrich) were injected at the concentration of 1M into the same areas of cockroach antennae described above.

Electroantennogram (EAG)

A male cockroach was restrained with one antenna fixed in an EAG setup, as previously described (Page and Koelling 2003). The electrical signals in response to ethyl acetate was conveyed to an amplifier (IDAC4, Syntech, The Netherlands) and processed with EAGPro software (Syntech). Prior to injection of tachykinin

and dsRNA, three to five pre-recordings with 5 min intervals for olfactory responses to ethyl acetate (1 sec stimulation), diluted to 10^{-2} (vol/vol) with mineral oil (Sigma-Aldrich), were measured. Five min after injection of tachykinin and dsRNA, 13 to 20 consecutive post-recordings were made to ethyl acetate delivered every 5 min. EAG amplitudes were normalized by dividing a mean amplitude value of pre-recording (before injection) from each olfactory amplitude during post-recordings (after injection). EAG recordings were conducted at CT5~6.

Single sensillum recording (SSR)

Olfactory responses using SSR was measured from single-walled type C sensilla by inserting the tip of glass electrode into sensillum basement. Odorant stimulation was the same as the EAG recording setup described above. Olfactory responses were measured by the number of spikes (action potentials) elicited by ethyl acetate stimulation by deducting spike numbers during 1 sec post-stimulation from 1-sec pre-stimulation. Three to five pre-recording prior to injection and 13 to 21 consecutive post-recording were employed (N=12).

Data analysis

Gene expression data were analyzed by Student’s t-test (SPSS, Version 20, IBM,

NY, USA). Comparisons of EAG and SSR responses between pre-injection and post-injection were analyzed using a one-way ANOVA test followed by Bonferroni correction with multiple comparisons (SPSS, Version 20, IBM, NY, USA). Time frames were divided as a group before and after injection during EAG and SSR. Statistical analysis was conducted among these groups.

Results

Cloning of octopamine receptor, tachykinin, and tachykinin receptor of *P. americana*

P. americana genome information have been reported to contain genes encoding octopamine receptor (*Pa*OA1) and TK (*Pa*TK) neuropeptides (Predel et al. 2005),(Bischof and Enan 2004). We subcloned these genes using gene-specific primer sets as shown in Table S1. In contrast, tachykinin receptor gene of *P. americana* (*Pa*TKR) was not available in GenBank. For this reason, the conserved regions of TKR protein sequences from several different species of insects were aligned, especially in transmembrane regions 6 and 7 (TM6 and TM7) (Fig. 1A). Specific primer sets for TKR gene were generated from partial mRNA (192bp) of *Pa*TKR as a novel gene (Table S1). The cloning of the full coding sequence of PaTKR gene remains to be accomplished.

Localization of *PaOA1*, PaTK, and *PaTKR* genes in antennae

PaOA1, PaTK and PaTKR in cockroach antennae were localized by using double-labeling studies with in situ hybridization and immunostaining (Fig. 1). First, PaOA1 mRNA were localized in the antennal neurons (Fig. 1B) compared to control (Fig. 1D). Neuronal clusters consisting of 5~6 neurons that expressed PaOA1 mRNA were found in each antennal segment near the area of antennal

nerves (arrowheads in Fig. 1B and C) where PaOA1 and PaTK (arrows in Fig. 1C) were co-localized in the same neurons of the antennae (double arrowheads in Fig. 1C). Immunohistochemistry using tachykinin antibody in the brain of the cockroach showed that tachykinin-positive neurons are located in the vicinity of antennal lobe (AL) and mushroom body (MB) calyces (Figure 1G). Double-label studies using an anti-sense RNA probe of the PaTKR and an antiserum to the D. melanogaster odorant receptor co-receptor (DmOrco) overlapped in ORNs of the antennae (Fig. 1E) when compared to control (Fig. 1F). DmOrco gene is well conserved among different insect species and forms heterodimers with ligand-specific conventional odorant receptors (ORs) in the ORNs, as reported previously (Jones et al. 2005). Intriguingly, our results demonstrated that most ORNs (arrowheads in Fig. 1E) also expressed the TKRs (arrows and double arrowheads in Fig. 1E).

Effects of tachykinin (TK) on olfactory sensitivity in antennae

In order to better understand the functional roles of TK on olfactory responses in antennae, 15 synthesized active TK peptides were used to identify the effects on olfactory responses in the antennae (see Fig. 2 legend). Among them, TK-2, TK-7 and TK-12 peptides represented to decrease the olfactory responses after TK application (Fig. 2, $p<0.05$), while no detectable differences from control saline and other TKs were found (Fig. 2 and Fig. 3A, $p > 0.6$). TK-7 injection

elicited the strongest decrease in EAG responses (Fig. 2 and Fig. 3B and G, $p < 0.05$). Notably, a significant decrease in EAG responses was observed at 25 min and the greatest reduction in EAG amplitude was shown 45 min after TK-7 injections (Phase II in Fig. 3B). At this point there was approximately a 30% reduction in amplitude after TK-7 application (Fig. 3B). Furthermore, recording from single trichoid sensilla after TK-7 injection also showed a decrease in the number of action potentials compared to saline injection as control (Fig. 4A, B, and G).

Alternation of olfactory sensitivity by dsRNA

dsRNA of each gene demonstrated that the transcript levels of both PaOA1 and PaTK mRNAs were prominently reduced for 2 hours after injection (Fig. 3H and I, $p < 0.05$). The RNAi effect was noticeable during a 12-hour period after dsRNA injection. dsRNA injections of PaOA1, PaTK, and PaTKR significantly increased EAG amplitudes about 50 minutes after injection (Phase III and IV), compared to “before injection” (Fig. 3D, E, and F, $p < 0.01$, ANOVA with Bonferroni correction test). Olfactory responses in single sensillum levels were also affected by dsRNA injection (Fig. 4). The number of action potentials was slightly increased after injection of PaOA1 dsRNA (Fig. 4D, $p < 0.01$). In contrast, although we did not observe a strong increase in the number of spikes overall before and after injection of PaTK dsRNA (Fig. 4E, $p > 0.2$, ANOVA,

Bonferroni correction test), we found significant differences in phase III (9~13th pulse numbers), compared to spike numbers in “before injection” (Fig. 4E, $p < 0.02$). Next, we tried to test whether antennal olfactory sensitivity might be affected by both tachykinin and octopamine. Obviously, the injection of octopamine alone showed the decrease of olfactory responses (inverted triangles in Fig. 3E, N=6, $p<0.01$). With co-injection of PaTK dsRNA and octopamine, we observed no significant increase or decrease in olfactory responses, compared to “octopamine alone” and “PaTK dsRNA alone” (empty circles in Fig. 3E). However, EAG responses after co-injection were significantly different, compared to “before injection” (N=4, $p<0.01$), indicating that octopamine concentration used in this experiment, 5 μl of 10mM octopamine, were rather high to compensate the effect of PaTK dsRNA. Spike numbers upon the dsRNA injection of PaTKR did not show significant differences throughout pulse numbers (Fig. 4F, $p > 0.7$). Spike frequency in response to ethyl acetate stimulation was significantly decreased by clonidine treatment (an octopamine agonist) (Fig. 5A, $p < 0.001$), whereas yohimbine (an octopamine receptor antagonist) elevated the olfactory responses after 6th to 9th odor pulses (phase II-b in Fig. 5B, $p < 0.001$). Other pulses showed no significantly differences. * indicates significant difference of the group compared to before injection.

Discussion

A variety of studies have examined the effects of octopamine on olfactory responses in insect antennae. Consistent with our findings here, EAG amplitude has been shown to be decreased by treatment with exogenous octopamine in *P. Americana* (Marianna I Zhukovskaya 2012), (M I Zhukovskaya and Kapitsky 2006). In contrast, while we found a decrease in action potentials in response to ethyl acetate stimulation, Zhukovskaya and Kapitsky (2006) reported an increase in nerve impulse responses recorded in single sensilla responses to sex pheromone following octopamine injection. Similarly, octopamine was reported to increase the response of pheromone sensitive ORNs in the hawk moth, *Manduca sexta* (Flecke and Stengl 2009), the silk moth, *Antheraea polyphemus* (Pophof 2000), and the cabbage moth, *Mamestra brassicae* (Grosmaitre et al. 2001). These findings raise the possibility that octopamine may differentially impact the response of sensilla tuned to respond to sex pheromone and those that respond to at least some other odors (e.g., ethyl acetate) and dominate the EAG response. This view is consistent with recent observations in P. americana that exogenous octopamine does have differential effects on both the behavioral (Marianna I. Zhukovskaya 2008) and olfactory receptor neuron responses (Marianna I Zhukovskaya 2012).

In the present study we also find evidence that tachykinin may also play a major role in olfactory modulation. We showed that tachykinin (TK) neuropeptides co-localized with octopamine receptors in antennal neurons in *P. americana*, and may act via tachykinin receptors (TKR) present in ORNs and may act to regulate olfactory sensitivity. These neurotransmitters have previously been found to act as neuromodulators in the visual system by altering the amplitude of responses to light (Lim-Kessler et al. 2008). Substance P, which is a member of TK peptide family in mammals, and octopamine (OA) efferent systems act in concert to increase electroretinogram (ERG) response and regulate the circadian rhythm in sensitivity in visual system of the horseshoe crab, Limulus polyphemus (Bolbecker et al. 2009). However, the chemical and electrophysiological studies of interactions between OA and substance P are largely unknown. Evidence for OA modulation in the moth *Bombyx mori* demonstrated that OARs were located somewhere near the base of sensilla hairs (Von Nickisch-Rosenegk et al. 1996), implying that OA plays an important role in fine tuning of the olfactory responses (Marianna I Zhukovskaya 2012). Interestingly, cockroach antennae have shown that OA is located in the antennal heart of a neurohemal area, which is secreted from octopaminergic dorsal unpaired median (DUM) neurons in *P. americana*.

In this regard, our study is the first to demonstrate the fact that OA- and TK-related signaling pathways affect the olfactory regulation of peripheral organs.

Co-localization of TK and OARs has suggested the possibility that OA regulates the production or release of TK, which induces the reduction of olfactory responses in ORNs. A previous study has demonstrated that TK-deficient flies exhibit defects in olfactory perception and behaviors responding to specific odorants (Winther, Acebes, and Ferrús 2006). Moreover, Drosophila tachykinins (DTKs) present in LNs of the ALs, a primary olfactory processing center in insect brains (Seki et al. 2010), exert a presynaptic inhibitory action to ORNs (Ignell et al. 2009). This neuronal circuit may regulate olfactory sensitivity to ORNs from AL neurons, modulating innate food searching behaviors under internal and external cues(Hewes and Taghert 2001). Given the importance of peripheral olfactory modulation by neuropeptides, food searching behaviors are also controlled by regulating the insulin signaling in ORNs (Root et al. 2011). It has also been reported that tachykinin receptors and short neuropeptide F receptors (sNPFR) are affected by starvation via insulin signaling in Drosophila ORNs (Root et al. 2011), (Birse et al. 2011). These studies also imply that low olfactory responses at a day time enhance the food searching behavior in fruit flies (Krishnan, Dryer, and Hardin 1999). Likewise, lower olfactory sensitivity has been demonstrated at dusk compared to day time and dawn in a cockroach at which cockroaches habitually show strong food preference (Page and Koelling 2003), thus they may share similar molecular and neural mechanisms modulating

olfactory sensitivity. Similar peptidergic pathways for the modulation of olfactory sensitivity also exist in vertebrate systems. In Mexican salamander (Ambystoma mexicanum), centrifugal peptidergic neurons modulate olfactory responses associated with hunger (Mousley et al. 2006). Moreover, the tetrapeptide Phe-Met-Arg-Phe-NH2 (FMRFamide) has shown to modulate the neural activities of ORNs in the olfactory epithelium of the mouse and the salamander (Ni et al. 2008), (Daesik Park, Zawacki, and Eisthen 2003). Recently, several studies has shed light on the olfactory modulation in ORNs in peripheral sites, where acetylcholine is released by micovillar cells of the olfactory epithelium (Ogura et al. 2015) and dopamine is released into the olfactory mucus triggered by exposure to irritants (Lucero and Squires 1998). Another study has found the hormone, leptin and its receptors in olfactory mucosa (Baly et al. 2007). Neuropeptide Y (NPY), associated with the regulation of satiety signal of hypothalamus in vertebrates, has also been shown to modulate olfactory responses to a food related odorant in rat olfactory mucosa by using electroolfactogram (EOG), where NPY application increase EOG amplitudes in starved rats but not in fed ones (Salesse et al. 2012). Unlike peripheral modulation, there are ample reports on olfactory sensitivity changes by central mechanisms focused on CNS (Ignell et al. 2009), (Chalasani et al. 2010), (Kawai et al. 2015). Our results have demonstrated that levels of TK regulate the olfactory responses in ORNs of the peripheral olfactory

organs. The results suggest that the reduction of olfactory responses by TK reflects either a decrease of the number of responding neurons or efficiency of olfactory signal transduction in ORNs. However, the mechanism by which TK alters olfactory sensitivity in ORNs remains unclear. Further experiments are necessary to demonstrate which olfactory signal transduction components are involved in TK-related signaling in ORNs. Our study has also demonstrated that TK-mediated neural circuits exist in antennal olfactory systems. Although neural circuits associated with sensory facilitation by peptidergic presynaptic modulation has been reported in the ALs of the brain (Ignell et al. 2009), our results indicate that TK may have an important role on the fine tuning of peripheral olfactory responses in ORNs, which in turn may subsequently facilitate olfactory perception and recognition. One potential hypothesis is that the release of octopamine into the hemolymph of the antennal heart ultimately activates receptors in antennal neurons which in response release tachykinin. Tachykinin, in turn, could activate its receptors in the ORN to modulate olfactory responses (Fig. 6). A recent study has also shown that the cyclic nucleotides cAMP and cGMP modulate the olfactory sensitivities in ORNs by octopamine and adaptation to pheromone concentration, respectively (Schendzielorz et al. 2012). Thus, it will be interesting to observe whether there are correlations between TKR components and these cyclic nucleotides in ORNs. Taken together, our study indicates that neuronal

networks are present in the peripheral olfactory organ in the cockroach, which is regulated by neuropeptides and biogenic amine systems.

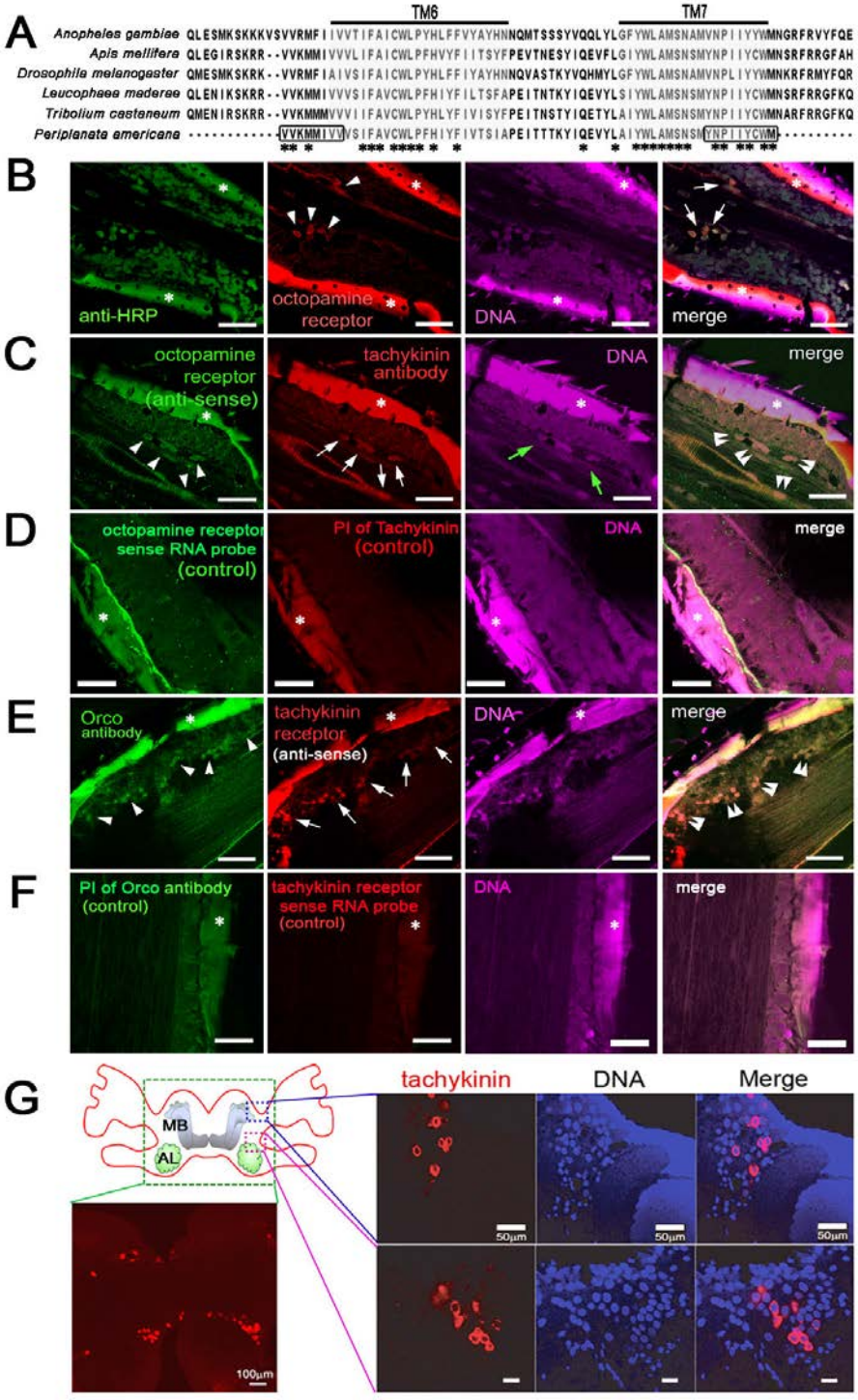

A
TM6
TM7
Anopheles gambiae
Apis mellifera
Drosophila melanogaster
Leucophaea maderae
Tribolium castaneum
Periplaneta americana
B
anti-HRP
octopamine receptor
DNA
merge
C
octopamine receptor (anti-sense)
tachykinin antibody
DNA
merge
D
octopamine receptor sense RNA probe (control)
PI of Tachykinin (control)
DNA
merge
E
Orco antibody
tachykinin receptor (anti-sense)
DNA
merge
F
PI of Orco antibody (control)
tachykinin receptor sense RNA probe (control)
DNA
merge
G
MB
AL
100μm
tachykinin
DNA
Merge
50μm

Figure 1. Localization of octopamine receptors, tachykinin, and tachykinin receptors in the antennae of *P. americana*. (A) Partial amino acid sequence of tachykinin receptors of *Periplaneta americana* (PaTKR) and its alignment with other insect tachykinin receptors present in mosquito (*Aedes aegypti*), honey bee (*Apis mellifera*), fruit fly (*Drosophila melanogaster*), red flour beetle (*Tribolium castaneum*), and Madeira cockroach (*Leucophaea maderae*). Transmembrane regions 6 and 7 are shaded in gray. Amino acid residues selected for gene cloning were indicated by boxes. (B) *In situ* hybridization with antisense RNA probes of octopamine receptor (red), where octopamine receptors (arrowheads) were co-localized with neuronal marker anti-HRP antibody (arrows). (C) Expression of tachykinin (arrows) in octopamine receptor neurons (green, arrowheads) were co-localized (double arrowheads). Green arrows indicate antennal nerves. (D) Control of in situ hybridization using sense RNA probes of octopamine receptor and pre-immune serum of tachykinin. (E) Tachykinin receptor (arrows) of *P. americana* was co-localized (double arrowheads) with odorant receptor co-receptor (Orco, arrowheads) in olfactory sensory neurons of the antennae. (F) Control of *in situ* hybridization using sense RNA probes of tachykinin receptor and pre-immune serum of Orco. Scale bars demonstrate 20 μm. (G) Immunostaining with tachykinin antibody in brain tissue of cockroach. A schematic diagram of the cockroach brain showed mushroom body (MB) and antennal lobes (AL) where tachykinin (red) was mainly localized. Images demonstrate tachykinin staining in the brain area indicated with a dotted box. Cell bodies were stained with DAPI (blue). Scale bars demonstrate 20 μm unless indicated. Asterisks demonstrate the autofluorescence from antennal cuticle.

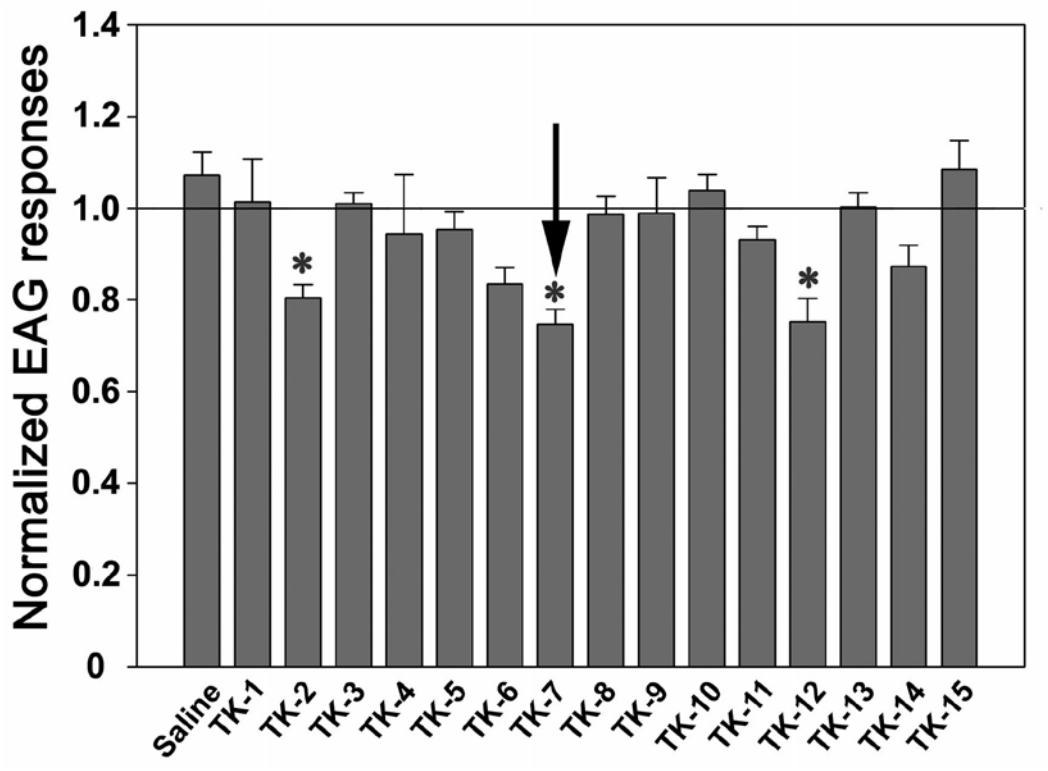


Figure 2. Normalized EAG responses of cockroach antennae to ethyl acetate (10-2 dilution) after injection of tachykinin (TK) peptides. EAG amplitudes were decreased after injection of TK-2, -7, and -12, compared to other TK peptides and control saline injection (N=4-8, ANOVA with Bonferroni correction, *=$p<0.05$). TK-7 (arrow) showed strongest effects on decrease of olfactory responses, which was in turn used for further experiments. Effects of other TKs on EAG responses were not significantly different from saline control injection. Information on synthesized TK peptides was as follows: PaTK1: APSGFLGVR-NH2, PaTK2: APEESPKRAPSGFLGVR-NH2, PaTK3: NGERAPASKKAPSGFLGTR-NH2, PaTK4: APSGFLGTR-NH2, PaTK5: APGSGFMGMRNH2, PaTK6: APAMGFQGVR-NH2, PaTK7: APASGFFGMR-NH2, PaTK8: VPASGFFGMR-NH2, PaTK9: GPSMGFHGMR-NH2, PaTK10: APSLGFQGMR-NH2, PaTK11: APNMGFMGMR-NH2, PaTK12: MGFMGMR-NH2, PaTK13: GPSVGFFAMR-NH2, PaTK14:

APSAGFMGMR-NH2, PaTK15: APSAGFHGMR-NH2.

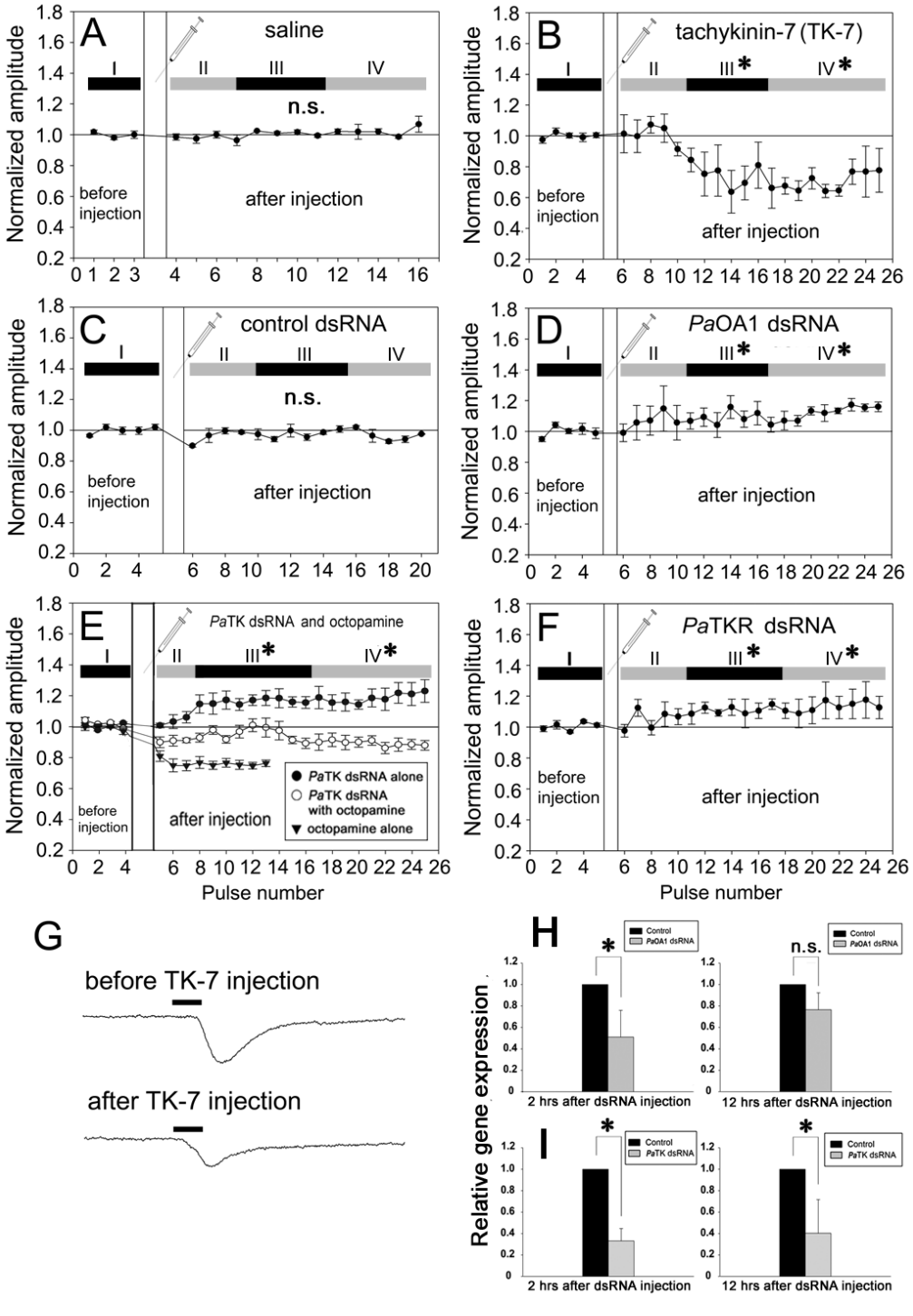
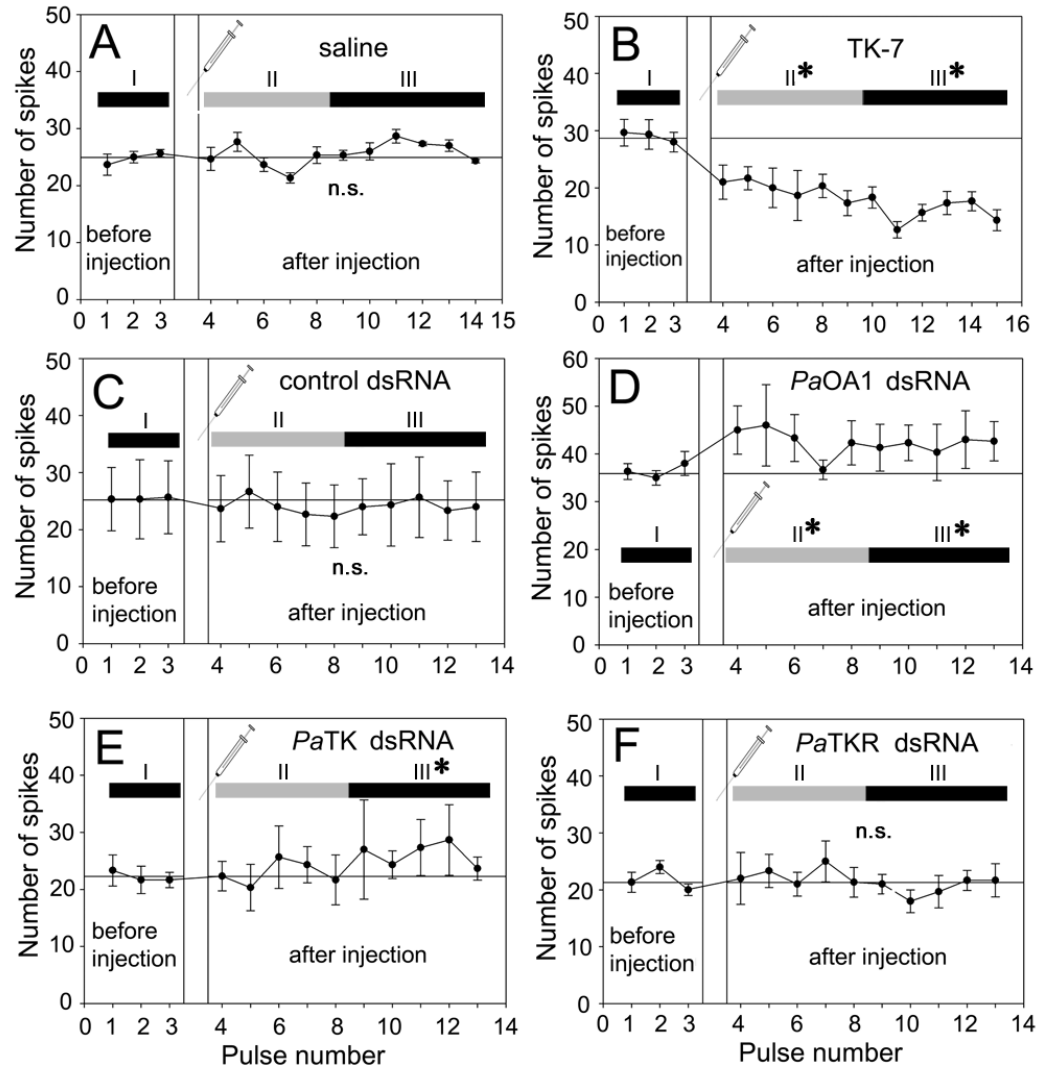

A
saline
n.s.
B
tachykinin-7 (TK-7)
C
control dsRNA
n.s.
D
PaOA1 dsRNA
E
PaTK dsRNA and octopamine
PaTK dsRNA alone
PaTK dsRNA with octopamine
octopamine alone
F
PaTKR dsRNA
I
II
III*
IV*
before injection
after injection
Normalized amplitude
Pulse number
G
before TK-7 injection
after TK-7 injection
H
Control
PaOA1 dsRNA
2 hrs after dsRNA injection
12 hrs after dsRNA injection
n.s.
I
Control
PaTK dsRNA
Relative gene expression

Figure 3. Effects of “before and after injection” of tachykinin, octopamine receptors, tachykinin receptors on olfactory responses (EAG) in antennae and quantitative real-time PCR after dsRNA injection of tachykinin and octopamine receptor. (A) There was no significant difference before and after a control injection of saline (N=3, $p>0.6$). (B) Tachykinin-7 (TK-7) injection produced a significant decrease in olfactory responses phase II and phase III after injection compared to before injection (phase I) (N=5, *=$p<0.001$). (C) Injection of control dsRNA from an expression vector (pQE30) did not significantly alter EAG responses (N=5, $p>0.7$). (D) Injection of octopamine receptor (PaOA1) dsRNA elicited significant increase in EAG amplitude in Phase III and IV, which continued to over 2 hours after injection (N=5, $p<0.001$). (E) Three EAG traces showed interaction between octopamine and tachykinin on the modulation of olfactory sensitivity in cockroach antennae. Injection of octopamine itself (inverted triangle) decreased EAG response after injection in Phase III and IV, while TK-dsRNA (filled circle) induced significant increase of EAG amplitude (N=5, $p<0.01$). Notably, the enhanced effect of TK-dsRNA on EAG responses was eliminated when co-injected with octopamine (empty circle). (F) Injection of tachykinin receptor (PaTKR) dsRNA had a significant positive effect on EAG amplitude (N=3, $p<0.001$). (G) Representative EAG traces before and after TK-7 injection, showing that TK-7 decreased EAG responses. (H) Relative RNA expression levels of octopamine receptor (PaOA1) in antennae after injection of octopamine receptor dsRNA were strongly reduced at 2 hours after injection but not significantly different 12 hours after injection (Student’s t-test: *, $P\leq0.05$). (I) Relative RNA expression levels of tachykinin gene (PaTK) in antennae showed significant reduction at 2 and 12 hours after injection. Values depict mean ± SE. One-way ANOVA test followed by Bonferroni correction for multiple comparisons was employed to test the difference in EAG responses, while Student t-test was used to test the difference of gene expression levels. Pulse number indicates the number of ethyl acetate stimulation every five minutes. Phase I depicts EAG responses during

“before injection” and phases II, III, and IV indicate EAG responses during “after injection”.


A
saline
I
II
III
n.s.
before injection
after injection
Number of spikes
B
TK-7
II*
III*
C
control dsRNA
D
PaOA1 dsRNA
E
PaTK dsRNA
F
PaTKR dsRNA
Pulse number

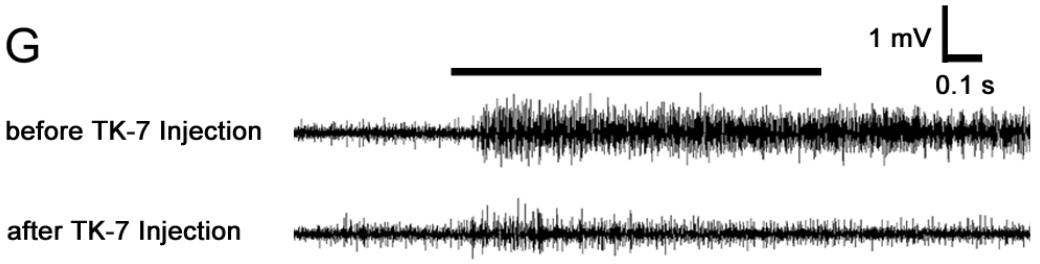

G
1 mV
0.1 s
before TK-7 Injection
after TK-7 Injection

Figure 4. Olfactory responses to ethyl acetate by TK-7 and other dsRNA treatment using single sensillum recording. (A) Saline injection caused no significant changes after injection (N=5, $p>0.5$). (B) Application of TK-7 induced a substantial decrease in the spike activities of olfactory receptor neurons (N=4, $p<0.001$). (C) Control non-specific dsRNA did not alter olfactory responses (N=4, $p>0.2$). (D) Octopamine receptor (PaOA1) dsRNA treatment resulted in significant increase in spike numbers (N=5, $p<0.01$). (E) Tachykinin (PaTK) dsRNA injection exhibited no significant changes in olfactory responses at phase II (4~8th pulses) (N=4, $p>0.9$) but significant increase in phase III (after 9th pulse) (N=4, $p<0.02$). (F) Tachykinin receptor (PaTKR) dsRNA injection did not show significant changes (N=5, $p>0.7$). (G) Representative olfactory response traces by SSR upon TK-7 injection. Significant decrease of spike trains was shown, indicative of decrease of olfactory sensitivity. One-way ANOVA test followed by Bonferroni correction for multiple comparisons was employed for statistical analysis.

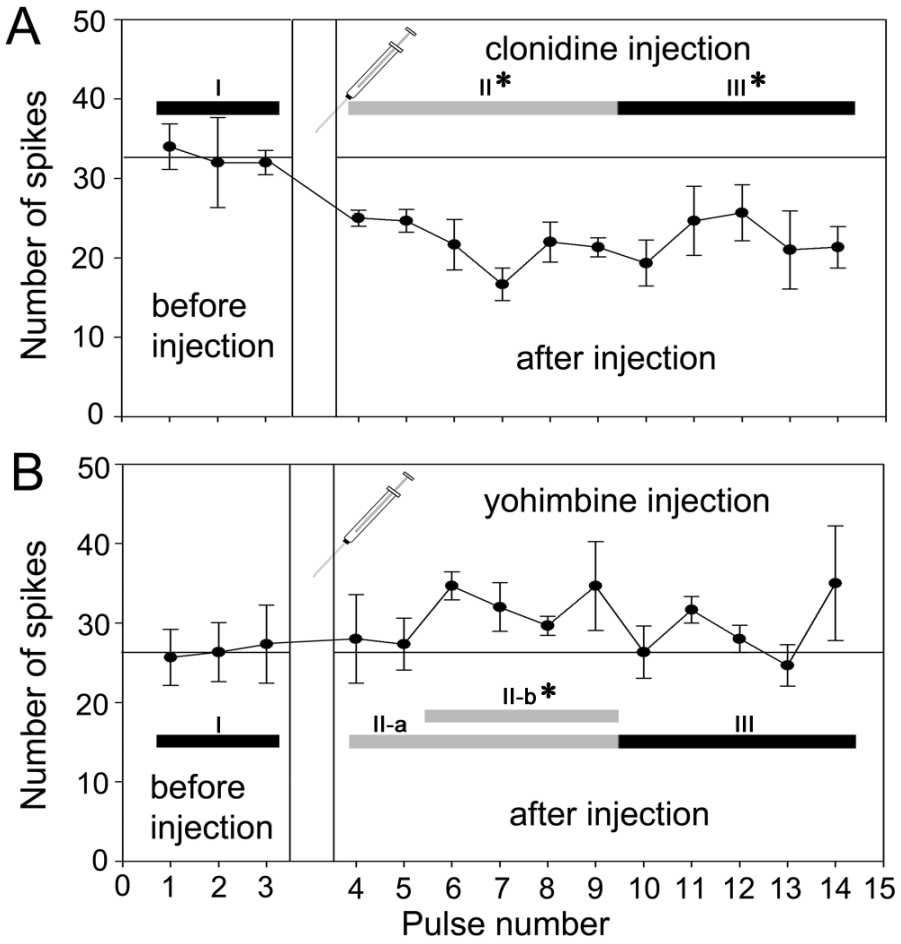


Figure 5. Effect of octopaminergic agonist and antagonist on olfactory responses using single sensillum recordings. (A) Injection of clonidine, an octopamine receptor agonist, into the antennae resulted in a drastic decrease in the number of spikes right after injection until the end of the experiment (Phase II and III), compared with "before injection" (Phase I) (N=5, $p<0.001$). (B) Injection of the octopamine receptor antagonist, yohimbine, induced a slight increase in the middle of time phase (II-b, 6th~9th pulses), compared with "before injection" (N=5, $p<0.001$). One-way ANOVA test followed by Bonferroni correction for multiple comparisons was employed for statistical analysis.

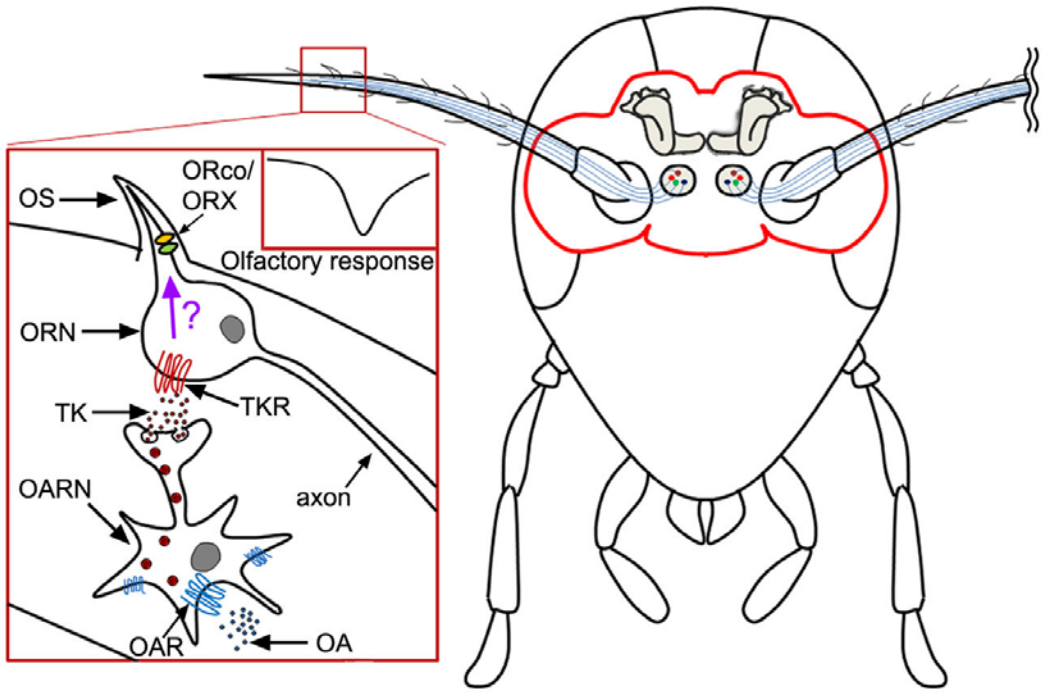


Figure 6. Schematic diagram of olfactory neuromodulation models in the peripheral olfactory organ in the cockroach. Tachykinin is produced from octopamine receptor neurons in antennae. The tachykinin displays the neuro-inhibitory function on olfactory sensitivity changes in the antennal ORNs by unknown mechanisms (arrow with a question mark). OA:Octopamine, OARN: Octopamine receptor neuron, ORN: Odorant receptor neuron, OS: Olfactory sensillum, TK: Tachykinin, TKR: Tachykinin receptor.

Table S1. Gene-specific primers used for qRT-PCR and preparation for RNA probes

Application		GenBank Access Number	Primer information	Product size (bp)
RNA probes	*Pa*OA1	AY333178.1	Forward primer (FP): 5’-CAACAGCTCCAAGAAGTCCAG-3’ Reverse Primer (RP): 5’-GCTGTCCTCTCCTACCGAGTT-3’	754
	*Pa*TK	AY766012.1	FP: 5’-CCCCATCACACAACAAGAGTT-3’ RP: 5’-CCCTCCATCTCTGAGTCCTTT-3’;	615
	*Pa*TKR	novel gene	FP: 5’-GGGTAGTGAAGATGATGATTGTGGTGG-3’ RP: 5’-TCATCCAACAGTAGATGATGGGATTGTAC-3’	192
qRT-PCR	*Pa*OA1	AY333178.1	FP: 5'-CTCTTCTGGCTGGGCTATTG-3' RP: 5'-TCCTTGCTAAAGAGGGCGTA-3'	
	*Pa*TK	AY766012.1	FP: 5'-GCAAGAAGGCACCATCAGC -3' RP: 5'-ATGCCCATAAACCCGGAAC-3'	
	*Pa*TKR	novel gene	FP: 5'- CAAGAGATGGCGAAGCAACAT -3' RP: 5'-CCCATAAACCCGGAAC-3'	
	*Pa*Act	AY116670.1	FP: 5'-GCTATCCAGGCTGTGCTTTC-3' RP: 5'-ACC GGAATCCAGCACAATAC-3'	

Table S2. Active forms of tachykinin peptides in *Periplaneta Americana*

Periplaneta americana (Pa)	
tachykinin (TK) peptide	Peptide sequence
PaTK1	APSGFLGVR-NH2
PaTK2	APEESPKRAPSGFLGVR-NH2
PaTK3	NGERAPASKKAPSGFLGTR-NH2
PaTK4	APSGFLGTR-NH2
PaTK5	APGSGFMGMR-NH2
PaTK6	APAMGFQGVR-NH2
PaTK7	APASGFFGMR-NH2
PaTK8	VPASGFFGMR-NH2
PaTK9	GPSMGFHGMR-NH2
PaTK10	APSLGFQGMR-NH2
PaTK11	APNMGFMGMR-NH2
PaTK12	MGFMGMR-NH2
PaTK13	GPSVGFFAMR-NH2
PaTK14	APSAGFMGMR-NH2
PaTK15	APSAGFHGMR-NH2

Chapter II

Structural and functional differences in the antennal olfactory system of worker honey bees of *Apis mellifera* and *Apis cerana*

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Abstract

Olfactory cues are important sensory modalities on individual discrimination, perception, and efficient orientation to food sources in most insects. In honey bees, which is well known as eusocial insects, olfactory cues are mainly used to maintain a colony. Although much research has been reported on olfactory systems in honey bee olfaction, little is known about the differences between two major honey bee species, the European honey bee *Apis mellifera* and the Asian honey bee *Apis cerana*. In order to understand the differences of olfactory characteristics in the two species, we compared distribution of sensory hairs on the antennae and antennal olfactory responses, using electron microscopy, electrophysiological recording and molecular expression level of odorant receptors. Our present study demonstrated that the antennae of *A. cerana* have more olfactory sensilla than *A. mellifera*, responding more strongly to various floral volatile compounds. At the molecular level, olfactory co-receptor (Orco), which makes heterodimers with other conventional olfactory receptors, is more abundantly expressed in the antenna of *A. cerana* than *A. mellifera*. These findings extend our understanding of the olfactory systems and behavioral responses to various ecological and biological signals in two closely related honey bee species.

Introduction

The complexity of olfactory systems in a given species may rely on its feeding ecology and chemosensory abilities involved in inter- and intra-specific communication and recognition in their environment (Pitts and Zwiebel 2006). The European honey bee, *Apis mellifera* and the Asian honey bee, *Apis cerana* have lived in distinct ecological niches and their social interactions have believed to be successfully evolved in their respective society (F. Ruttner 1988). *A. mellifera* is widely distributed and used in beekeeping by producing a large proportion of honey and royal jelly (Jianke et al. 2010), while *A. cerana* is bred locally in Asia including Korea, China, and Japan as well as southeastern Asian countries. Although *A. cerana* provides lower quantity of honey and royal jelly compared to *A. mellifera*, this species has shown strong resistant to diseases, wasps, and bee mites (Peng et al. 1987),(Su et al. 2005). Despite the similarity in morphological characteristics of both two species, no hybrids were observed even when colonies of both types were kept in a same palce, implying that the two bee species had allopatric speciation in evolution (Friedrich Ruttner and Maul 1983). Recent studies of the two species have shown that geographical isolation and evolutionary divergence occurred by representing between the two species in biological differences such as their ecosystems, chemosensation, and

morphological structures (Büchler, Drescher, and Tornier 1992).

In social insects like honey bees, chemical communication plays a critical role in colony maintenance, development, behavioral regulation, and caste transition, as well as defense behaviors (Crespi and Yanega 1995),(Denison and Raymond-Delpech 2008),(Sandoz 2011). For instance, hygienic behaviors are most important in social immune behaviors where adult bees detect and remove diseased broods in their colony mainly using olfactory sensation from the antennae (Rothenbuhler 1964),(Wilson-Rich et al. 2009). Moreover, honey bees are sensitive to pheromones that are important olfactory cues involved in colony maintenance including the division of labor (Crespi and Yanega 1995),(Robinson 2002). It has been reported that *A. cerana* is more efficient to remove ectoparasitic mites such as *Varroa jacobsoni* mainly based on olfactory detection abilities, compared to *A. mellifera* (Peng et al. 1987). Despite many differences in olfactory-driven behaviors such as anti-parasite and hygienic behaviors as well as food preference between *A. mellifera* and *A. cerana*, little is known about the structural and functional differences on olfactory perception in regards to their specific species.

The antenna, a main peripheral olfactory organ of the honey bee, is equipped with numerous olfactory or non-olfactory sensilla by bees can detect many different sensory modalities (Galizia and Szyszka 2008). In order to

understand the structural and functional differences of olfactory systems in two honey bee species, here we mainly centered our aims on the characterizing distribution patterns of olfactory sensilla, olfactory responses to floral odorants, and the expression level of Orco in antennae by employing electron microscopic observation, electrophysiological approaches, and western blot analysis. This comparative study on olfactory systems of antennae of two major honey bee species may provide better understanding of differences in olfactory functions and behaviors in the future.

Materials and methods

Insect preparation

Two honey bee species, *A. mellifera* and *A. cerana*, were maintained on apiaries of Seoul National University campus surrounded by the Gwanak mountains in Seoul, Korea. The apiaries of each species were geographically segregated. For experiments pollen-foragers of both species were captured directly at the entrance of three hives.

Observation of antennal sensilla using SEM

Collected bees were placed in the freezer for 1 hr, after which they were decapitated and antennae were cut at their base. The antennae were then cleaned for 1 hour in PBS solution and followed by dehydration through a graded ethanol series of 25, 50, 70, 90, 100 % for 10 min each. After drying in the oven at 40^{o}C for 24 hours, the scape of the antennae was attached to double side sticky tape (3M Korea, Seoul, Korea). All samples were coated with a thin layer of gold on all sides and each antenna was fixed on a holder in the sample chamber. The samples were scanned with a SUPRA 55VP, Field-Emission Scanning Electron Microscope (Carl Zeiss, Germany). Antennal images were captured from the anterior and posterior side. We scanned only flagellar segments 3 to 10, because

there are no olfactory receptors on the first and second segments of honey bee flagellum (Frasnelli et al. 2010). Each segment from 3rd to 10th was scanned longitudinally at a 500X magnification. The orientation was determined by the direction in the honey been head, which was also identical when antennae samples were placed on the holder for SEM observation (Fig. 5A, and B). Both anterior and posterior antennal sides were thoroughly observed to eliminate any biases. Olfactory sensilla, which were S. trichodea type A , B (Fig. 1A and B), S. placodea (Fig. 1C), and S. basiconica (Fig. 1D), and non-olfactory sensilla, which were S. campaniformia (Fig. 1E), S. coeloconica (Fig. 1F), S. ampullacea (Fig. 1G) and S. chaetica (Fig. 1H), were identified according to their specific morphological characteristics as described in Frasnelli et al. Each type of sensilla was counted from all selected images by using image analysis tool in Adobe Photoshop (CS5).

Surface areas and densities

Flagella 3-10 were viewed using bright field optics at 500X magnification. The length of each species flagella was measured with an adobe photoshops digital scale bar, and the width was measured at the middle of each flagellum length. The average values of these measurements were calculated for 6

individuals per species. The surface areas (length × diameter × pi) of each flagellum were also calculated. Sensilla densities were estimated by the mean number of sensilla divided by the total mean surface area.

Electroantennogram (EAG)

EAG responses were amplified and recorded from left antennae of *A. cerana* and *A. mellifera* foragers with IDAC4 with EAGPro software (Syntech, Hilversum, The Netherlands). Bees were immobilized by cooling and then restrained in a pipette tip, holding head and appendages in place for electrophysiological measurements (Fig. 6). The first two segments of the antenna were fixed with wax to hold their flagellum and the very tip of 10^{th} antennal segment was carefully removed, after which a recording electrode was immediately placed over the 10^{th} antennal segment. The reference electrodewas placed into the base of antenna. Electrodes were filled with a glass micropipette filled with KCl (0.1M). The test odorant compounds were selected based on floral scents as reported previously (Farina et al. 2007). Benzaldehyde, linalool, nerol, α-pinene, geranylacetate, ethyl acetate, 1-hexanol, citral, α-terpineol, geraniol, myrcene, β-caryphyllene, farnesene, benzylacetate, and methyl salicylate were purchased from Sigma-Aldrich (>98% purity, Milwaukee, USA). For each compound, 1 ml of a diluted odorant in mineral oil (10^{-2}, vol/vol) were absorbed

onto a filter paper (1 cm^2 diameter, Hyundae, Seoul, Korea), which was then inserted into individual Pasteur pipettes. Each odorant was delivered by 1 s air pulse through constant air flow (40 ml/s) to the antennae of a honey bee, which was automatically controlled by a stimulus controller (CS-55, Syntech, Hilversum, The Netherlands). Mineral oil was used for control. An interval of at least 60 seconds was allowed between odorant stimulation.

Western blot

Rabbit polyclonal antibodies against Am/Ac OR2 (AmOrco) were generated by the Ab Frontier company (Seoul, Korea). Based on peptide information of AmOrco and AcOrco (GenBank: AmOrco: XP_001121145.1; AcOrco: AEN71859.1) and the sequence alignment of these two peptides (Fig. S1), a target epitope region was chosen as NH_2-TKKQEMLVRSAIKYWVER-C to produce polyclonal anti-Am/Ac Orco antibody. Rabbits were immunized three times with 0.5mg of the synthesized Am/Ac Orco peptide, after which serum-specific antibody was affinity-purified on columns using immobilized antigen peptides.

For Western blotting, honey bee antennae (n=15) in each sample were homogenized in 1 ml of ice-cold 0.1M Tris-HCl buffer (pH=7.8 with 0.5% Triton X-100 and 1μl protein inhibitor cocktail). The homogenized mixture was

centrifuged at 12,000g for 10 min at 4℃. The supernatant was filtered through glass wool (Sigma, St. Louis, USA) to remove excess lipids and used for biochemical assays. Electrophoresis was carried out with a vertical electrophoresis unit (Novex mini cell, Invitrogen). Protein preparation from the antennae of two species was seperated by SDS-PAGE (10%) in triplicate at 130V for 100min in a chamber. Proteins separated on the gel were transferred onto a nitrocellulose membrane (Whatman, USA) by electroblotting. After blocking in PBS buffer containing 0.1% Tween-20 and 5% fat-free dry milk for 1 hour at room temperature, the membrane sheets were incubated for overnight at 4^{o}C with primary Am/Ac Orco antibody. The membrane was then incubated with the horseradish peroxidase conjugated with anti-rabbit IgG secondary antibody for 3 hours. The antigen-antibody complex was visualized using a chemiluminescence kit according to manufacturer's instruction (Young In Frontier Co., Seoul, Korea).

Data analysis

All statistics were performed using SPSS version 17.0 (USA) statistical software package version 17.0. Data expressed as mean±standard errors (SE) for each experiment were analyzed by analysis of variance (ANOVA) with antenna, type and segment of sensilla. ANOVA with Duncan's Test was employed to

compare the differences of membrane potential between two groups (*A. cerana* and *A. mellifera*). Student's *t*-test was also used to compare means among the relative protein expression level.

Results

Differences of Sensilla Number and Density in Antennae

Prior to characterizing the abundance of olfactory and non-olfactory sensilla on antennae from two honey bee species, we investigated antennal length and surface area. It turned out that the length and surface area of antennae had no significant differences between *A. cerana* and *A. mellifera* (Table 1). The overall number of olfactory sensilla was significantly higher in the antennae of *A. cerana* compared to *A. mellifera* (Fig. 2A, $p<0.01$). For non-olfactory sensilla such as S. campaniformia, S. coeloconica, S. ampullaca, and S. chaetica, *A. mellifera* antennae had more non-olfactory sensilla than *A. cerana* (Fig. 2B, $P<0.05$). This demonstrated that the olfactory sensilla in *A. cerana* seemed to be more abundant than *A. mellifera*. S.placodea demonstrated highest density, whereas S.basiconica showed lowest density in the antennae of both honey bee species.

Number of Olfactory Sensilla in Antenna Segment

Next we counted the olfactory sensilla on antennae of two honey bee species. We identified S. placodea, type A and B of S. trichodea, and S. basiconica with SEM images, which were characterized to have olfactory responses (Frasnelli et al. 2010), demonstrating that these sensilla were abundantly distributed on the antennae of *A. cerana* than *A. mellifera* ($P<0.01$, Fig. 3A).

Among these sensilla, S.placodea sensilla were observed with highest level of abundance on the antenna of two honey bee species (Fig. 3A). In constract, non-olfactory sensilla, S. ampullaceum and S. campaniform, were more abundant in *A. mellifera* than *A. cerana* ($p<0.05$). No significant differences were found in the numbers of S. coelocapitula and S. chaetica between the antennae of two species (Fig. 3B, $p>0.1$).

All types of olfactory sensilla were observed in every flagellum between 3rd and 10th segments in both honey bee species. Especially, the distal segment, 10th segment, were observed to have more olfactory sensilla compared to other segments (Fig. 4, $P<0.05$), except for S. placodea that were evenly distributed on each segment of antennae of *A. cerana* and *A. mellifera* (Fig. 4C). The pattern of numbers of S. trichodea type B and S. basiconica was increased from proximal to the distal flagella (Fig. 4B, D). S.trichodea type A demonstrated to be equally distributed in number across the antennal segments in *A. cerana*, whereas this type of sensilla in *Apis mellifera* showed to be distributed in higher number on the distal segment than proximal segments (Fig. 4A). Subsequently, we compared distribution patterns of these sensilla on the anterior and posterior sides of the antennae (Fig. 5A and B). The anterior side of the antennae showed significantly more sensilla than the posterior side (Fig. 5A). The number and distribution patterns of each type of sensilla were similar in both *A. cerana* and *A. mellifera*

(Fig. 5 B-E).

Olfactory Responses Measured by Electroantennogram (EAG)

We measured via electroantennography the summed depolarization potential of the antenna's olfactory receptor neurons after odor presentation in both species. Odorants were grouped into common floral compounds and components of pheromone blends (Riffell, Lei, and Hildebrand 2009). The experimental set up is depicted in Fig. 6A. The response to mineral oil was not significantly different from the response to the air stimulus (data not shown). Robust olfactory responses to different floral odorants were recorded which elicited fast downward voltage changes, in antennae of both species. In general, the olfactory amplitudes from EAG of both species were similar patterns for each odorant, suggesting that the proportion of respective sensory neurons for the 15 substances is similar in the two species (ANOVA, $N=12$, $P<0.05$, Fig. 6B). In both species, the strongest response was for α-terpineol and the weakest response was for α-pinene. EAG responses elicited by 15 tested compounds on the antennae of *A. cerana* were significantly larger than *A. mellifera* (ANOVA, $P<0.05$, Fig. 6B).

Protein Expression of Orco

Based on the olfactory responses measured by EAG, we next investigated the expression level of odorant receptor proteins in antennae of *A. cerana* and *A. mellifera*. The expression level of odorant receptor co-receptor (Orco) was

detected by polyclonal antibodies. Evidently, Orco protein expression levels demonstrated that Orco is more highly expressed in the antenna of *A. cerana* than *A. mellifera* (Fig. 7A, $P<0.05$).

Discussion

A. mellifera and *A. cerana* were thought to be separated from their ancestral line about 1 and 2 million years ago for which the latest time in genus *Apis* (F. Ruttner 1988). *A. cerana*, inhabited in wild conditions, while, *A. mellifera* is by far the most common domesticated honeybee in the world (Goulson 2003). Therefore, *A. cerana* may be exposed to more wild plants and natural enemies than *A. mellifera*, being easily attacked by numerous parasites and pathogens (Joshi et al. 2000). Therefore, this ecological separation throughout evolutionary time may result in different host preference and olfactory sensitivity to certain chemicals such as pheromones and floral scents. The distribution patterns of olfactory and non-olfactory sensilla types on the flagellum of honey bees have been compared previously (Haase et al. 2011), where olfactory sensilla were more distributed in the distal segments of the antennae except 10^{th} segment in *A. mellifera*. Our study scrutinized each type of olfactory sensilla on the antennal segments 3~10 in *A. mellifera* and *A. cerana.* The arrangement pattern of each sensillum type was similar in two species. However, the mean numbers of olfactory and non-olfactory sensilla are significantly different between two species. Based on location and structure, S. chaetica is considered to have a gustatory function, due to their tip pores and their perpendicular orientation with

respect to the antennal cuticle surface (A. T. Whitehead and Larsen 1976), whereas S. campaniformia may have specific thermohydroreception function (DIETZ and HUMPHREYS 1971). S. trichodea type A, B and S. basiconica were considered to have an olfactory function due to the possession of many wall pores (Ågren and Hallberg 1996)(Gupta 1986)(Dweck 2009) The multiporous sensilla placodea are the largest and the most noticeable sensilla type on the forager antennae. Its function is assumed to be olfactory, because they possess a multiple cuticular pore system (Bleeker et al. 2004)(Slifer and Sekhon 1961)(Gao, Luo, and Hammond 2007) Our present study has demonstrated that the higher number of S. placodea on the antennae of the worker *A. cerana* may result from their poreplate density, as the size and surface area of the antennae in the two species appears to be almost identical (Table 1). However, previous observation reported that the number of S. placodea on the drone antenna of *A. mellifera* is 3-fold higher than in *A. cerana*, which might result from an increase of flagellar surface and poreplate density (Woltedji et al. 2012).

In honey bees, the differences of olfactory sensitivity are associated with morphological and electrophysiological properties of antennae (Anfora et al. 2010). The described structural differences in the peripheral system of *A. cerana* and *A. mellifera* foragers may indicate strong differences in odor detection and also in chemical communication in both species. A previous study has

demonstrated that *A. florea* drones have fewer poreplate sensilla than *A. mellifera*, and a much smaller number of olfactory sensory neurons (Brockmann and Brückner 2001). However, it was not examined how antennal sensilla numbers affect the olfactory sensitivity and perception yet. In the present study, olfactory responses by EAG in *A. cerana* have shown to be higher than *A. mellifera*. Our EAG data has demonstrated that 1-hexanol, citral, and α-terpeneol elicited the largest olfactory responses. Among these volatile compounds, 1-hexanol is known to exist in alarm pheromones of honey bees and are highly effective in releasing alarm behaviors (Collins and Blum 1983). Volatile terpenoid compounds seem to occur frequently in flower aromas of the compositae family of the plants (Buttery et al. 1986). These chemicals may be partly involved in flower recognition by bees (Waller, Loper, and Berdel 1974) (Pham-Delegue et al. 1986).

Since EAG records the sum of responses of all olfactory receptor neurons housed in the sensilla on antenna (K.-E. Kaissling 1971), significant differences in olfactory sensitivity between each respective forager of the *A. mellifera* and the *A. cerana* are most likely derived from their respective odorant receptor protein expression levels. Taken together, our present study has shown that *A. cerana* has more olfactory sensilla on the antenna and stronger olfactory responses to floral odorants, compared to *A. mellifera.* Aligned with ongoing genomics analysis on olfactory-related genes in the future such as olfactory receptor repertoires in *A.*

cerana, this study provides an platform for future comparative study on molecular and neural mechanisms underlying olfaction in these two species, which is likely to contribute behavioural preference to nectar sources in nature.

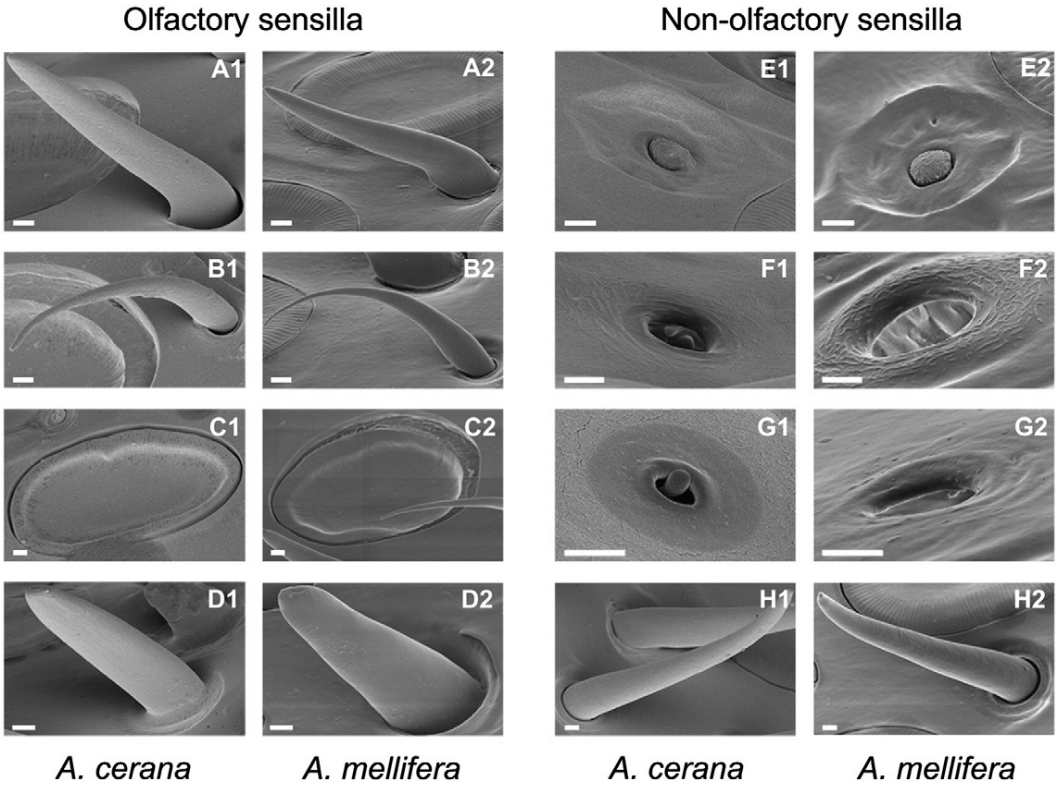


Figure 1. Scanning electron micrographs of sensilla on the antennae of *Apis cerana* (1) and *mellifera* (2). (A1, 2) sensillum trichodeum type A. (B1, 2) Sensillum trichodeum type B. (C1, 2) Sensillum placodeum. (D1, 2) Sensillum basiconicum. (E1, 2) Sensillum campaniformia. (F1, 2) Sensillum coeloconicum, (G1, 2) sensillum ampullaceum. (H1, 2) Sensillum chaetica. Scale bar depicts 1 μm.

Table 1

Characterization of the abundance of olfactory and non-olfactory sensilla on the antennae of two honey bee species, *A. cerana* and *A. mellifera*.

	Apis cerana	*Apis mellifera*	P
Flagellum length	2.65 mm ± 0.016	2.66 mm ± 0.026	n.s.
Flagellum surface	1.38 mm^2 ± 0.017	1.38 mm^2 ± 0.028	n.s
Trichodeum type A density	1697.82 ± 8.05	1435.81 ± 46.60	$P < 0.05$
Trichodeum type B density	1005.58 ± 51.82	722.68 ± 51.42	$P < 0.05$
Placodeum density	1810.87 ± 3.94	1697.10 ± 62.31	$P < 0.05$
Basiconicum density	89.98 ± 1.62	80.62 ± 2.08	$P < 0.05$

Sensilla density above indicates sensilla numbers per mm^2.
n.s.: no difference.

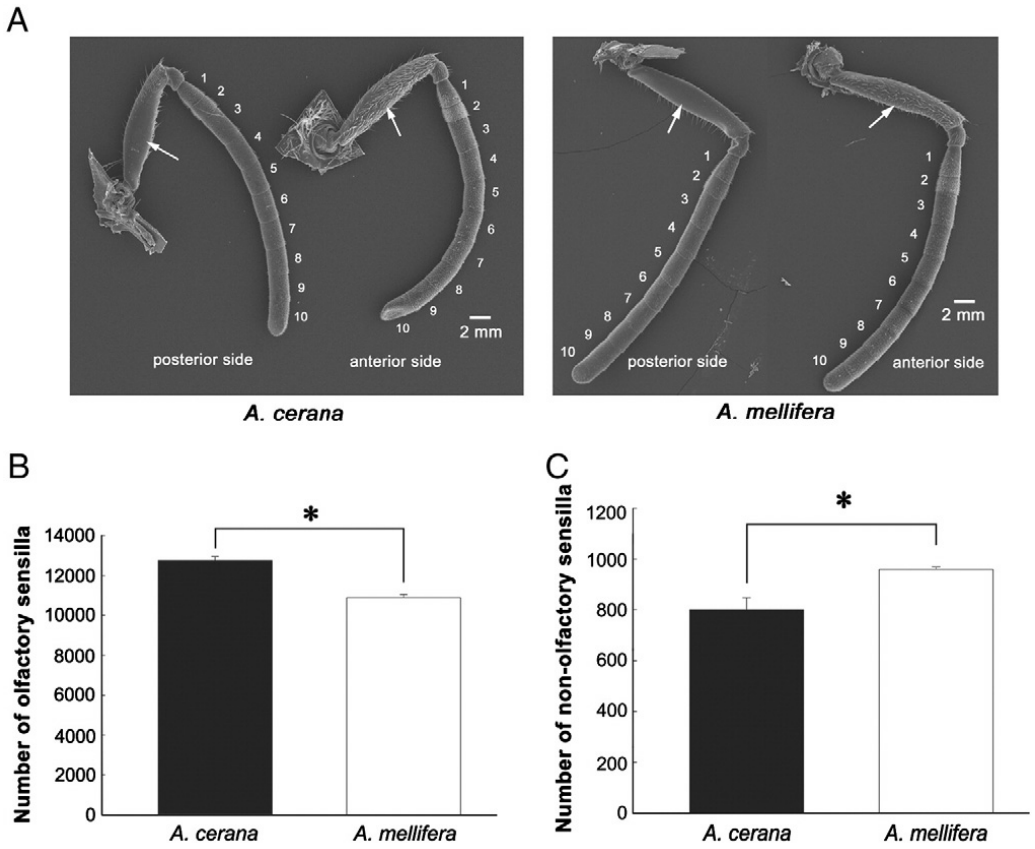


Figure 2. The number of olfactory sensilla (A) and non-olfactory sensilla (B) of *A. cerana* (black bar) and *A. mellifera* (white bar). (A) Putative olfactory sensilla: placodea, trichodeum types A and B, basiconica. A. cerana has larger number of olfactory sensilla than A. mellifera. (B) Non-olfactory sensilla were more numerous in *A. mellifera* than in *A. cerana* (N = 12bees from one species). Significance was determined by Student's t-tests (*= P b 0.01). Error bars depict mean ± SE.

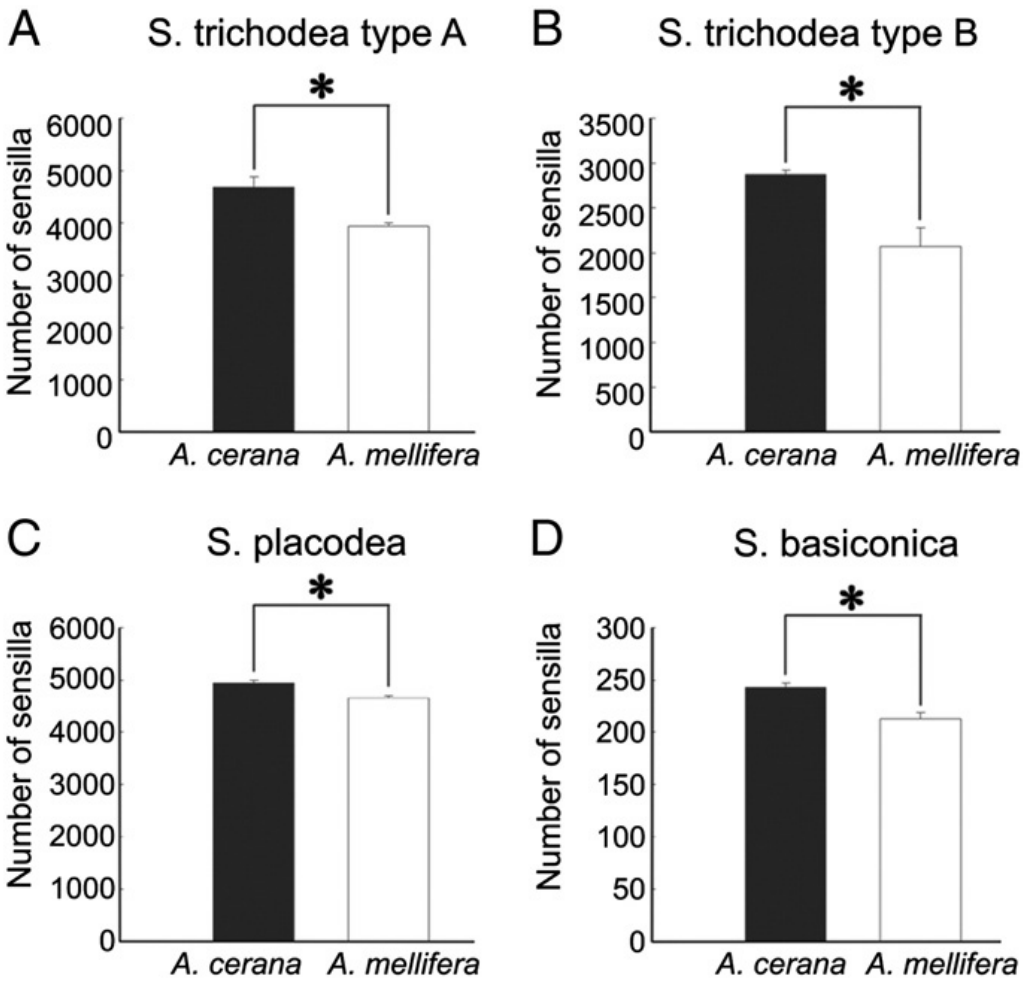


Figure 3. The average number of olfactory sensilla type on the antenna for *A. cerana* and *A. mellifera*. Four classes of olfactory sensilla are significantly more abundant on *A. cerana* antenna compared to *A. mellifera*. (A) TA, sensillum trichodeum type A, (B) TB, sensillumtrichodeumtype B, (C) Pl, sensillumplacodea, (D) Ba, sensillumbasiconica. Significance was determined by Student's t-tests (*=P b 0.01). Error bars depict mean±SE.

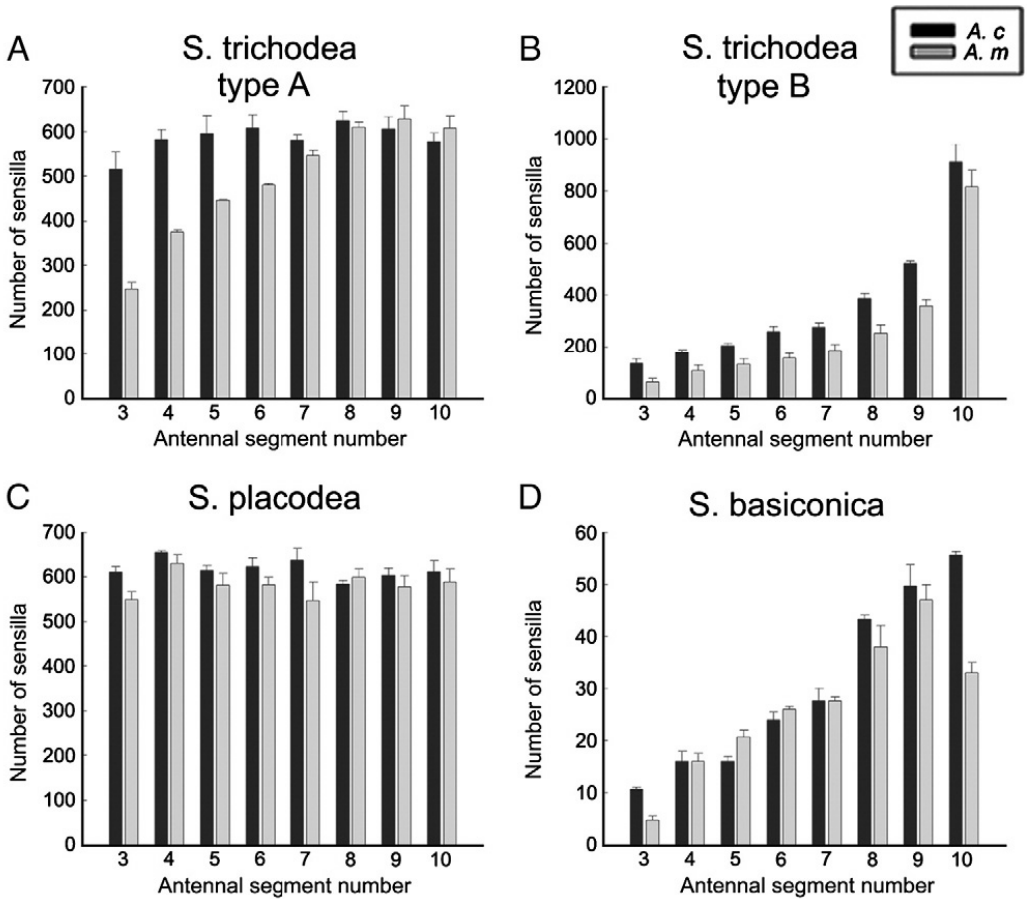


Figure 4. Number of olfactory sensilla per antenna segment in *A. cerana* and *A. mellifera*. Mean number of sensilla on the *A. cerana* (black bars) and on the *A. mellifera* (gray bars) is expressed with their SE. The distribution pattern of each sensilla type was conserved among individuals within a single species and was also conserved between two species. However, the mean number of sensilla per antenna segment is likely to differ between these two species. (A) TA, sensillum trichodeum type A, (B) TB, sensillum trichodeum type B, (C) Pl, sensillum placodeum, (D) Ba, sensillum basiconicum. Statistical significance was determined by one-way ANOVA with Duncan's test (* = P b 0.01).

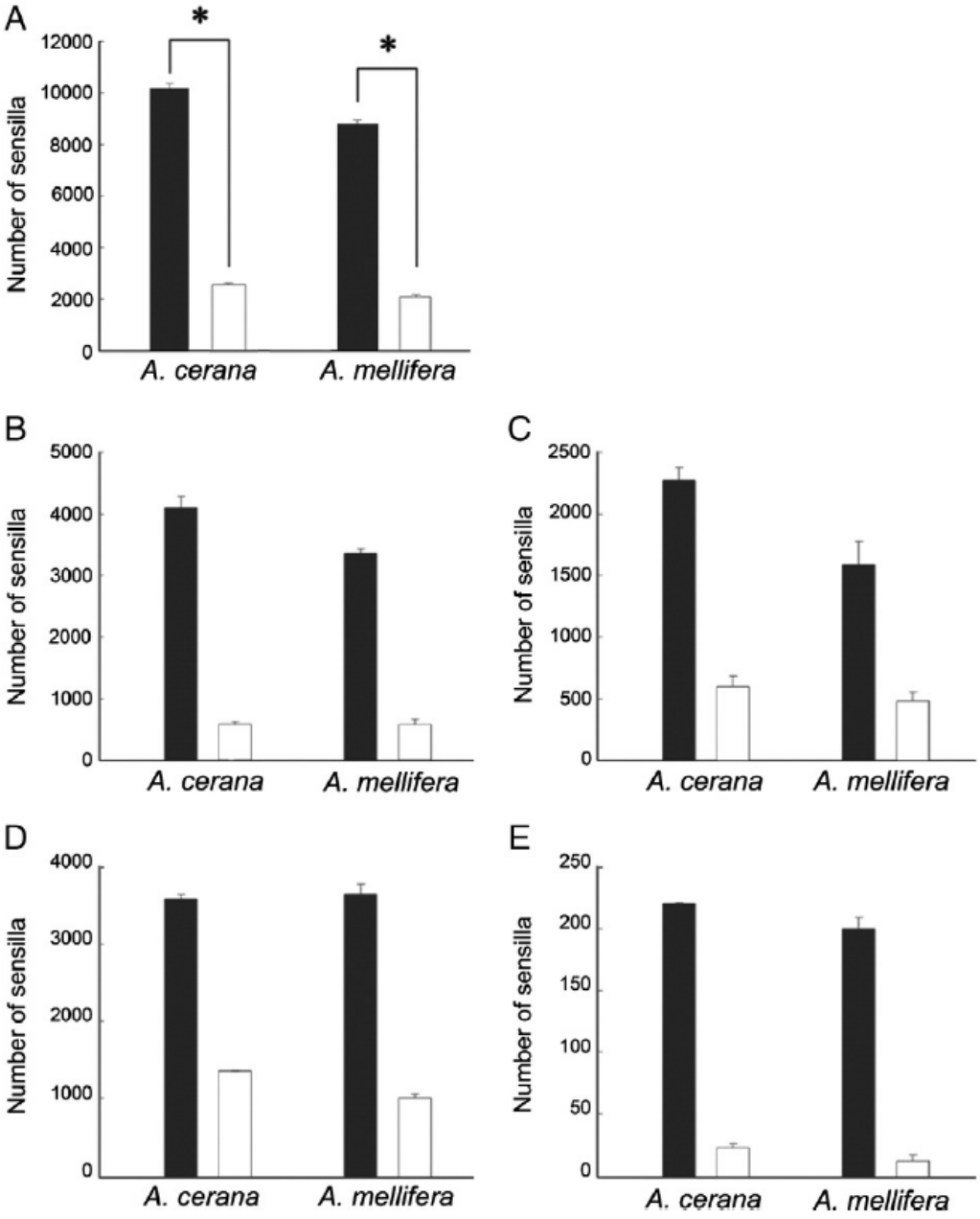

A
*
*
12000
10000
8000
6000
4000
2000
0
Number of sensilla
A. cerana
A. mellifera
B
5000
4000
3000
2000
1000
0
Number of sensilla
A. cerana
A. mellifera
C
2500
2000
1500
1000
500
0
Number of sensilla
A. cerana
A. mellifera
D
4000
3000
2000
1000
0
Number of sensilla
A. cerana
A. mellifera
E
250
200
150
100
50
0
Number of sensilla
A. cerana
A. mellifera

Figure 5. The number of olfactory sensilla at anterior and posterior aspects. (A) The two species show similar pattern: The number of olfactory sensilla directed anterior part is higher than posterior aspects (N=12). (B)–(E) The average number of olfactory sensilla type on the antenna at anterior and posterior aspects in both species A. cerana and A. mellifera. (B) TA, sensillum trichodeumtype A, (C) TB, sensillum trichodeumtype B, (D) Pl, sensillum placodeum, (E) Ba, sensillum basiconicum. Significance was determined by Student's t-tests (*=P b 0.01). Error bars depict mean ± SE.

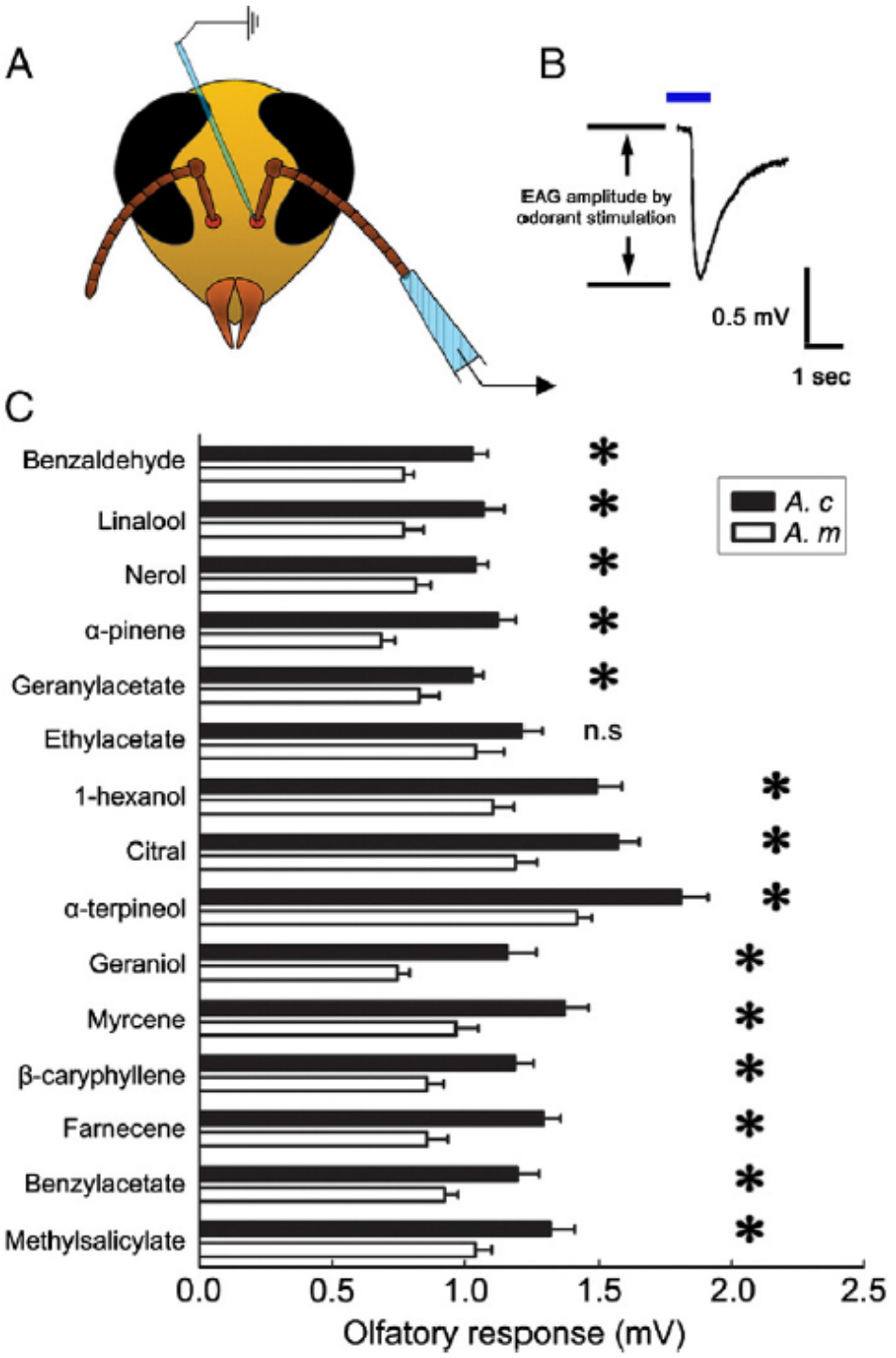

A
B
EAG amplitude by odorant stimulation
0.5 mV
1 sec
C
Benzaldehyde
Linalool
Nerol
α-pinene
Geranylacetate
Ethylacetate
1-hexanol
Citral
α-terpineol
Geraniol
Myrcene
β-caryphyllene
Farnecene
Benzylacetate
Methylsalicylate
n.s
A. c
A. m
0.0
0.5
1.0
1.5
2.0
2.5
Olfatory response (mV)

Figure 6. Electroantennogram responses in the antennae of *A. cerana* and *A. mellifera* foragers (A) Schematic drawing of the compound exposure system for honeybee antennal sensilla. (B)Measurement of EAG responses from a trace. Olfactory responses were measured from the baseline of the trace to the peak apex after onset of odorant stimulation (bar above the EAG trace). (C) Significant differences of olfactory responses to most floral odorants were found (Student's t-test, * = P b 0.01). Error bars depict mean ± SE.

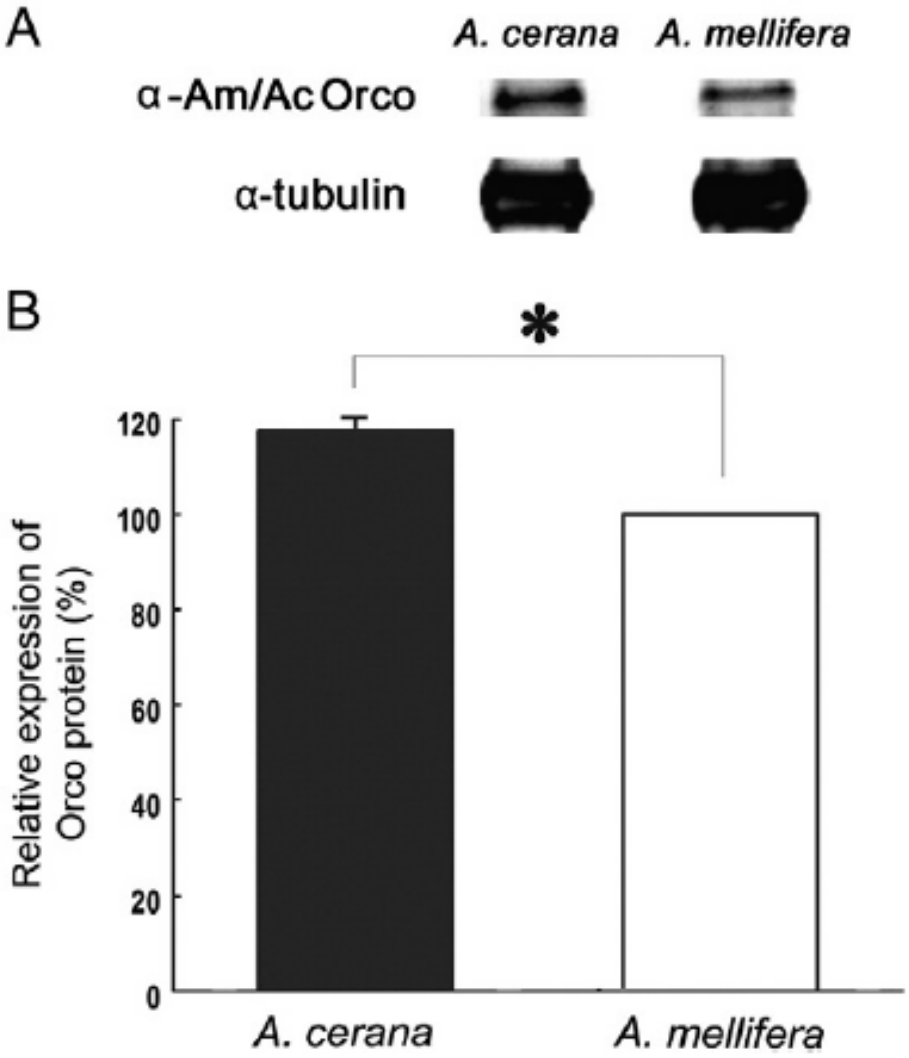


Figure 7. Am/Ac Orco protein expressions in the antennae of *A. cerana* and *A. mellifera*. (A) Western blotting was performed on protein fractions from these two species antenna homogenates (N = 60). A 52 kDa protein was recognized by the anti-Orco protein antiserum. (B) The protein expression level of A. cerana is higher than that of A. mellifera. *P b 0.05 as determined by Student's t-test.

Chapter III

Identification and characterization of sugars and amino acids receptors in the western honey bee, *Apis mellifera*

This chapter was published in Journal of Asia-Pacific Entomology and is due to be submitted shortly

Jung et al., (2015) *JAPE*. 18(1): 19-26

Abstract

The sense of taste is responsible for the choice of profitable food sources and nest mate recognition in honeybees. Taste detection for food sources occurs within cuticular hairs located on the antennae, on the mouthparts, and on the tarsi of the forelegs. The gustatory sensilla, which are composed of cuticular hair, respond to sugars, salts, and amino acids. In the honey bee, although sugar detection is a crucial factor in determining the acceptability of nectar and pollen for collection, little is known about the molecular and neural correlates underlying sugar perception. Here we identified the function of sugar receptors in *Apis mellifera* that is most closely related with sugar receptors in *Drosophila melanogaster* such as DmGr64a-f and DmGr5a. We determined that gustatory receptor 1 of *A. mellifera* (AmGr1) responded to sucrose, glucose, trehalose, and maltose in a dose-dependent manner, and that the expression patterns of AmGr1 and AmGr2 are distinct from those in *Drosophila*. AmGr1 alone showed full functionality. AmGr1 and AmGr2 were co-localized or not in the antennal neurons, and especially AmGr1 was highly expressed at the distal segment of the antennae. We find that AmGr10 is a broadly tuned L-amino acids receptor but not to respond other compounds such as sweet and bitter taste substances.

Keywords: taste, gustatory receptors, honeybee, peripheral taste detection,

Introduction

The sense of taste plays a critical role in evaluating and identifying potential food and drink by discriminating various chemical compounds in food sources (Yarmolinsky, Zuker, and Ryba 2009). Taste and gustatory systems are critical for detecting and responding to sweet, sour, salty, bitter, and umami stimuli (Zhao et al., 2003; Zhang et al., 2013). They are also capable of distinguishing between these various taste modalities to generate innate behavioral responses. In insects, gustatory systems also play a critical role in multiple behaviors, including feeding, toxin avoidance, courtship, mating, and oviposition (Sato, Tanaka, and Touhara 2011). Gustatory organs are widely distributed over the entire surface of the body, enabling insects to efficiently detect nonvolatile chemosensory information such as potential foods or toxic compounds (Sato, Tanaka, and Touhara 2011). Taste substances are recognized by gustatory sensory neurons that express putative seven-transmembrane proteins in the gustatory receptor (Gr) family. The Gr family is encoded by many related but diverse genes, and genome projects have revealed 68, 13, 76, and 65 Gr genes in the fruit fly (Clyne 2000), honeybee (Weinstock et al. 2006), mosquito (Hill et al. 2002), and silkworm moth (Wanner and Robertson 2008), respectively.

Especially the detection of sugar is crucial in determining whether a food

source is an edible, energy-rich food that is an ingestible sweet substance for nutrition. The sugar fraction constitutes an essential part of honeybee-collected nectar and pollen. Despite its critical importance to the survival of the species, little is known about the molecular and neuronal basis of sugar taste receptor function in the honeybee. In addition, no functional study is so far available to determine the tastant specificity of any of the 10 gustatory receptors of the honeybee.

Perception of amino acids is important taste modality for a variety of animals. Like mammals, insects such as honeybee need to ingest proteins from foods because they cannot synthesize several amino acids. Insects depend on nectar sugars for energy substrate, primarily flight. Although free amino acids are second most abundant compounds in nectar after carbohydrates (Gardener and Gillman 2001), the biological significant roles of their presence is still being unknown. Previous study showed that quality and quantity of amino acids are believed to enhance insect longevity and fecundity. Increasing evidence supports the preference of insects for sugar solutions enriched with amino acids. Honeybee also prefer natural or artificial sucrose solutions including amino acids(Arenas and Farina 2012),(Bertazzini et al. 2010). These preferences for nectar containing high abundant amino acids could arise from taste cues which can be perceived by taste receptors in gustatory organ. Studies in the fleshfly and the blowfly have indicated

that the labellar sensilla respond to amino acids(Shimada and Tanimura 1981). Other study have demonstrated that taste cells in the mosquito, tsetse fly and fruit fly also respond to amino acids (Dimond et al., 1956; Van der Goes van Naters and Den Otter, 1998)(Toshima and Tanimura 2012). By contrast, it is unknown whether the gustatory receptor neurons of honeybee can recognize to amino acids and the gustatory receptors for amino acids have not yet been identified in honeybee.

The goal of the current study was to identify the receptor for amino acids of honeybee. To achieve this, we cloned full length *A. mellifera* cDNAs encoding candidate amino acids receptors from honeybee gustatory organs. We then report that AmGr10, which is one of the conserved social insect gustatory receptor, is expressed in external and internal organs of honeybee. We performed the functional characterization of this receptor in vitro using heterologous expression in *Xenopus* oocytes and two electrode voltage-clamp recording. We find that AmGr10 is a broadly tuned L-amino acids receptor but not to respond other compounds such as sweet and bitter taste substances. We also showed that addition of the 5’ribonucleotide IMP potentiated the response, which server as a hallmark of umami taste, distinguishing it from a more general sensing of glutamate using Ca^{2+} imaging assay. IMP was not agonist of AmGr10 but rather bind and stabilize the receptor in the glutamate-bound state. In addition, we have

employed *in situ* hybridization and an immunostaining method to investigate the anatomical organization of sweet taste receptors in the honeybee antenna. We also examined the characterization and identification of *A. mellifera* sweet taste receptors using a heterologous expression system. We demonstrated that AmGr1 functions as a sweet receptor responding to sucrose, glucose, trehalose, and maltose but not to fructose and that AmGr2 may function as a co-receptor for variability of sugar sensitivity in honeybees. In addition, we report observations on the electrophysiological responses to sweet tastants of gustatory sensitivity in the antenna of *A. mellifera*. The identification of sugar receptors generates powerful molecular tools to investigate the function of sweet taste receptor cells and also the logic of sweet taste perception in honeybees.

Materials and Methods

Insect preparation

Honeybees of *A. mellifera* were maintained on apiaries of Seoul National University campus, which is surrounded by the Gwan-ak mountain range in Seoul, Korea. For electron microscopy and immunohistochemistry, honey bees of the species were captured directly from three hives regardless of age. For electrophysiology experiments, free-flying honeybee foragers were caught in the morning of every experimental day upon return to the hive entrance. They were placed in glass vials and cooled in ice until they stopped moving.

Scanning Electron Microscopy (SEM)

Collected bees were placed in the freezer for 1 h, after which they were decapitated and antennae were cut at their base. The antennae were then cleaned for 1 h in PBS solution followed by dehydration through a graded ethanol series of 25, 50, 70, 90, and 100% for 10 min each. After drying in the oven at 40^{o}C for 24 h, the scape of the antennae was attached to double side sticky tape (3M Korea, Seoul, Korea). All samples were coated with a thin layer of gold on all sides and each antenna was fixed on a holder in the sample chamber. The samples were

scanned with a SUPRA 55VP, Field-Emission Scanning Electron Microscope (Carl Zeiss, Germany). Antennae were imaged from the dorsal and ventral side. We scanned only flagella segments 3 to 10 because there are no olfactory receptors on the first and second segments of honey bee flagellum. Each segment from 3rd to 10th was scanned longitudinally at a magnification of 500 times. Two images per segment were collected. Sensilla chaetica were identified according to specific morphological characteristics as described in a previous study (Frasnelli et al. 2010). Each type of sensilla was counted from all selected images by using an image analysis tool (UTHSCSA ImageTool Version 3.0).

RNA Isolation and cDNA synthesis

Total RNA was isolated from the honeybee antenna using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Using 1 µg of total RNA, cDNA was synthesized with oligo-dT with Invitrogen Superscript III enzyme (Grand Island, NY, USA). Then, using a template of 1 µl of synthesized cDNA, polymerase chain reaction (PCR) amplification was performed with gene specific primer sets for the target genes AmGr1 (XM_006567733.1) and AmGr2 (XM_397125.5). PCR conditions were performed by procedures at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min 30 sec, and a final

extension at 72 °C for 5 min using an Applied Bioscience Thermal Cycler (Foster City, CA, USA). *Amrps49* gene was used as a control for normalization. All experiments were in triplicate. Primer information for qRT-PCR is described in Table S1.

Quantitative real-time PCR (qRT-PCR)

Complementary DNA synthesis was conducted as described above. Quantitative real-time PCR was carried out with StepOne Plus (Applied Biosystems, Foster City, CA, USA) using SYBR green qRT-PCR Master Mix (Fermentas, Ontario, Canada). Primer information for qRT-PCR is described in Table S1. Quantitative analysis was employed by StepOne plus Software V. 2.0 (Applied Biosystems, Foster City, CA, USA). Results were normalized to a validated control gene, actin, using the ΔΔCt method. The pQE30 vector sequence was used as a control.

Gene cloning of gustatory receptors 1, 2, and 10

Full-length coding sequences of candidate sugar gustatory receptors of *A. mellifera* were PCR-amplified from pools of total cDNA prepared from worker *A. mellifera* antennae using TaKaRa Ex-Taq (Takara Shuzou, Kyoto, Japan). Amplification reactions (25 µl) included 0.3 µl TaKaRa Ex-Taq, 2.5 µl 10 x Ex-

Taq buffer, 2 µl 2.5 mM dNTP mixture, 2 µl 5 pmol of each primer, 1 µl template cDNA, and 17.2 µl sterilized distilled water. All amplification reactions were carried out using a 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA.) under the following conditions: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 58-68 °C for 30 s, 72 °C for 1.5 min; and 72 °C for 10 min. PCR amplification products were run on a 1.0% agarose gel and verified by DNA sequencing. PCR primers (Table S1) were designed based on nucleotide sequences reported previously (Krieger et al. 2004).

In situ hybridization, immunostaining, and imaging

RNA probes of *AmGr2* for *in situ* hybridization were prepared with DNA clones obtained as described in Table S1 by the procedures as described previously (H.-W. Kwon et al. 2006). Antennae of worker *A. mellifera* were fixed with 4% paraformaldehyde (PFA) solution overnight, after which antennae were cut to 3-5 mm lengths. Tissues were then washed with PBS buffer (pH 7.4), followed by dehydration and rehydration using an ethanol series from 25%~100%. Tissues were then hybridized with a hybridization solution containing Dig-labeled RNA probes for 20 h at 58 °C. After several washes with PBS buffer containing 0.2% Tween 20, tissues were incubated with peroxidase (POD)-conjugated anti-DIG antibodies (Roche, Indianapolis, IN, USA) in a blocking reagent (Roche)

overnight at 4 °C, followed by a signal visualization process using a tyramide signal amplification (TSA) kit in accordance with the manufacturer's instruction (PerkinElmer, Waltham, MA, USA). After *in situ* hybridization, immunostaining using several different antibodies was performed as described previously (Jung et al. 2013). Tissue preparations after *in situ* hybridization were subsequently incubated with rabbit anti-gustatory receptor 1 antibodies (1:200 in PBS with 0.1% Tween 20, PBSTw) at 4 °C overnight. After 3-4 times washing with PBSTw, anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) conjugated with cy2 fluorophore were incubated overnight at 4 °C. Cell and neuronal staining was conducted with TOTO-3 (Invitrogen, Carlsbad, CA, USA). Stained tissue preparations were dehydrated with an ethanol series and 100% acetone to embed into Spurr's epoxy resin (Jung et al. 2013). Resin-embedded tissues were incubated at 60 °C in an oven overnight and sectioned at 20 μm thickness by using an automatic sliding microtome (HM 355S; Microm, Thermo Scientific). Sectioned tissues were mounted with Serva fluoromount (Crescent Chemical Co). Images were captured with a LSM 510 confocal microscope (Zeiss, Thornwood, NY) and were processed with ZEN 2009 software. Rabbit polyclonal antibodies against AmGr1 were generated by Abfrontier Company (Seoul, Korea). Based on AmGr1 peptide information (XP_006567794.1), NH2-

TWEKLEKELSQRHRKISKIS-C was chosen as the target epitope region to produce anti-AmGr1 antibody. Rabbits were immunized three times with 0.5 mg of the synthesized peptide, after which serum-specific antibody was affinity-purified on columns using immobilized antigen peptides.

Receptor expression in *Xenopus* oocytes and two-electrode voltage-clamp electrophysiological recordings

In vitro transcription of cRNA was performed by using a mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Plasmids were linearized with EcoRI, and capped cRNA was transcribed using SP6 RNA polymerase. The cRNA was purified and resuspended in nuclease-free water at a concentrar0ion of 1 μg/1 μl and stored at -80 ℃ in aliquots. Matured female *Xenopus laevis* frogs were purchased from Xenopus (Ann Arbor, MI, USA). Frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester (Sigma, USA) and oocytes were removed surgically. Oocytes were freed from the follicle cells by treatment with collagenase A for 1 h at room temperature and incubated for 24 h in modified Barth's solution (Sato, Tanaka, and Touhara 2011). cRNA was microinjected (27.6 ng) into *X. laevis* oocytes at stage v or vi. The oocytes were then

incubated at 17 ℃ for 3~5 days in Barth's solution sterilized by filtration. A two-electrode voltage-clamp technique was employed to observe tastant-induced currents at a holding potential of -70 mV. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT), low-pass filtered at 50 Hz, and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1322A (Axon Instruments, Forster City, CA, USA) and software pCLAMP 10 (Molecular Devices, LLC, Sunnyvale, CA).

Tip recordings of antennal sensilla

For tip recordings from antennal sensilla, the flagellum was immobilized and the bee's head including the antennae and the recording electrode was surrounded by a humid chamber. Before recordings, the antennal tip was washed using tissue paper moistened with distilled water. Recordings from gustatory sensilla were made from so-called sensilla chaetica (Esslen and Kaissling 1976) at the tip of the antenna in the ventral zone devoid of olfactory sensilla. These sensilla can be easily identified by their external morphology (A. T. Whitehead and Larsen 1976). A grounded reference electrode filled with 1 mM KCl was inserted into the compound eye. The five sugars selected for tip recording testing (sucrose, glucose, fructose, maltose, and trehalose; Sigma) are the most common sugars

encountered in plant nectar (Perret 2001). For stimulation, 1 mM KCl with sugar concentrations of 10 mM and 1 mM KCl alone were used. Stimuli were applied for approximately 5 s with an interstimulus interval of 3 min. Only a few of the taste hairs did not respond to sugar family and also did not show mechanoreceptor responses. These sensilla were excluded from the data analysis. The recording electrode was connected to a preamplifier (Taste Probe; Syntech, Kirchzarten, Germany) in conjunction with a 100-3000 Hz band-pass filter. Recordings of action potentials were acquired at a 12 kHz sampling rate and analyzed with Autospike 3.1 software (Syntech, Kirchzarten, Germany). The responses of antennal sensilla to all tested solutions were quantified by counting the number of spikes after stimulus onset.

Phylogenetic analysis

The amino acid sequences of the *A. mellifera* gustatory receptors (AmGrs) and *Drosophila melanogaster* gustatory receptors (DmGrs) were downloaded from NCBI and previous work (Kent and Robertson 2009). All amino acid sequences used for phylogenetic analysis were aligned with Clustal X. Phylogenetic analysis was constructed with the 16 AmGrs and 55 DmGrs by using the MEGA5 Neighbor-Joining method. Bootstrap analysis was conducted by using

1000 Neighbor-Joining replications.

Immunofluorescence analysis

For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS (PBST), blocked with 2% normal goat serum and 1% bovine serum albumin (BSA) and labeled with an anti-AmGr10 antibody (1:500 dilution) at 4 ℃ overnight. After being washing three times with PBST, cells were incubated with a cy3 goat anti-rat antibody (1:1000) for 2 h at room temperature. To detect the expression of AmGr10 in the cells, images were obtained by confocal laser scanning microscopy (LSM700, Carl Zeiss, German) with excitation and emission set to 569 nm and 623 nm, respectively.

Intracellular Calcium assay

For calcium signaling assay, HEK-293 cells expressing AmGr10 were cultured for more than 3 days, and Fluo-4 NW dye mix solution (Molecular probes) was loaded into the cell in 96-well microplate. After incubation at 37℃ for 1 hour, the fluorescence signal upon the addition of MSG (50, 100, 300 and 500 mM) was measured at 516 nm by excitation at 494 using a spectrofluorophotometer (Perkin Elmer, USA). To normalize the response, the changes of fluorescence ratio were divided by maximal fluorescence changes induced by each compounds.

Scanning electron microscopy (SEM)

Galea of honeybee mouth parts were prepared by removing only the proboscis part (labium and shaft). Samples were washed by using PBST 0.1% and were dehydrated with a series of alcohol to 100%, after which each galea was desiccated in a 40°C dry oven. The basal side of the galea was attached to a strut-coated double sided adhesive tape (Agar Scientific, Essex, United Kingdom). Resolution of a scanning electron microscopy (SUPRA 55UP, Zeiss, Oberkochen, Germany) was about 1.0 nm (15kV) to 1.7 nm (1kV).

Calcium imaging

Transfected cell lines were subsequently cultured on confocal dishes (SPL, Pocheon, Korea). Before experiments, the culture media was removed and 100 μl of 2 μM Fluo-4 AM were loaded and directly replaced (Invitrogen, Grand Island, NY, USA), after which the cells were incubated in the dark for 90 min at room temperature. After incubation, 1X HBSS buffer was added onto the confocal dish, which was then directly placed in the LSM700 inverted confocal microscope for observation (Zeiss, Oberkochen, Germany). Images were captured maximum 100 frames per every two second interval. Test chemicals were dissolved in HBSS at various concentrations. Calcium influx into the transfected cells upon ligand binding was monitored and analyzed with ZEN software (Zeiss, Oberkochen,

Germany). Fluorescent intensity changes were calculated as follows34: Intensity F = (Fafter – Fbefore) / F max.

Data analysis

Relative gene expression, sensilla and spike counting were analyzed by Student's t-test (SPSS, Version 20, IBM, NY, USA). Comparisons of voltage clamp responses and spike number were analyzed using a one-way ANOVA test followed by Bonferroni correction with multiple comparisons (SPSS, Version 20, IBM, NY, USA).

Result

Differences of number of sensillum chaetica per antennae segments

Based on location and structure, s. chaetica are considered to have a gustatory function because of their tip pores (Fig. 1) and their perpendicular orientation with respect to the antennal cuticle surface (A. T. Whitehead and Larsen 1976). We investigated characterizing the abundance of s. chaetica on antennae from *A. mellifera*. S. chaetica were observed in every flagellum from 3^{rd} to 10^{th} segments in the antennae of honeybees. Approximately 500 s. chaetica were found distributed over the antennal flagellum (Fig. 1). Especially, the distal segment was observed to have a greater abundance compared with other segments ($P<0.05$, Fig. 1). This result demonstrated that the antennal distal segment plays an important role for the detection of nonvolatile chemosensory information such as potential food sources and toxic compounds.

Responses of contact chemoreceptors to sugars

We recorded responses of GRNs located in chaetic sensilla of the tip of antennae upon 5-s stimulation with sucrose, glucose, fructose, maltose, and trehalose. Data were obtained for responses for the five tastants from 7 bees. The recording provides evidence that in most cases a single sensory cell was stimulated by sugars (Fig. 2). The solution of KCl failed to stimulate spike activity

in any of the sensilla tested (Fig. 2D). Responses to sucrose in the 500 ms of stimulation ranged from 13 spikes at a concentration of 10 mM to 22 spikes at 30 mM and 19 spikes at 100 mM (Fig. 2A). Sucrose caused higher firing rates at a given concentration than any compound tested and had a much lower threshold (Fig. 2A). For average spike counts in the first 500 ms after stimulus onset the concentration-response curve showed saturation above 100 mM sucrose (Fig. 2A); however, the sensilla chaetica in antenna had much lower firing rates for other sugars than sucrose with the relative responses to lower concentrations being trehalose < maltose < glucose < fructose < sucrose. The average number of spikes with 1 M fructose is similar to that of the 30 mM sucrose responses (Fig. 2B). We could not characterize the responses of 100 mM fructose solutions (Fig. 2B) in sensilla chaetica. In addition, the glucose, maltose, and trehalose solutions at 500 mM failed to affect a response in all but one or two hairs tested (Fig. 2C). The cell responding to sugars maintained a high rate of firing, adapting steadily to 70-75% of the initial firing rate over the first 100 ms of stimulation (Fig. 2D). The difference in adaptation rate between sugars was not significant.

AmGr1 **and** ***AmGr2*** **are highly expressed in the distal segment of antennae**

We examined sugar sensing receptors of the 10 *A. mellifera* Grs that were most closely related to gustatory receptor genes in other insects (Robertson

and Wanner 2006b). Two of these receptors, AmGr1 and AmGr2, cluster confidently with the eight candidate sugar receptors in each of the flies (Fig. 3), based on the role of DmGr5a as a trehalose receptor (Chyb et al. 2003). We determined whether *AmGr1* and *AmGr2* genes were critical for contact chemoreception in the antenna distal segment where honeybees respond to different kinds of substances such as pollen and nectar from the antennal-segment specific expression patterns of *AmGr1* and *AmGr2* by using quantitative real time PCR. These two receptors were significantly enriched in the distal segment compared with the sum of other segments of antennae ($P<0.05$, Fig. 4).

AmGr1 is a specific receptor for sugar compounds

To identify the function of the two candidate sugar receptors of *A. mellifera*, we examined *Xenopus* oocytes expressing candidate gustatory receptor genes, *AmGr1* by using two-electrode voltage-clamp recording. *Xenopus* oocytes injected with *AmGr1* cRNA was stimulated with sweet tastants that have been reported to have responsiveness in honey bees (Gabriela et al. 2011). These tastants are major sugar components in nectar and pollen that provide honey bees with carbohydrates and protein, which are necessary for survival (Gabriela et al. 2011). The oocyte expressing AmGr1 responded robustly to sucrose, glucose, maltose, and trehalose in a dose dependent manner but not to fructose (Fig. 5A and B); however, the oocyte expressing AmGr2 did not show any response to

sugar substances (Fig. 5C).

Fructose is a specific ligand for an AmGr3

We expressed AmGr3 in HEK293T cells and performed Ca^{2+} imaging. HEK293T cells expressing AmGr3 exhibited Ca2+ responses to D-fructose in a dose-dependent manner with an Ec50 value of 32mM and a threshold concentration of 3mM (Fig. 6 A and B). AmGr3 belongs to one of the functionally known members of the insect Gr family that forms a highly confident single lineage on the phylogenetic tree (Fig. 3). Previous study showed that DmGr43a and BmGr9 which are orthologs with AmGr3 exhibited Ca2+ responses to D-fructose in a dose-dependent manner (Sato, Tanaka, and Touhara 2011).

Localization of sugar receptors AmGr1 and *AmGr2* in antennae

AmGr1 and *AmGr2* in antennae were localized by using double-labeling studies with *in situ* hybridization and immunostaining (Fig. 7). First, *AmGr2* genes were localized in the antennal neurons (Fig. 7) and compared with the control (data not shown). Immunostaining images using an anti-sense RNA probe of the *AmGr2* and an antiserum to AmGr1 were overlapped in gustatory receptor neurons (GRNs) of the antennae (Fig. 7). It also has been reported that *Dmgr64f* is co-expressed with other sugar receptors *Dmgr5a* and *Dmgr61a*; however, there is also a fraction of cells with non-overlapping expression of AmGr1 or *AmGr2* in

the antennae (Fig 7). This result suggests that *A. mellifera* sugar receptors function as a heterodimer or homodimer and that AmGr2 is required as a co-receptor for a dynamic range of sugar tastes.

AmGr10 is ubiquitous expressed in external and internal organs of honeybee

Previous study showed that the bee genome encodes very few gustatory receptors compared with *Drosophila melanogaster* (Robertson and Wanner 2006b). RT-PCR experiments demonstrated that here are 10 Gr genes in gustatory organs of honeybee(Robertson and Wanner 2006b). Four Grs (AmGr6-10) have no apparent orthology to any of the fly lineages which were unable to identify amino acid receptors, suggesting that one of the four Gr genes of honeybee may be candidate of amino acid receptors. Expression of AmGr10 was significantly enriched in gustatory organs and was expressed at high levels in internal organs such as fatbody, brain, and hypophrangeal gland (Fig. 8), so it may be expressed in internal taste organs of gustatory tract.

L-amino acids are ligands for an *A. mellifera* gustatory receptor, AmGr10.

We examined 4 of the 10 *A. mellifera* Grs (AmGrs) that were closely related to Gr genes in other insects. These 4 Grs were expressed in *Xenopus laevis* oocytes for functional analysis. Using the two electrode voltage-clamp technique,

oocytes injected with Gr cRNA were stimulated with L-amino acids. One of them, oocytes injected with AmGr10 cRNA were recognized by glutamate, aspartate, arginine, asparagine, glutamine, and lysine (Fig. 9B) while they did not respond to other compounds including sugar and bitter compounds (Fig. 9A). On the basis of the dose-response curve, the EC50 value of compounds were ----mM for AmGr10. These results suggested that specific L-amino acids were ligands for AmGr10 and that AmGr10 triggered by ligands activate to the generation of a depolarizing receptor potential in oocytes.

We also transfected AmGr10 in HEK293T cells and carried out intracellular Ca2+ assay. First, we performed an immunocytochemistry to confirm the expression of AmGr10 in HEK293T cells. Almost transfected HEK293 cells expressing AmGr10 showed red fluorescence at cell surface (Fig. 10A). In contrast, HEK293T cells without AmGr10 did not show the any signal, suggesting that the red fluorescence was specific for AmGr10 (Supplementary Fig.1). This result implies that AmGr10 was successfully expressed at the lipid membrane of HEK-293 cells. To examine the response profiles to L-amino acids, HEK293T cells expressing the AmGr10 were stimulated with 100mM concentrations of each amino acid, and the fluorescence intensities following the receptor activations were compared among 17 types of amino acids. AmGr10 exhibited the highest response intensities to L-Glu and L-Asp of the 17 evaluated amino acids (Fig. 10).

AmGr10 also recognized significant responses to L-Arg, L-Asn, L-Lys, and L-Gln, which is consistent with our oocyte expression of AmGr10 research.

IMP can strongly potentiate the umami taste intensity in AmGr10

Previous electrophysiological studies showed that taste responses to L-amino acids are considerably potentiate by purine nucleotides such as inosine monophosphate (IMP) in rats (Yoshii, Yokouchi, and Kurihara 1986). Also, mammalian taste receptor, T1R1 and T1R3 play a role in function as a broadly tuned L-amino acid receptors sensing to almost of the 20 amino acids combined to IMP(Nelson et al. 2002). To assay the effect of IMP, HEK293T cell expressing AmGr10 were stimulated with MSG in the presence or absence of IMP. Sensing Ability of AmGr10 to L-amino acids were dramatically enhanced by low doses of IMP (Fig. 11 A and b). In the presence of 2.5mM IMP, the EC50 of AmGr10 for L-Glutamate was shifted –fold. However, IMP alone did not activate AmGr10, even at high concentration tested in our assay. The effect of IMP on AmGr10 was saturable (Fig. 11), and selective; AmGr1 which functions as a sweet taste sensor was not activated by L-amino acid in the presence of IMP, and IMP did not enhance the response of AmGr1 to sweet taste stimuli (Fig. 11).

Discussion

Identification of sugar-stimulated gustatory receptors of *A. mellifera*

In the honeybee, the antennae constitute the major chemosensory organs together with the fore tarsi and the mouthpart. Antenna gustation plays a pivotal role in appetitive food sensing as shown by the proboscis extension reflex being elicited by stimulation of the antennae with sucrose solution (Matsumoto, Sandoz, and Giurfa 2013); however, less is known about which gustatory receptors are processed by sugar sensation at the peripheral nervous system. This study is the first to demonstrate that the gustatory receptor 1 (*AmGr1*) gene is activated by several sugar substances, including sucrose, glucose, maltose, and trehalose. Although genetic studies using *Drosophila* have suggested that co-expression of gustatory receptors is responsible for sensing from sugar family members such as sucrose, glucose, and trehalose (Jiao et al., 2007; Jiao et al., 2008) to bitter compounds (Moon et al., 2006; Lee et al., 2009) and to CO_2 (Kwon et al., 2007; Jones et al., 2007), AmGr1 seems not to require the expression of other Grs to show responsiveness to sucrose, glucose, maltose, and trehalose because *AmGr2*, which from phylogenetical study is one of the candidate genes in sugar sensing receptors, did not respond to sugar substances. Co-expression of AmGr1 and AmGr2 did show different sensitivity to members of the sugar family (Jung et al.

2015); therefore, we suggest that AmGr1 may exhibit different ligand sensitization properties depending on the AmGr2-expressing neuron. AmGr2, as shown with the odorant receptor system (Benton et al., 2006; Larsson et al., 2004), may interact with ligand specific Grs to form heterodimeric complexes that are necessary for stabilization of sensitization of ligand specific Grs. Our study showed that the responses of co-expression with AmGr1 and AmGr2 are more stable than the responses of AmGr1 alone, supporting the role of AmGr2 in stabilization. In addition, we find evidence that *AmGr1* co-localized with *AmGr2* in antennal neurons in sensilla chaetica, implying that AmGr2 plays an important role in the fine tuning of the dynamics of sweet taste responses. In case of the honeybee, it has been reported that sucrose responses between different hairs on the same antenna showed a high degree of variability (Haupt 2004) in a similar manner to our results. Based on localization patterns of *AmGr1* and *AmGr2*, we suggest that combinational expression of these two sugar receptors may provide honeybees with a dynamic range of sugar perception over a large range of concentrations in nectar and pollen.

Meanwhile, AmGr1 and co-expression of AmGr2 did not recognize D-fructose, suggesting that the fructose receptor may recognize very selectively and discriminate between other sugars. AmGr3 and its orthologs, including BmGr9 and DmGr43a, form a distinct Gr subfamily that is not included in the sugar or

bitter receptor subfamilies (Robertson and Wanner 2006b). Previous studies demonstrated that both BmGr9 and Dm43a showed a response to D-fructose (Sato et al., 2011; Miyamoto et al., 2012), implying that AmGr3 may play a role in D-fructose sensitization. This study showed that D-fructose is a ligand for AmGr3.

Response properties of antennal taste hairs

Many electrophysiological studies have focused on sucrose specialized sensilla located on the mouth parts (T. Whitehead and Larsen 1976), antennae (Haupt 2004), and fore tarsi (de Brito Sanchez et al. 2014); however, no study has analyzed the perception in antennae of other sugar family members such as glucose, fructose, maltose, and trehalose. Our results show that honeybee are responsive in the antennae to 5 sugars that occur naturally in nectar or honeydew, that the taste receptors initiating proboscis extension are located in the tip of the antennae (Fig. 1), and that there is high expression of gustatory receptor genes in the tip of the antenna (Fig. 4). Using tip recording analysis to provide information about the variability of antennal taste hair sugar responses, it was shown that there was considerable response variability among different types of sugar (Fig. 2) with the relative responses to lower concentrations being trehalose < maltose < glucose < fructose < sucrose. These results reflect evolutional adaption to distinct ecological niches and food sources. Nectar is mainly composed of three sugars, two hexoses (glucose and fructose) and their combination into a disaccharide

(sucrose), and the amounts and relative concentrations vary among species (Percival, 1965). Honeybee are nectar users and a hierarchy of sugar sensitization and threshold is to be expected in their receptors because most nectars contain combinations of these three sugars and in varying concentrations from 10 to 60% (Rosati, Caporali, and Paoletti 2012). The response of the antenna sensilla chaetica of the honeybee to sucrose, glucose, and fructose concentration is different to that found in the galeal sensilla chaetica of the honeybee by Whitehead (T. Whitehead and Larsen 1976). They report that sucrose provides a more effective stimulus at all concentrations and that glucose is more effective than fructose at low doses of about 1 M; however, they also report that fructose is more effective than glucose at high concentrations. These different tissue specific sensitivities to sugar compounds may depend on the expression of fructose receptors, and there is a need to identify fructose receptors.

In addition, we propose that AmGr10, a honeybee Gr, constitutes an amino acid –activated receptor that is inactivated by other sweet and bitter compounds. Although mammalian studies have suggested that coexpression of T1R1 and T1R3 is necessary to respond to L-amino acids such as L-glutamate and L-Aspartate (Nelson et al. 2002), AmGr10 appears not to require the expression of other Grs to respond L-amino acid in vitro. However, we cannot rule out the possibility that AmGr10 is capable of responding different ligand combined with

other Grs because AmGr10 expressing neuron may coexpress other Grs among 10 *AmGr* genes.

While AmGr10 was highly conserved among hymenopteran species (Doori Park et al. 2015), its orthologs form a distinct Gr subfamily that not categorized in the sugar or bitter receptor families and were disappeared within nonsocial insect species(Robertson and Wanner 2006b),(Werren et al. 2010), implying that AmGr10 may have a unique function within hymenoptera. We demonstrated that AmGr10 responded to L-amino acids, suggesting that the AmGr10 orthologs appear to represent a Gr subgroup that is crucial for perception of amino acids. Expression patterns of AmGr10 were significantly enriched in internal organs such as fatbody, brain, and hypopharangeal gland (Fig. 8), so it plays an important role in monitoring amino acid concentration and the involvement in intestinal absorption or some metabolism. Despite *Drosophila* are known to detect amino acids, its orthologs were not present in the Drosophila genome, indicating that they may have evolved novel molecular mechanisms similar to the fatty acid sensing mechanism found in PLC pathway in sugar sensing neuron (Masek and Keene 2013).

While heterologous expressed T1R1+T1R3 functions as a broadly tuned L-amino acid receptor in mice, these receptors can more narrowly tune umami taste-amino acids in humans (Nelson et al. 2002),(Li et al. 2002). We performed

two in vitro heterologous expression experiments to analyze responses of amino acid stimuli in cells and oocytes transfected with AmGr10 (Fig. 9 and 10). Heterologously expressed AmGr10 responds to some amino acids among polar group of amino acids. The sensation of amino acids may be due to the detection of specific side chains of amino acids; the carboxylic acid (Aspartate and Glutamate) and the other one containing nitrogen (Arginine, Lysine, Asparagine, and Glutamine), in AmGr10 present on the honeybee. Additionally, the responses of AmGr10 to acidic amino acids are much stronger than those to other amino acids, suggesting that recognition of AmGr10 to amino acids may be related to its chemical properties.

The next question is whether AmGr10 is an umami receptor. Unique sensory properties of umami indicate synergic effect between L-amino acids and purine nucleotides such as IMP and GMP(S Yamaguchi and Ninomiya 2000). Several studies have revealed that there were several candidate umami taste receptors including the T1R1/T1R3(Nelson et al. 2002), mGluR1 (Toyono et al. 2003), mGluR4 (Toyono et al. 2002), taste-mGluR1(San Gabriel et al. 2005), and taste-mGluR4 (Chaudhari et al. 1996), (Chaudhari, Landin, and Roper 2000). Our result demonstrated that AmGr10 was able to function as a tuned amino acid receptor. In addition, AmGr10 which showed the responses to MSG, L-AP4 and other amino acids was dramatically potentiated by purine based-5'ribonucleotide.

Therefore, we propose that AmGr10 is a constituent of the umami response. Several research groups tried to find umami taste receptors functional domain and demonstrated multiple binding sites within a large extracellular Venus flytrap (VTF) domain of mGluRs and T1Rs (Kunishima et al. 2000), (Muto et al. 2007), (F. Zhang et al. 2008b). The VFT domain consists of two lobes and the binding site is located in a hinge region between the two lobes. The L-Glu binding sites of T1R1/T1R3 localized in the hinge region of the VFT domain in T1R1 and the IMP-binding site is the opening region of the VFT of T1R1(F. Zhang et al. 2008b). In addition, previous study have identified five amino acids which bind to L-Glu at the hinge region and these amino acids are conserved between human and mouse (F. Zhang et al. 2008b). However, we could not identify the VFT domain and five amino acids as a critical for L-Glu binding on AmGr10, suggesting that the additional residues that are critical for L-Glu and other amino acid recognition remain to be identified in AmGr10. Research should focus on determining the binding site of L-amino acids of AmGr10 using molecular modeling based on the structures of AmGr10 and site-directed mutagenesis assay.

In conclusion, our research begins to overcome challenges in the study of the gustatory tuning of the chemosensory receptors of the honeybee and provides an extension of studies about sweet taste perception in the antennae of the honeybee. We have focused on determining the natural ligands of gustatory

receptors to understand the gustatory system of honeybees. We identified and characterized two sugar-sensing receptors of the honeybee, gustatory receptor 1 and 2. Furthermore, we characterized broadly the response properties of individual members of the sweet chemicals using tip recording. Comparative analyses between queens, workers, and drones should be performed for sugar perception because sugar stimuli may play pivotal roles in the life of the honeybee. Different castes may show different expression levels of gustatory receptor genes depending on their different gustatory environment. Although the insect Grs and mammalian Grs do not resemble each other at the amino acids sequence, our finding demonstrate that these insect gustatory system have a common mechanism for sensing amino acids in the external environment. Our finding in identification the amino acids responsiveness of honeybee Gr paves the way for characterizing the other insect Grs and for a better understanding of the contribution of many Grs to taste perception and the modulation of feeding behaviors in insects

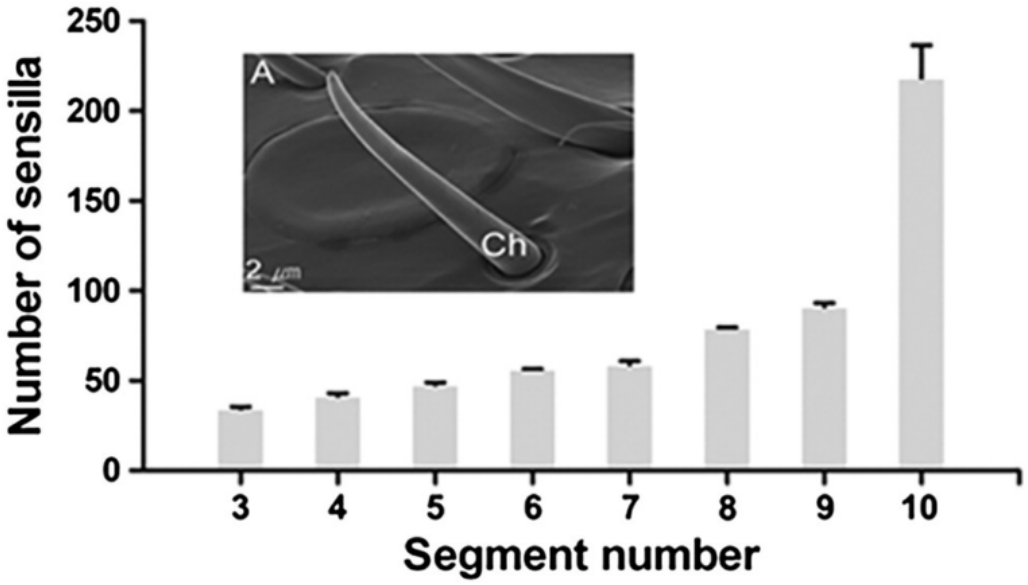


Figure 1. The mean number of sensillum chaetica of *A. mellifera* per antenna segment. (A) Details of sensillum chaetica. Number of sensillum chaetica is higher on the distal segment of antennae. Error bars indicate SEM (n= 3).

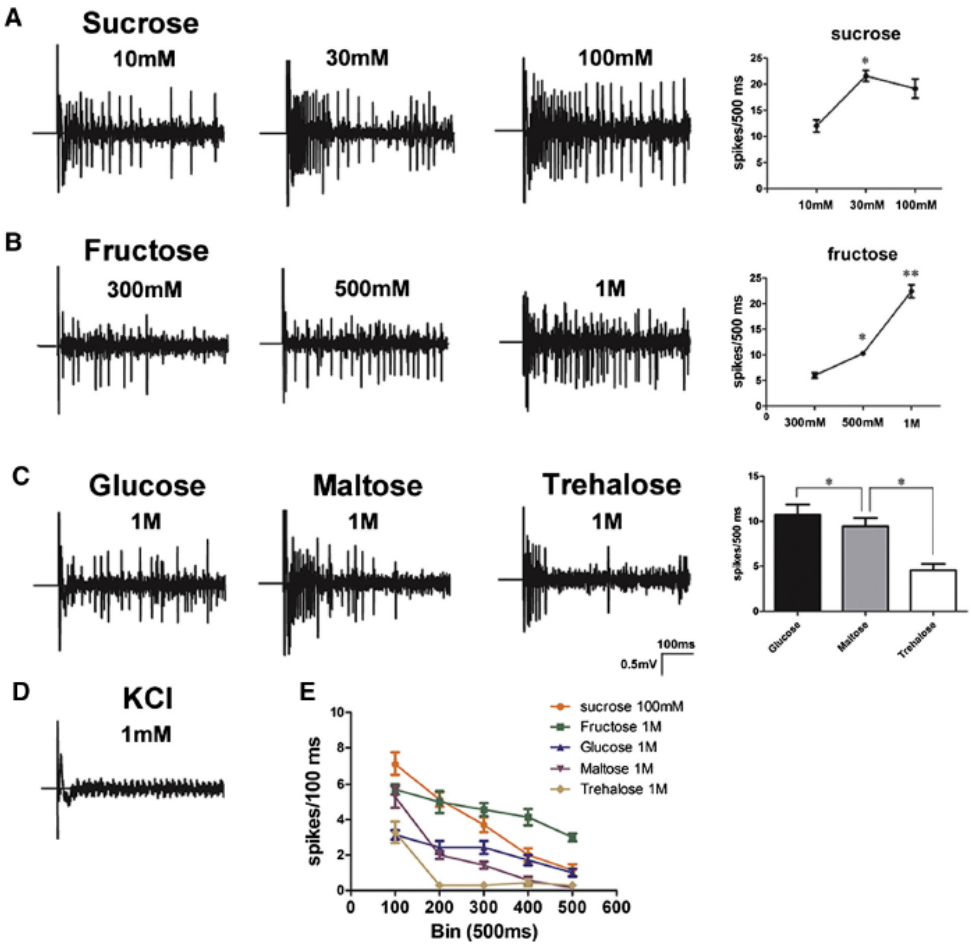


Figure 2. Representative traces of nerve firings from sensilla chaetica and quantification of the mean action potentials induced by the indicated concentration of sugars in the tip of the antenna. (A) Sucrose (10 mM, 30 mM, and 100 mM); (B) Fructose (300 mM, 500 mM, and 1 M); (C) Glucose, Maltose, and Trehalose (1 M); (D) KCl 1 mM; (E) Adaptation of the cell in the first 500 ms of sugar stimulation, expressed as the number of spikes during the first 100 ms (n = 7).

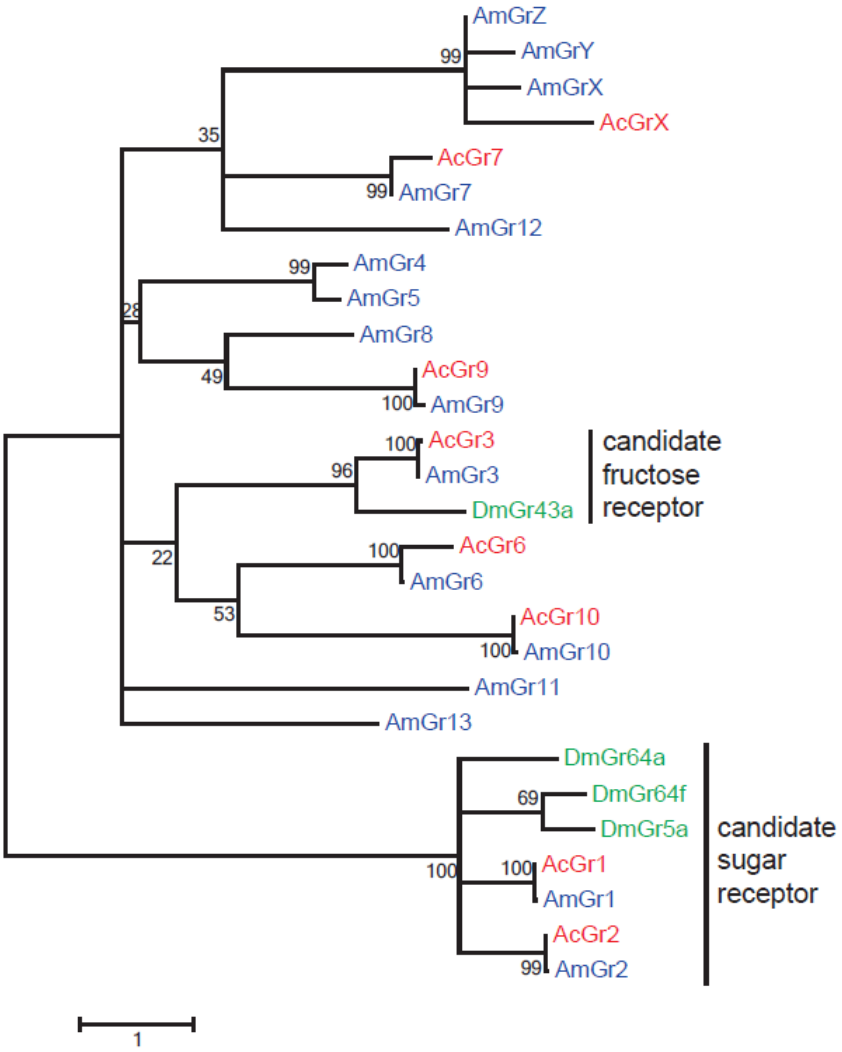


Figure 3. Phylogenetic tree of gustatory receptor proteins of *Apis mellifera*, *Apis cerana* and *Drosophila melanogaster* generated in MEGA5 by using a Neighbor-Joining method (Doori Park et al. 2015). The *Apis mellefera* gustatory receotors in red. Numbers indicate bootstrap values (%). AmGr1 and AmGr2 cluster with *Drosophila melanogaster* sugar receptors family.

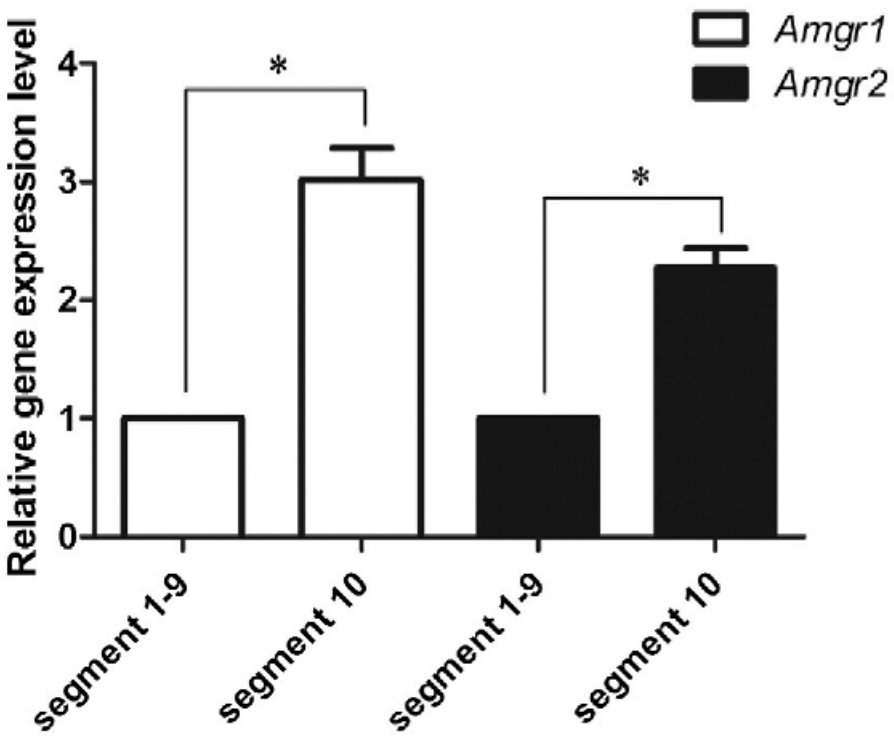


Figure 4. Relative RNA expression levels of gustatory receptor genes 1 and 2 (*AmGr1* and *AmGr2*) in antennae showed significant increases in the distal segment. Values depict mean ± SE. Student t-test was used to test the difference of gene expression levels.

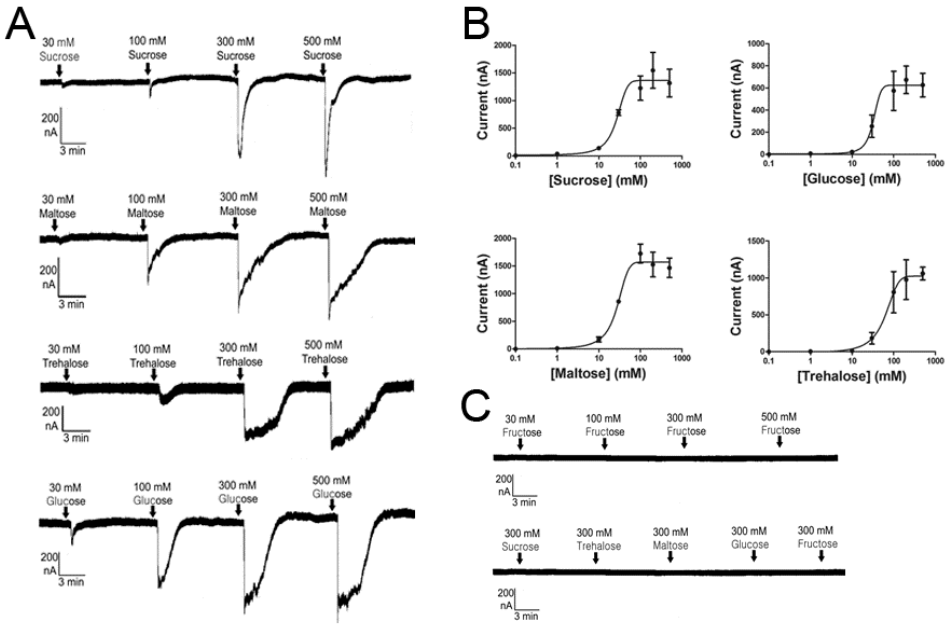


Figure 5. Current traces of Xenopus oocytes expressing AmGr1 alone stimulated with a range of sugar concentrations. (A) Current with AmGr1 was dependent on the dose of the sugars except for fructose. (B) Dose-dependent responses of Xenopus oocytes expressing AmGr1 to sugar substances. Error bars indicate SEM (n= 6). (C) The oocyte expressing AmGr2 showed no response to the sugars. The current responded to stimulations of the indicated concentrations of sugars. The oocyte was stimulated for 10 s as indicated by the black

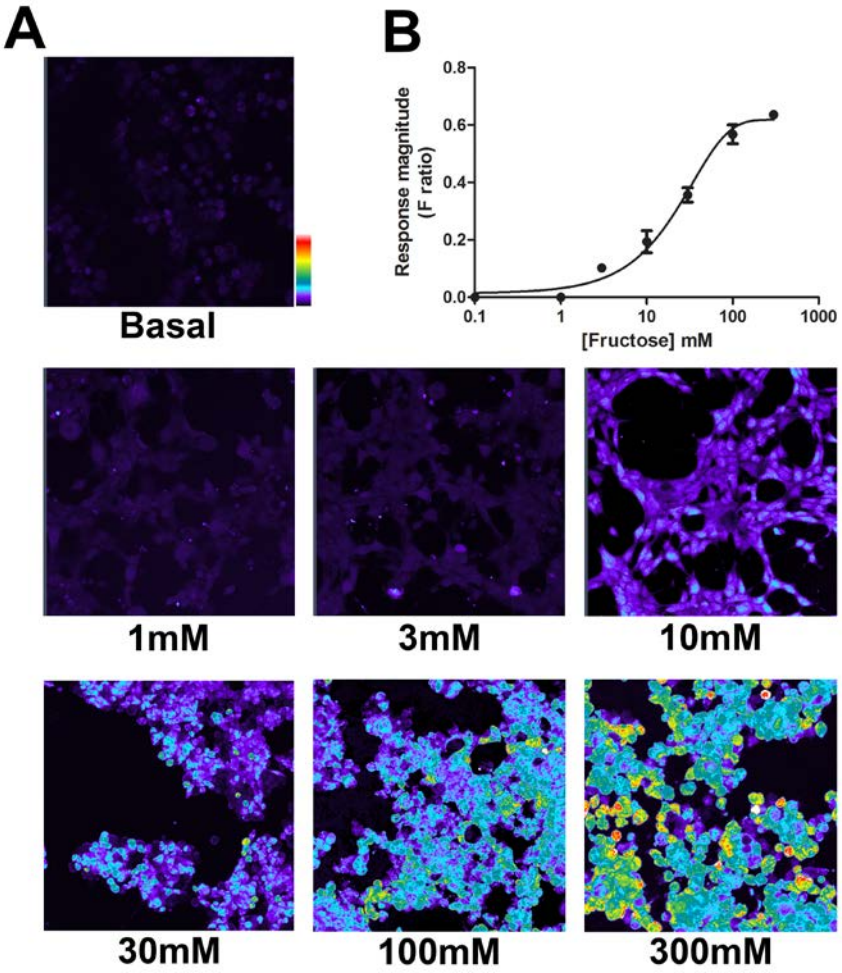


Figure 6. D-Fructose is a ligand for an *A. mellifera* gustatory receptor. (A) Dose-dependent Ca2+ responses of HEK293T cells expressing AmGr3 to D-fructose. The pseudocolored images demonstrate the changes in fluo-4 fluorescence with increasing concentrations of D-fructose where red indicates a cell that shows the greatest response. (E) Dose-response curve of AmGr3 to D-fructose based quantitative analysis of Ca2+imaging. The curve was fitted to the Hill equation (n=7; EC50= 32mM).

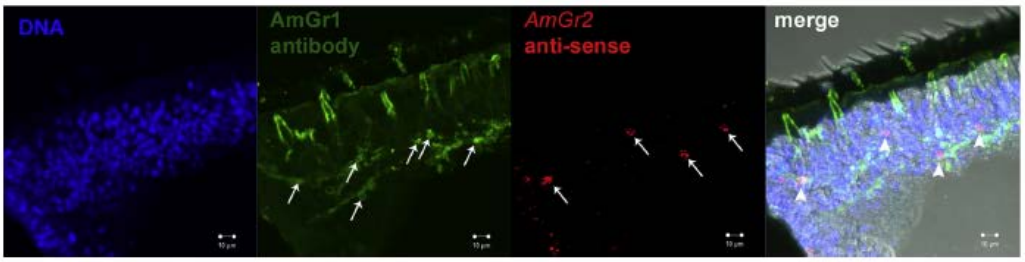


Figure 7. Localization of gustatory receptors 1 and 2 in the antennae of A. mellifera. In situ hybridization with antisense RNA probes of *gustatory receptor 2* (red, white arrows), where gustatory receptor 2 of *A. mellifera* was co-localized with gustatory receptor 1 (green) in the antennae of honeybee; however, there is also a fraction of cells with non-overlapping expression of AmGr1 or AmGr2 in the antennae. Scale bars demonstrate 10 μm.

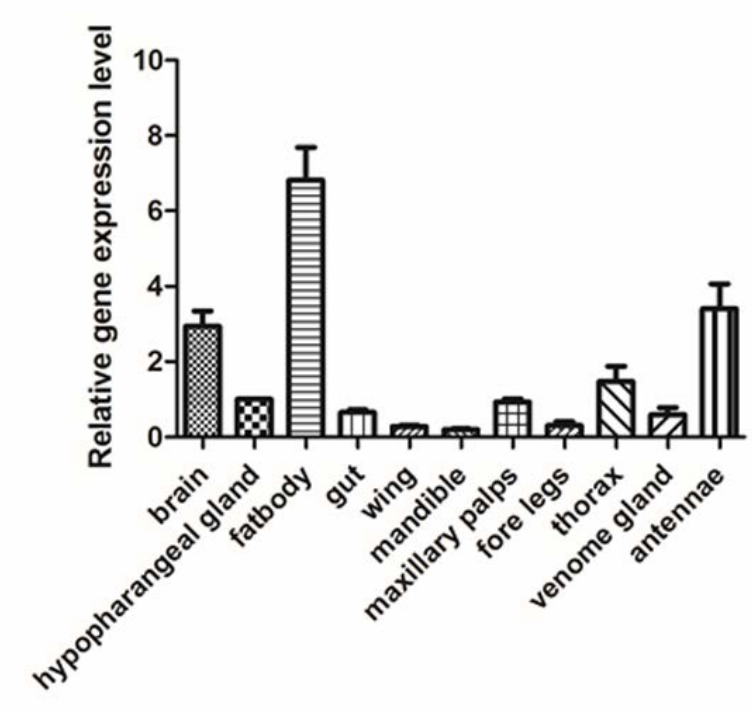
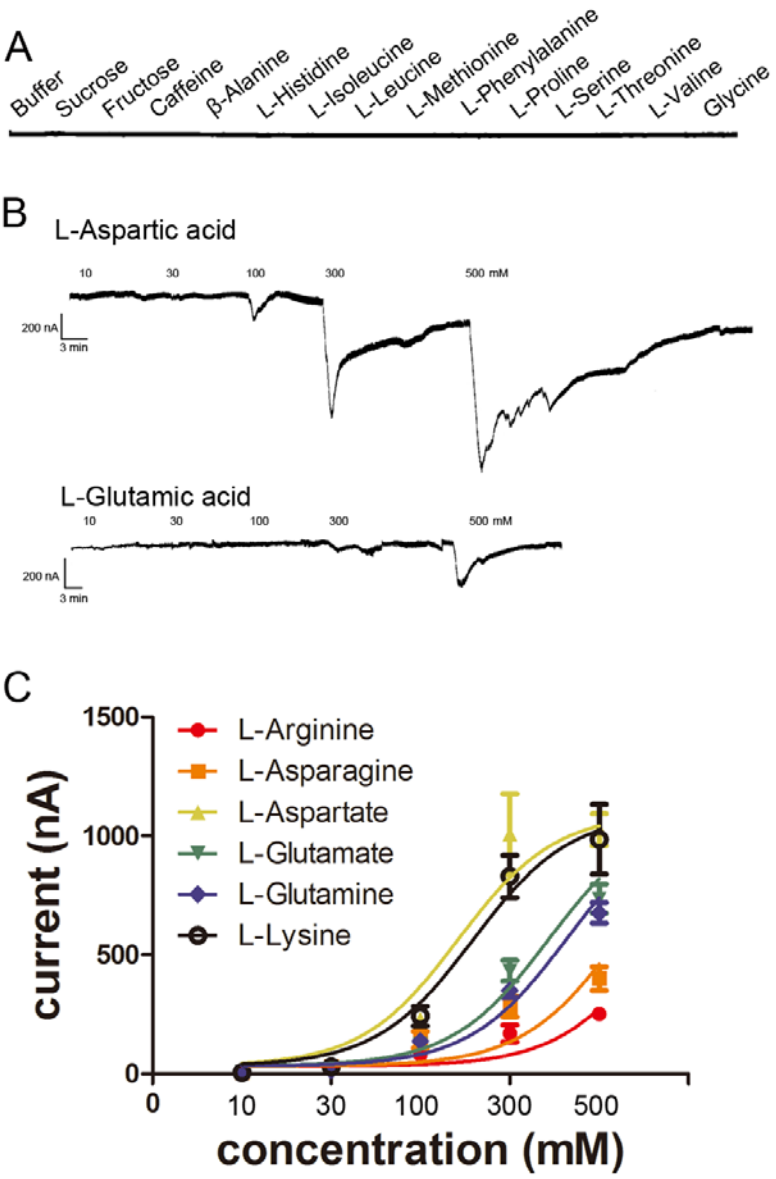


Figure 8. Gustatory receptor 10 gene expression in various tissues in *Apis mellifera*. Quantitative real-time PCR analysis of AmGr10 in organs of worker bees including brain, hypopharangeal gland, fat body, gut, wing, mandible, maxillary palps, fore legs, thorax, venome gland, antennae.


A
Buffer
Sucrose
Fructose
Caffeine
β-Alanine
L-Histidine
L-Isoleucine
L-Leucine
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Valine
Glycine
B
L-Aspartic acid
10
30
100
300
500 mM
200 nA
3 min
L-Glutamic acid
10
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100
300
500 mM
200 nA
3 min
C
1500
1000
500
0
current (nA)
L-Arginine
L-Asparagine
L-Aspartate
L-Glutamate
L-Glutamine
L-Lysine
0
10
30
100
300
500
concentration (mM)

Figure 9. Resoponses of *Xenopus* oocyte with AmGr10 to stimulation with amino acids. (A) The current traces recorded from AmGr10-expressing Xenopus oocytes with sequential application of various tested compounds. AmGr10 *Xenopus* oocytes fail to respond to any of sugar, bitter substances and some amino acids. (B) Inward current responses of AmGr10 *Xenopus* oocytes stimulated with a range of L-Asp and L-Glu concentration at the holding potential of -70mV. (C) Dose-response profile of AmGr10 *Xenopus* oocytes to 6 amino acids. The curve was fitted to the Hill equation. Error bars indicate SEM (n=7).

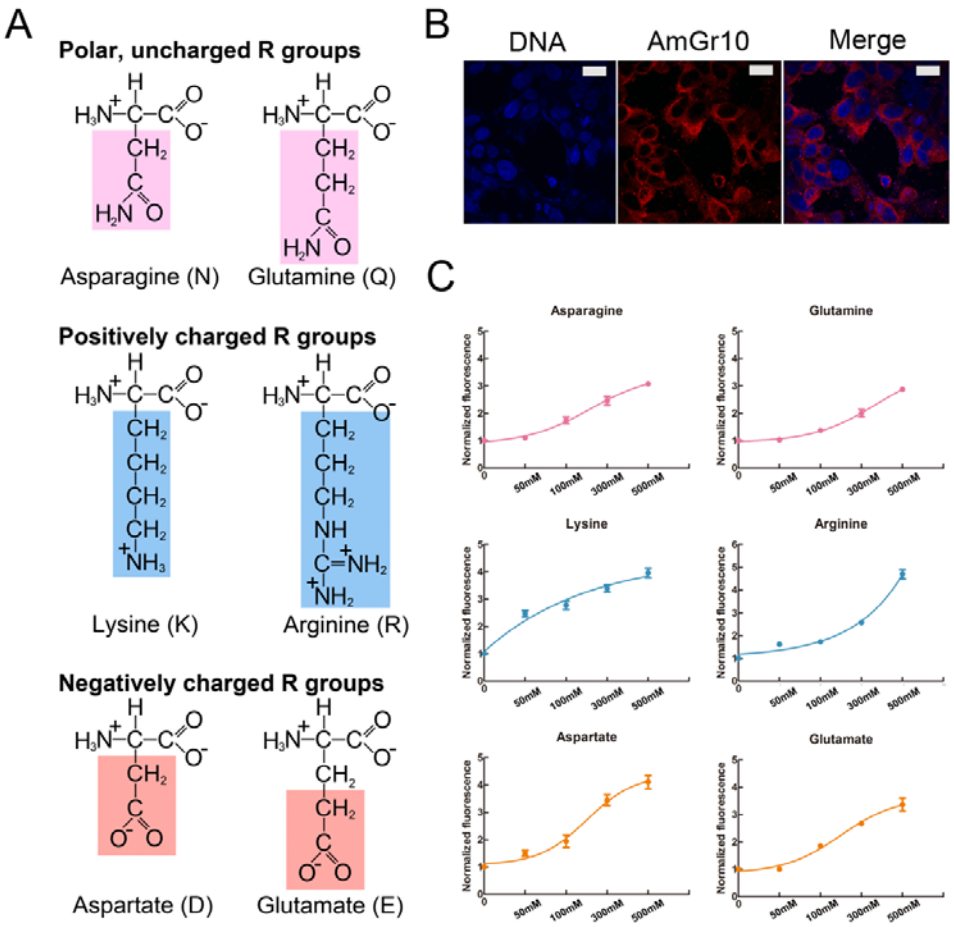

Figure 10. Expression of AmGr10 protein in cells and Ca2+ signaling assay. (A) Structure of 6 amino acids. (B) Immunofluorescence of AmGr10-expressing HEK293T cells. The red fluorescence represents AmGr10, which indicates apparent staining in the plasma membrane. (C) Dose-dependent measurement of intracellular calcium changes using Fluo-4 in HEK293T cells expressing AmGr10 stimulated with 6 amino acids. The Y axis represents the normalized response which is shown as the change of fluorescence ratio relative to the ratio of control (n=9). Each point represents the mean ± SEM.

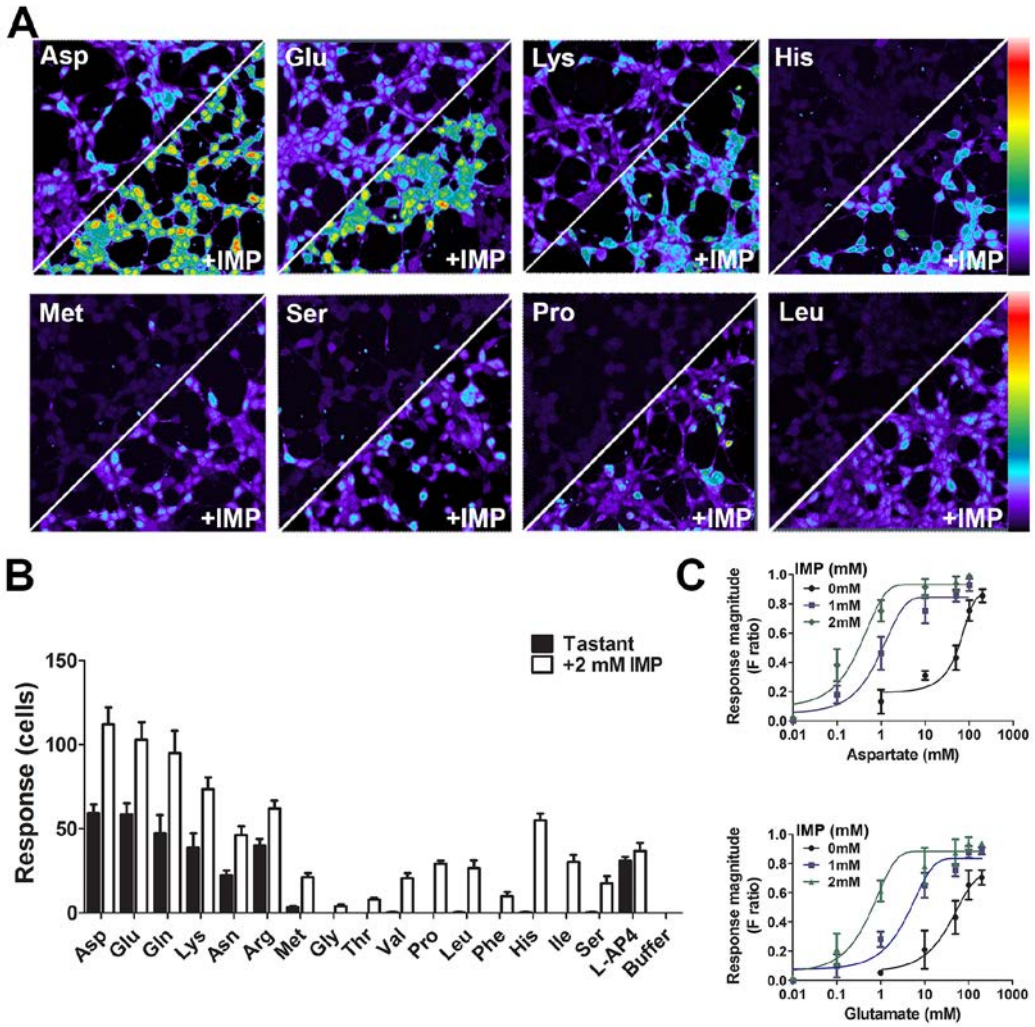

Figure 11. AmGr10 responds to L-amino acids. (A) HEK293T cells expressing AmGr10 was activated by L-amino acids (left) and responses were potentiated by IMP (right). Amino acids were 50mM and IMP was 2mM; the color scale indicates the F ratio. (B) Quantification of amino acid responses for AmGr10. (C) Dose responses of AmGr10 to L-asp, L-Glu, and IMP. The presence of 2mM IMP shifts the responses by at least one order magnitude to the left. Each point represents the mean ± SEM of ten assays.

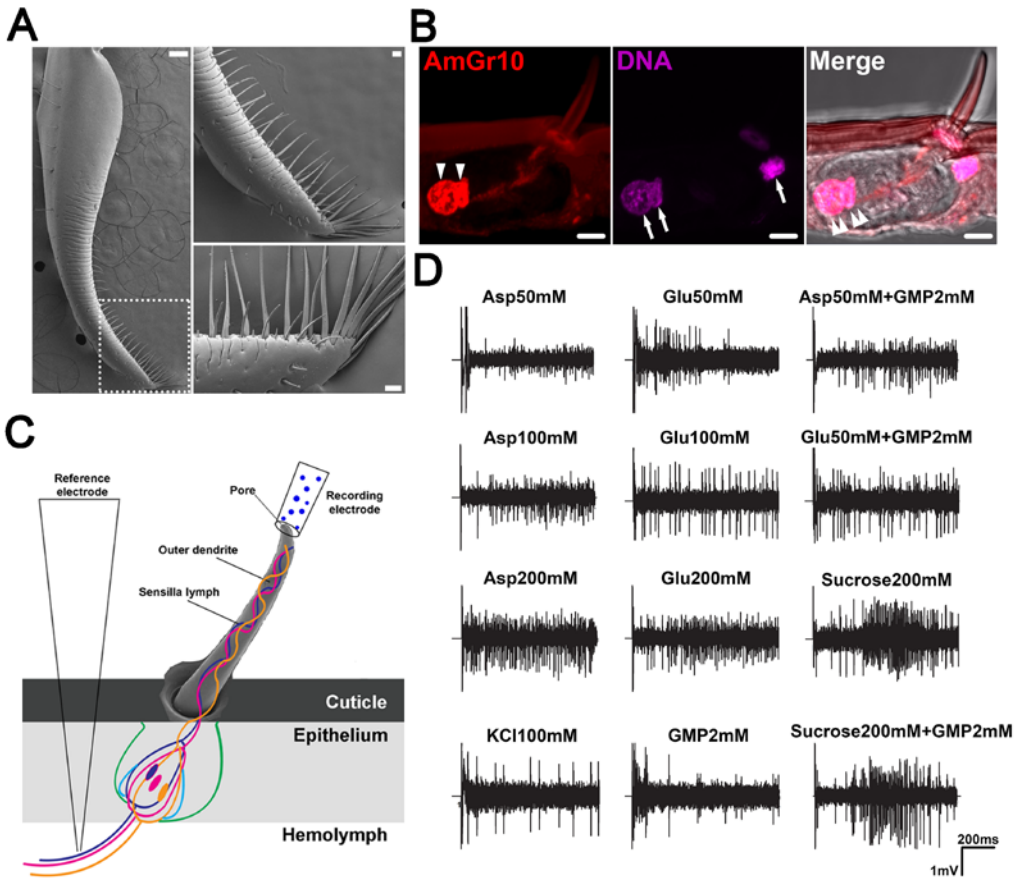

Figure 12. Honeybees are sensitive to L-amino acids in KCl solution. (A). The structure of the honeybee's proboscis revealed by scanning electron microscopy: the galea of the two maxillae, and the labium comprised of the two labial palps (LP) attached to the glossa. (B) AmGr10 antibody (red) was localized to the distal part of galea, where sensillum chaetica was innervated by Gr10-expressing neurons. (C) Schematic diagram of honeybee tip recording experiment. (D) one-second tip recordings were made from the galeal sensilla. Honeybee are more sensitive to the stimuli amino acids with IMP than amino acid alone.

Chapter IV

Discrimination of Umami Substances using Floating Electrode Sensor mimicking Insect Taste Systems

This chapter was submitted in ACS NANO.

Abstract

We report a floating electrode sensor mimicking insect taste systems for the detection and discrimination of umami substances. Here, carbon nanotube field-effect transistors (CNT-FETs) with floating electrodes were hybridized with nanovesicles containing honeybee umami taste receptors, AmGr10. This strategy enables us discriminate between umami and non-umami substances. This sensor could also be utilized for the detection of L-Monosodium glutamate (MSG) in real samples. Moreover, we demonstrated the synergism between MSG and disodium 5'-inosinate (IMP) for the umami taste using this platform. This floating electrode sensor mimicking insect taste systems can be a powerful platform for the detection of various umami tastants. Furthermore, our research could facilitate various applications in food industry and give people information about insect taste systems.

Keywords: umami, carbon nanotube, nanovesicle, honeybee umami taste receptor, bioelectronic sensor

Introduction

The sense of taste is closely associated with human life, because it provides important information for the research of nature and food quality, and so on. Moreover, it would have a wide range of applications in the food industry (Nelson et al. 2002),(Song et al. 2014). Sweet, sour, salty, and bitter tastes are well known to many people as basic taste qualities. Umami, however, was not recognized as one of the basic taste qualities for more than 100 years because its biological mechanism was discovered in the early 2000s even though it was first described in 1908 by Kikunae Ikeda.(Blacquière et al. 2012),(Nakamura 2011). In humans and specific species of animals, L-Monosodium glutamate (MSG) and 5'-ribonucleotides such as disodium 5'-inosinate (IMP) and disodium 5'-guanylate (GMP) are known to cause the umami taste (Nakashima et al. 2001). These are widely present in natural foods such as meat and fish. The most noticeable feature of the umami taste is a synergism between MSG and IMP or GMP. It indicates that 5'-ribonucleotides can strongly enhance the umami taste intensity. In other words, the 5'-ribonucleotidescould decrease the threshold of response to MSG.(Nakashima et al. 2001), (F. Zhang et al. 2008a). Recently, various MSG sensors based on nanomaterials have been intensively developed.(Khan, Gorski, and Garcia 2011)(Koh et al. 2008). However, these methods sometimes suffered

from some limitations. For example, in a previous method, carbon nanotubes were functionalized with glutamate oxidase to detect MSG, but this method should be performed in only around physiological pH conditions (~pH 7.4) due to the enzyme function. And, this sensor could detect MSG down to 200uM (Koh et al. 2008). Moreover, these previous methods did not show the synergism that is the hallmark of the umami taste. However, it is important to detect MSG with high sensitivity because it has been informed that excessive exposure to MSG could cause health problems such as numbness, weakness and so on.(Geha et al. 2000)(Krishna et al. 2010)(Williams and Winfield, n.d.)(Khan, Gorski, and Garcia 2011). On the other hand, it has been reported that the combination of floating electrode structures and a CNT-FET could enhance the sensor sensitivity, caused by increased number of Schottky barriers (J. Lee et al. 2012). Thus, the floating electrode sensor could be utilized for discrimination of umami substance with high sensitivity.

Taste and the gustatory sensory system in insects play an important role in identifying potential foods by discriminating nutritious rich substrates, such as sugars and amino acids that promote feeding, from harmful, mostly bitter-tasting compounds that inhibit feeding (Yarmolinsky, Zuker, and Ryba 2009). Although mammalia and insecta have the independent evolutionary origins for taste sensory systems and distinct anatomic and molecular pathways, the cellular metabolism to

identify nutritional food sources and avoid intoxication is largely conserved (Amrein and Thorne 2005)·(Liman, Zhang, and Montell 2014). Honeybee which has been a central insect research model for the study of chemosensory perception and learning can detect a repertoire of taste qualities similar to human. Honeybee gustatory receptors are expressed in gustatory receptor neurons (GRNs), which can discriminate between sweet and bitter tastes, respectively (A. Dahanukar et al. 2007), (Jiao, Moon, and Montell 2007)·(Sato, Tanaka, and Touhara 2011), (Y. Lee, Moon, and Montell 2009)·(Wang et al. 2004). Although, the project of genome of honeybee has revealed 10 gustatory receptor genes (Weinstock et al. 2006), the study on determining the natural ligands of these gustatory receptors remains unknown. Recently, our previous study showed that in *Apis mellifera*, gustatory receptor 1 and 2 (AmGr1 and Gr2) responded to sweet taste substances(Jung et al. 2015). However, taste receptors for bitter and umami substances are unknown in honeybee species. Here we identify a honeybee umami taste receptor. Using calcium imaging assay, we find that *Apis mellifera*, gustatory receptor 10 (AmGr10) a selectively tuned umami substances but not to respond other compounds.

Herein, we report floating electrode sensor mimicking insect taste systems for the discrimination of umami substances through hybridization with nanovesicles containing honeybee umami taste receptors. First, we fabricated

carbon nanotube field effect transistor (CNT-FET) with floating electrodes by standard photolithography processes. Then, the floating electrodes on the device were functionalized with the nanovesicles containing AmGr10. This sensor discriminated MSG from non-umami substances with high sensitivity and selectivity. Also, we could detect MSG in real samples without any additional treatment. Significantly, we investigated the synergism of various umami substances, which indicated that the synergism enables the mixture of MSG and IMP to react to AmGr10 at lower concentrations than MSG only. Furthermore, our research should provide the broader opportunity to develop various applications in food industry and to study the insect taste systems.

MATERIALS AND METHODS

Materials

Single-walled semiconducting 99% carbon nanotubes (ssCNTs) were purchased from NanoIntegris Inc. (USA) and used in our experiments. MSG, IMP, sucrose, glucose, PTC, and other chemical reagents were purchased from Sigma-Aldrich (USA) and used.

Honeybee preparations

Honeybee species, *A. mellifera* were maintained on apiaries of Seoul National University campus surrounded by the Gwan-ak mountain range in Seoul, Korea. For cDNA synthesis, worker honey bees were captured directly from three hives regardless of age. They were placed in glass vials and cooled in ice until they stopped moving.

HEK-293 cell culture

Human embryonic kidney-293 (HEK-293) cells were cultured at 37 ℃ under 5% CO_2. Dulbecco's modified Eagle medium (DMEM, Invitrogen, CA, USA) containing 10% fatal bovine serum and 0.5% penicillin-streptomycin (Invitrogen, CA, USA) was used to culture HEK-293 cell.

RNA extraction, cDNA synthesis, RT-PCR

Total RNAs were extracted from the antennae of honeybee using a Qiagen

RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA), after which RQ1 RNase-free DNase I was applied according to the manufacturer's instructions (Promega, Madison, WI, USA). Reverse transcription procedures were carried out as described previously (Jung et al. 2013).

Heterologous expression of AmGr10 into HEK-293 cells

The expression vector was synthesized by inserting the cDNA for AmGr10 into the multiple cloning site of the pcDNA3.1 vector using the restriction enzymes EcoRI and NotI (Koscamco, Anyang, Korea). Template pDNA for pcDNA3.1-Gr10 and primer were then mixed with kit solutions and the total PCR reaction volume and conditions were followed as described earlier (Jung et al. 2015). 1 μg of the vector as a combination AmGr10 was transfected to the HEK293 cells by using Lipofectamine 2000 (Invitrogen, CA, USA). Transfection method was according to the manual. In brief, the Opti-MEM (Invitrogen, CA, USA) was mixed with Lipofectamine at the rate of 2 $\mu\ell$ in 50 $\mu\ell$ Opti-MEM and incubate for 5 min on room temperature. The plasmid DNA 1 μg also blended with 50 $\mu\ell$ Opti-MEM media. Two mixed media was combined and incubated for 20 min on room temperature. The solution was applied on the HEK cells. After 24 h, The cell was selected using concentration of 200 μg/ml of zeocin antibiotics (Invitrogen, CA, USA). Selected cell were cultured on blended media with 20 μg/ml zeocin.

Construction of nanovesicles from HEK-293 cell expressing honeybee umami receptor

Suspended HEK-293 cells expressing honeybee umami receptors in DMEM containing cytochalasin B (20 μg/mL, sigma, USA) were incubated at 37 ℃ with 300 rpm agitation. Cells and cell debris were separated by centrifugation at 2000g for 20 min, and nanovesicles were collected by centrifugation at 12000g for 30 min. The nanovesicles were suspended in PBS containing a protease inhibitor cocktail (Sigma, USA). Produced nanovesicles were used immediately or stored at -80 ℃for the subsequent experiments.

Western blot

Rat polyclonal antibodies against AmGr10 were generated by the Abclone company (Seoul, Korea). Based on peptide information of AmGr10 (GenBank: NP_001229923.1) and the sequence alignment of peptide, a target epitope region was chosen as NH2- SMNTQILIFVCILFLIE -C to produce polyclonal anti-AmGr10 antibody. Rats were immunized three times with 0.5 mg of the synthesized AmGr10 peptide, after which serum-specific antibody was affinity-purified on columns using immobilized antigen peptides. For western blotting, transfected cells containing honeybee umami taste receptors were homogenized in 1 ml of ice-cold 0.1 M Tris–HCl buffer (pH =7.8 with 0.5% Triton X-100 and 1 μl

protein inhibitor cocktail). Electrophoresis was carried out with a vertical electrophoresis unit (Novexmini cell, Invitrogen). Protein preparation from the cell was separated by SDS-PAGE (10%) in triplicate at 130 V for 100 min in a chamber. Proteins separated on the gel were transferred onto a nitrocellulose membrane (Whatman, USA) by electroblotting. After blocking in PBS buffer containing 0.1% Tween-20 and 5% fat-free dry milk for 1 h at room temperature, the membrane sheets were incubated for overnight at 4 °C with primary AmGr10 antibody. The membrane was then incubated with the horseradish peroxidase conjugated with anti-rabbit IgG secondary antibody for 3 hrs. The antigen antibody complex was visualized using a chemiluminescence kit according to the manufacturer's instruction (Young In Frontier Co.,Seoul, Korea).

Intracellular Calcium assay

For calcium signaling assay, HEK-293 cells expressing AmGr10 were cultured for more than 3 days, and Fluo-4 NW dye mix solution (Molecular probes) was loaded into the cell in 96-well microplate. After incubation at 37℃ for 1 hour, the fluorescence signal upon the addition of MSG (30, 50, 100, 300 and 500 mM) was measured at 516 nm by excitation at 494 using a spectrofluorophotometer (Perkin Elmer, USA). To normalize the response, the changes of fluorescence ratio were divided by maximal fluorescence changes induced by each compounds. To

perform calcium assay of nanovesicles, nanovesicles expressing AmGr10 were immobilized on poly-D-lysine-treated 96 well plates by incubation at 37℃ for 2hrs. The procedure of calcium assay upon 10mM MSG was same for the cells expressing AmGr10 described above.

Calcium imaging

Transfected cell lines were subsequently cultured on confocal dishes (SPL, Pocheon, Korea). Before experiments, the culture media was removed and 100 μl of 2 μM Fluo-4 AM were loaded and directly replaced (Invitrogen, Grand Island, NY, USA), after which the cells were incubated in the dark for 90 min at room temperature. After incubation, 1X HBSS buffer was added onto the confocal dish, which was then directly placed in the LSM700 inverted confocal microscope for observation (Zeiss, Oberkochen, Germany). Images were captured maximum 100 frames per every two second interval. Test chemicals were dissolved in HBSS at various concentrations. Calcium influx into the transfected cells upon ligand binding was monitored and analyzed with ZEN software (Zeiss, Oberkochen, Germany). Fluorescent intensity changes were calculated as follows(Sato, Tanaka, and Touhara 2011): Intensity F = (Fafter – Fbefore) / F max.

Fabrication of a CNT-FET with Floating Electrodes

First, ssCNTs were dispersed in 1,2-dichlorobenzene using ultrasonic cleaner for 5

hours. The concentration of ssCNT solution was 0.05 mg/ml. For assembly ssCNTs, an octadecyltrichlorosilane self-assembled monolayer with nonpolar terminal groups was patterned on SiO2 substrate (3000Å) using photolithography as previously reported (M. Lee et al. 2006). For selective adsorption CNTs, the substrate was placed in the solution of ssCNTs for 3 s and rinsed with 1,2-dichlorobenzene.Afterward, source, drain, and floating electrodes were fabricated using the photolithography and thermal evaporation (Pd/Au 10 nm/15 nm). The CNT network channel has dimensions of 3 um width and 154 um length. Each floating electrode has dimensions of 10 um width and 200 um length. Lastly, the source and drain electrodes were covered by a passivation layer (photoresist DNR) to eliminate leakage current during the electrical measurements in aqueous environments.

Immobilization Nanovesicles on the Floating Electrodes of the CNT-FET

Nanovesicles containing AmGr10 were dissolved in 2-iminothiolane•HCl solution (100 mM in Dulbecco`s Phosphate Buffered Saline) for the formation of thiol groups. And the2-iminothiolane•HCl solution including the nanovesicles were incubated for 1.5 hours at room temperature. Then, the CNT-FETs with floating electrodes were incubated in the solution containing AmGr10 expressed

nanovesicles for 3.5 h 4 ℃ in order that the nanovesicles were selectively attached onto the gold floating electrodes of the CNT-FETs. As a result, the nanovesicles with thiol groups were successfully immobilized oh the floating electrodes of the CNT-FETs.

Electrical Measurements

For the detection of signal derived from various target materials, a CNT-FET with floating electrodes was connected to a Keithley 4200 semiconductor analyzer. And a 9 μL droplet of DPBS buffer was placed on the channel region. A 100 mV bias voltage was applied and maintained during electrical measurements. Then, source-drain currents were monitored while introducing various target materials. In our experiments, G/G_0 was defined as the conductance change over the original conductance of the device.

Preparation of Tastants

MSG, IMP, sucrose, glucose, and PTC used in our experiments were dissolved in DPBS solution. For chicken stock solutions, the chicken stock which is available commercially was diluted at different ratios with the DPBS solution. Also, the mixture of MSG and IMP was prepared to measure the synergism according to the previously reported method (Shizuko Yamaguchi et al. 1968).

RESULTS AND DISCUSSION

Figure 1 shows a schematic diagram describing a floating electrode sensor hybridized with nanovesicles containing honeybee umami taste receptors, AmGr10, for the detection of umami substances. First, we fabricated a CNT-FET with floating electrodes by conventional photolithography processes. Briefly, octadecyltrichlorosilane self-assembled monolayer was patterned on SiO_2 substrate using photolithography for the alignment of CNTs. And, the substrate was placed in a solution of CNTs for the selective adsorption of CNTs. Then, source, drain, and floating electrodes were fabricated by photolithography and thermal deposition.The size and the number of floating electrodes can be easily adjusted by the photolithography process. Finally, the source and drain electrodes were covered by a passivation layer to prevent leakage currents. Following the fabrication of the CNT-FET with floating electrodes, we immobilized the nanovesicles on the floating electrodes of the device. In here, HEK293 cells were transfected with a pCDNA3.1 vector containing AmGr10 and the cells expressing AmGr10 were selected using antibiotics. To extract and obtain the nanovesicles from the cells expressing AmGr10, these cells were treated by cytochalasin B and were incubated with agitation. After a centrifugation, nanovesicles were collected and suspended in Phosphate buffered saline (PBS).

Figure 2a is the field emission scanning electron microscopy (FE-SEM)

image of nanovesicles immobilized on a gold surface. The nanovesicles were lyophilized and then coated with platinum (10 nm) using sputter coater. This result indicates that the nanovesicles were immobilized relatively uniformly on the gold surface with a uniform diameter of ~200nm.

Figure 2b shows a western blot analysis for the confirmation of AmGr10 expression. In brief, we expressed AmGr10 in HEK-293 cells and performed calcium assay and imaging to investigate the activation of intracellular Ca2+ influx. First, the expression of AmGr10 protein in HEK-293 cell was confirmed from the lysates of transfected with HEK-293 cells by using western blot analysis. Lane 1 represents the data from AmGr10-expressing cells or nanovesicles derived from them, while lane 2 represents the data from control cells or nanovesicles derived from them. The 42kDa band was expressed in the membrane from the AmGr10-expressing cells and nanovesicles compared with non-transfected with control cells and nanovesicles. These data indicates that honeybee taste receptor composed of AmGr10 was expressed in HEK-293 cells and nanovesicles. When the AmGr10 was successfully expressed in HEK cells, we determined that the cell derived-nanovesicles contain a sufficient amount of taste receptors. To investigate the functional activity of AmGr10, we carried out the measurement of intercellular calcium changes in AmGr10-expressing HEK-293 cells upon stimulation with various taste ligands. The HEK-293 cells expressing AmGr10 exhibited Ca2+

responses to MSG in a dose-dependent manner and a threshold concentration of 30mM which was measured by calcium indicator Fluo-4 using spectrofluorophotometer (Figure 2c). However, intracellular signal transduction did not occur to sugar and bitter compounds in transfected cells with AmGr10, as shown in Figure S1 of the Supporting Information. We performed calcium image analysis to identify whether AmGr10-mediated Ca2+ influx could also activate calcium signal in nanovesicles that were constructed from the HEK-293 cells expressing AmGr10. Note that the treatment of MSG (10mM) to AmGr10-expressing nanovesicles triggered the immediate increase in fluorescence ratio compared to the control experiment data, nanovesicles without receptor (Figure 2d). These data suggested that AmGr10 expressed on the plasma membrane of the cell was functional, and thus, the AmGr10-expressing nanovesicles could respond to the characterized agonist. We could not observe the recovery of calcium signaling to the baseline in nanovesicles that lacked ion pumps and calmodulines which were necessary to restore the Ca2+ concentration (Dong, Dunn, and Lytton 2002). Figure 3a shows the real-time response to various concentration of MSG obtained by utilizing our floating electrode sensor hybridized with nanovesicles containing AmGr10. A bias voltage was applied and maintained during electrical measurements. Then, source-drain currents were monitored after the introduction of the MSG solution to the sensor. As shown in the Figure 3a, the introduction of

MSG resulted in the increase of channel conductance. Our sensor began to respond to MSG at a concentration of 100pM. Meanwhile, the response to MSG was saturated with MSG at a concentration of 10mM.

Figure 3b shows the normalized sensitivity of the floating electrode sensor hybridized with AmGr10-expressing nanovesicles to various concentrations of MSG. Here, the normalized sensitivity was calculated following the method reported previously (Jun et al. 2012). The normalized sensitivity increases as the concentration of MSG increases, and it was saturated at high concentrations.

We performed experiments for the determination of selectivity. Figure 3c shows the real-time responses of our sensor to various tastants such as sweetener, sucrose, and bitter compound, phenylthiocarbamide (PTC). As shown in this graph, the injection of MSG (10 pM) caused sharp increase in the conductance of ~ 3%, while the additions of the sucrose and PTC with high concentrations (100 nM) did not have effects on the conductance at all. Also, the addition of the other sweetener, glucose (100 nM), did not result in the conductance change, as shown in Figure S1 of the Supporting Information.

To demonstrate the capability of our floating electrode sensor mimicking insect taste systems for practical applications, we carried out the experiments to detect MSG in real samples, chicken stock, without any additional treatment. The

chicken stock solution which is easy to get was diluted with Dulbecco`s Phosphate Buffered Saline (DPBS) to make watery chicken stock. Figure 4d shows that the responses of our sensor to diluted chicken stock at different ratios. The addition of the chicken stock solution caused a significantly increase in the conductance. This indicates that the result of real samples is similar to the result of real-time response to MSG in Figure 3a. It suggests the possibility that our floating electrode sensor mimicking insect taste systems will utilized as a sensor for practical applications.

Furthermore, we investigated the synergism between MSG and IMP which is the outstanding characteristic of umami (Yoshii, Yokouchi, and Kurihara 1986),(Nelson et al. 2002). IMP can strongly potentiate the umami taste intensity in AmGr10. Previous electrophysiological studies showed that taste responses to L-amino acids are considerably potentiate by purine nucleotides such as IMP in rats (Yoshii, Yokouchi, and Kurihara 1986). To examine the effect of IMP, HEK-293 cells expressing AmGr10 were stimulated with MSG in the presence or absence of IMP (Figure 4a). HEK-293 cells expressing AmGr10 showed Ca2+ response to MSG in a dose-dependent manner with an EC50 value of 75.0 mM and threshold concentration of 5.0 mM. Indeed, AmGr10 responses to MSG were dramatically enhanced by low doses of IMP. However, IMP alone had no effect on responses to MSG stimuli.

We also examined the synergism by utilizing the floating electrode sensor mimicking insect taste systems. Figure 4b shows the normalized sensitivity of the sensor to the mixture of MSG and IMP compared with MSG only. To verify the synergism, we prepared the mixture of MSG and IMP as following the previous research (Shizuko Yamaguchi et al. 1968). Here, the concentration of IMP remained constant which is 2.5 nM, and that of MSG increased. This result indicates that the synergism can lead responses between AmGr10 and MSG at lower concentrations. As shown in Figure 4d, the responses to MSG could enhanced significantly by the synergism between MSG and IMP. Therefore, the floating electrodes sensor mimicking insect taste systems successfully demonstrated the synergism which is the hallmark of umami.

CONCLUSIONS

We successfully developed the floating electrode sensor mimicking insect taste systems for the discrimination of umami substances using CNT-FETs and nanovesicles containing honeybee umami taste receptors, AmGr10. This sensor recognized MSG down to 100 pM with high sensitivity, and discriminated between MSG and other non-umami substances with high selectivity. Also, the sensor could perceive the presence of MSG in real samples. Importantly, we could verify the synergism between MSG and IMP using this sensor. In light of these, our floating electrode sensor mimicking insect taste systems could be utilized as a sensor with high sensitivity and selectivity in many various fields. Our research could get over the limitations of previous works in terms of the sensitivity and less restrictive experiment conditions. Moreover, our research could lead to more opportunities to develop various applications in food industry and to study the insect taste systems.

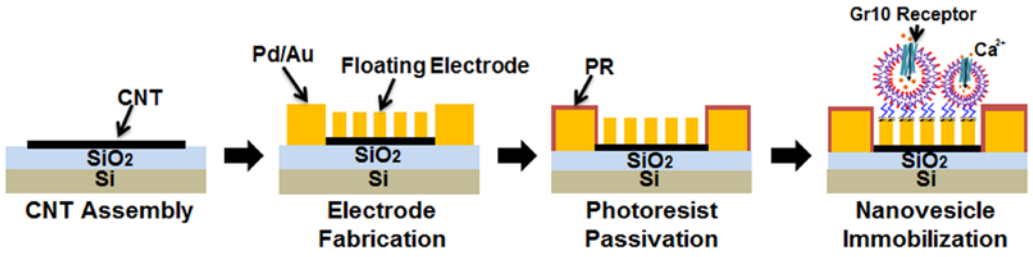

Figure 1. Schematic diagram depicting the fabrication process of floating electrode sensors hybridized with nanovesicles containing honeybee umami taste receptors (AmGr10). CNT-FETs with floating electrodes were fabricated by simple photolithography processes. The nanovesicles containing AmGr10 functionalized with thiol groups were immobilized on the gold floating electrodes.

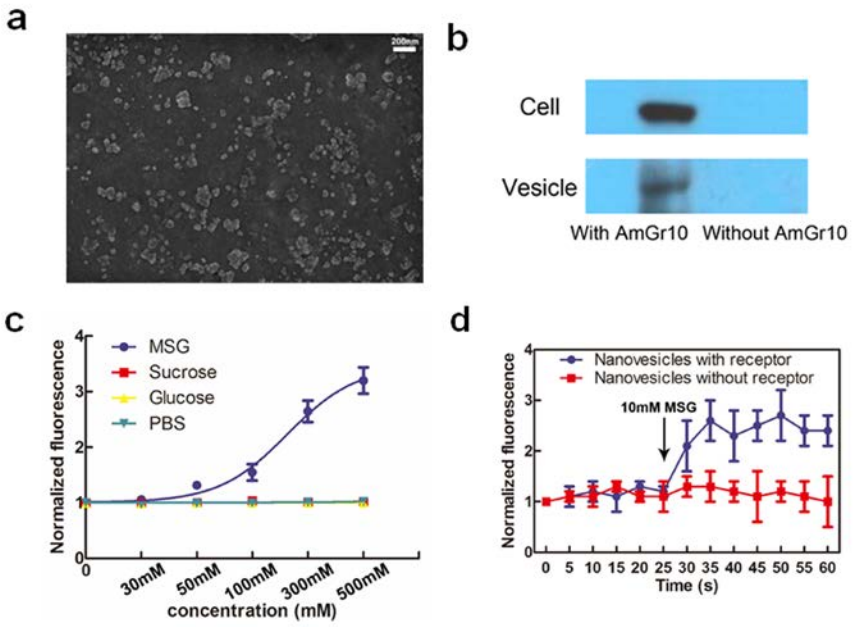

Figure 2. Expression of AmGr10 in HEK-293T cell and nanovesicles. (a) FE-SEM image of nanovesicles on the gold substrate. (b) Western blot analysis of AmGr10 protein expression in HEK-293T cell transfected with AmGr10 and the cell-derived nanovesicles. AmGr10-transfected cell and nanovesicles (with AmGr10) showed the specific band corresponding to the molecular weight of AmGr10, while the control cells and nanovesicles (without AmGr10) did not show the band. (c) The dose-dependent intracellular Ca2+ assay using fluo-4 in HEK-293T cells expressing AmGr10. The fluorescence intensity caused by Ca2+ influx increased as the concentration of MSG increased in the HEK-293T cell. (d) Real-time measurement of Ca2+ assay in the nanovesicles containing AmGr10. The addition of 10 mM of MSG resulted in the increase of fluorescence intensity compared with control nanovesicles.

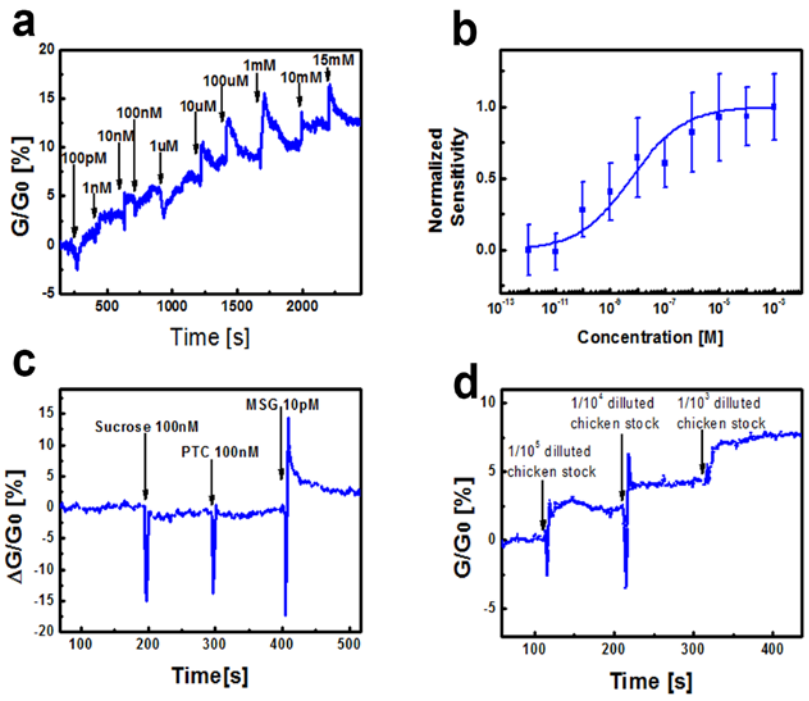

Figure 3. Detection of MSG with floating electrode sensor hybridized with nanoveislces containing AmGr10. (a) Real-time electrical measurement of MSG. The introduction of MSG caused an increase in the channel conductance. The channel conductance began to increase at after the addition of MSG with 100 pM. (b) Dose-dependent response of the floating electrode sensor to MSG. The normalized sensitivity increased as the concentration of MSG increased, and it saturated at around 1 μM of MSG. (c) Real-time responses of the floating electrode sensor to various tastants. The non-umami tastants (sucrose and PTC) caused negligible conductance changes, while the introduction of MSG resulted in the increase in channel conductance. (d) Real-time responses of the floating electrode sensor to MSG in chicken stock. This result indicated that the responses to MSG in the real sample are similar to those in the Figure 3a.

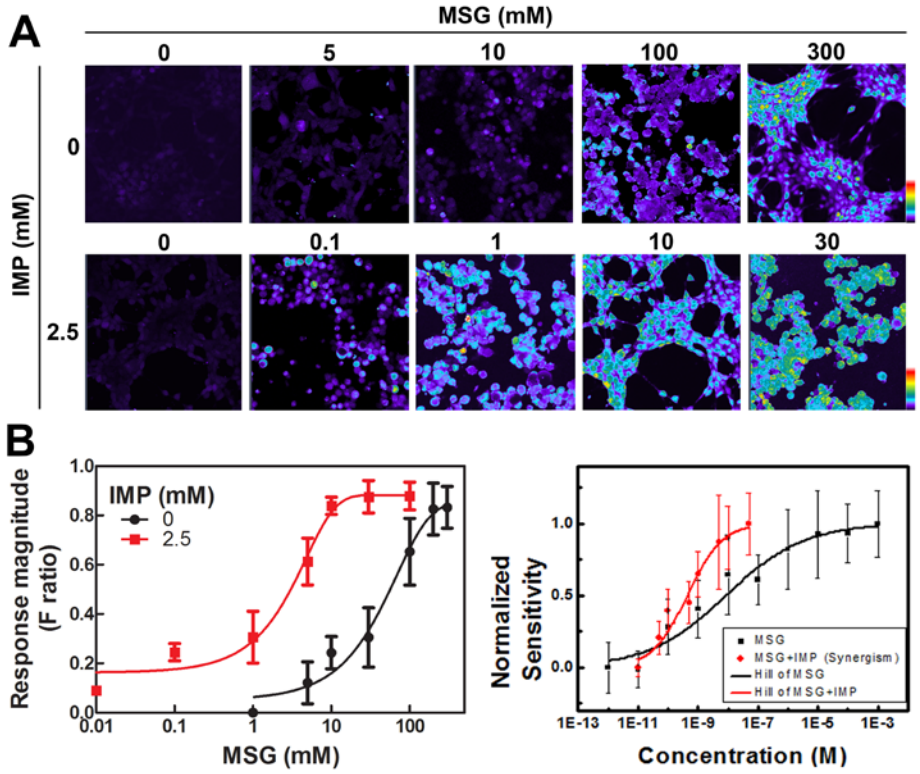

Figure 4. Synergism between MSG and IMP in HEK-293T cell and nanovesicles. (a) Dose responses of AmGr10 to MSG and IMP in HEK-293T cells. The HEK-293T cells expressing AmGr10 were stimulated by MSG with and without IMP (2.5 mM). The results indicated that AmGr10 was activated by MSG and the responses were potentiated by IMP. (b) Dose-response curve of AmGr10 to MSG and MSG+IMP based on quatitative analysis of Ca2+ imaging. The curve was fitted to the hill equation (Left). Normalized sensitivity of the floating electrode sensor in the presence and absence of 2.5 nM IMP (Right). We estimated equilibrium constant K between AmGr10 and MSG as 1.77 x 108 M-1 and the equilibrium constant K_s which shows synergetic effect by MSG and IMP as 2.30 x 109 M-1, respectively. The result showed that the responses to MSG could be enhanced by the synergism between MSG and IMP.

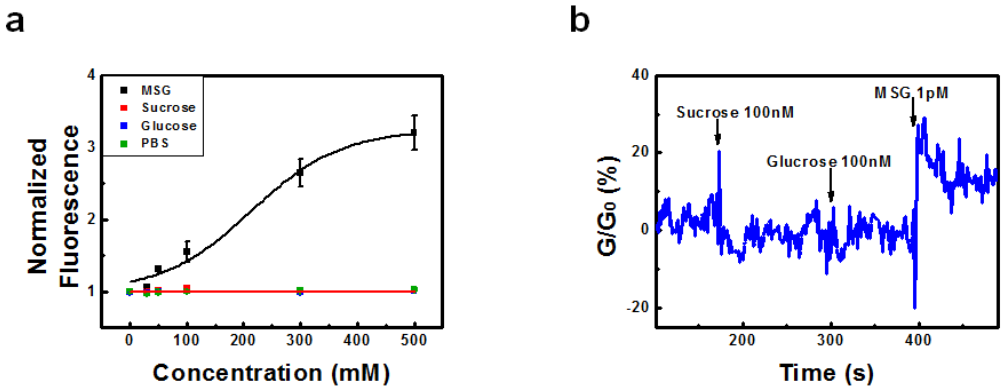

Figure S1. Detection of MSG to verify the selectivity. (a) The dose-dependent intracellular Ca2+ assay in HEK-293T cells expressing AmGr10. Only the addition of MSG caused Ca2+ influx in the HEK-293T cell. (b) Real-time detection of sweeteners. These sweeteners did not cause the conductance change.

General Conclusions

In the four chapters presented in this thesis, I have described 1) structural and functional differences in antennal olfactory system of honeybees of *Apis mellifera* and *Apis cerana,* 2) how neuromodulators alter the olfactory sensitivity in antennae of *Periplanata americana*, 3) identification of sweet and amino acids taste receptor neurons of honeybee in two heterologous expression systems, 4) a study on the applicability of artificial biosensor to detect umami taste compounds using nanovesicles containing honeybee gustatory receptor.

Two closely related honeybee species, *Apis mellifea* and *Apis cerana* have different the number of olfactory sensilla in the antenna, which results in different antennal olfactory responses to floral volatile compounds (Chapter I). This differences of odorant recognition can be caused by an expression of neuropeptide levels in the antenna and triggers a shift in olfactory sensitivity to food source odors (Chapter II).

The cellular function of honeybee gustatory receptors was tested by two heterologous expression in HEK293T cells and *Xenopus* oocytes. Cells and *Xenopus* oocytes expressing *Apis mellifera gustatory receptor genes* (*AmGr1*, *AmGr3*, and *AmGr10*) are responsive to sugar substances such as sucrose, glucose, maltose, trehalose (*AmGr1*), fructose (*AmGr3*) and amino acids (*AmGr10*) in a

dose-dependent manner (Chapter III). Finally, we developed a bioelectronics tongues using honeybee umami taste receptors for the detection and discrimination of MSG (Chapter IV).

Based on these findings, future studies of olfactory system in insects will be aimed at elucidating additional modulatory mechanisms, how these affect odor-driven behavioral changes in the olfactory system and how this related to the coding of odors. With the advancement in molecular techniques available for the study of insect chemical ecology, a multitude of facets can be explored. Characterization of olfactory receptors as well as receptors involved in synaptic transmission will offer great advances in our understanding of how olfactory sensitivity is different between two *Apis* species. This can then be related to the species specific olfactory behavioral output such as olfactory learning and memory, of ecological and physiological state.

In this thesis, molecular and electrophysiological studies in the *Apis mellifera* revealed that the honeybee gustatory receptor system covers a wide ligand spectrum of sugars and amino acids that are common to mammals. However, study on the identification of bitter and salt taste receptor neurons and the central mechanism to perceive and discriminate taste information is not yet elucidated. Future studies on the central mechanism of taste in the honeybee brain

are necessary to understand how insect gustatory neurons process chemical information and control behavioral outputs.

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KOREAN ABSTRACT

꿀벌과 바퀴의 후각 및 미각 인지에 관한 분자신경생리학적 특성 고찰과 바이오센서로의 응용

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초록

곤충은 외부 환경의 변화를 인지하고 이의 변화에 적절히 대응함으로서 생명현상을 유지한다. 이러한 외부 환경의 인지는 감각기관을 통해서 이루어지는데 Chemosensory (화학감각: 후각과 미각)는 외부환경의 화학물질을 인지하는 감각으로서, 후각은 공기 중에 있는 휘발성의 물질을 감지하는 반면 미각은 생명체가 섭취하는 음식에 있는 화합물을 확인하는 역할을 한다. 곤충의 화학 감각수용체는 화학감각기관을 통해 화학물질을 인지함으로서 섭식 및

교미 행동 뿐만 아니라 개체들 간의 의사소통에 있어서 핵심적인 역할을 하고 있는 것으로 알려져 있으므로 화학감각수용체의 기능 분석 및 정보전달 기작에 관한 연구는 곤충의 신경 생리 및 행동을 이해하는데 매우 중요하다.

후각 기전 연구에 관한 기존의 연구는 중추신경계를 중심으로 이루어져 왔다. 본 연구 (Chapter I)는 신경전달물질에 의한 후각 정보 전달의 기전을 말초신경계에서 규명하기 위해 진행되었다. 신경펩타이드 중의 하나인 Tachykinin 과 신경호르몬인 Octopamine 의 수용체가 후각 기관인 곤충의 더듬이에서의 발현함을 확인하였고, 면역염색기법을 이용하여 Octopamine 수용체와 tachykinin 분비 세포가 함께 위치하고 있는 것을 확인하였다. 이는 Octopamine 수용체 신경세포에서 tachykinin 의 분비가 조절되고 있음을 의미한다. 또한 tachykinin 수용체가 후각수용체 뉴런에 분포함으로서 tachykinin 의 분비에 의해 후각수용체의 활성이 영향을 받을 것이라고 예상하였다. 위의 가정을 증명하기 위해 tachykinin 펩타이드를 더듬이에 직접 주입한 후 EAG 기법을 통해 후각반응을 확인해 본 결과 냄새 물질에 대한 후각 반응의 감소현상이 나타난 반면, Tachykinin RNAi 를 주입했을 때는 후각 민감도가 높아짐을 확인하였다. Octopamin 에 의한 억제성 후각 기전은 선행 연구로 보고된 바 있으므로, Octopamine 이 tachykinin 의 분비함으로서 억제성 후각 반응이 나타난 것인지 확인해 보기 위해 Octopamine 과 Tachykinin RNAi 을 함께 더듬이에 주입 후 EAG 반응을 확인하였다. Octopamine 이 분비하는 tachykinin 은 직접 주입한 Tachykinin RNAi 과 함께 반응, 상쇄하여 반응값이 control 과

유사한 결과를 얻을 수 있었다. 위 결과는 신경전달 물질이 중추신경계 뿐만 아니라 말초신경계인 더듬이에서도 화학감각 신경반응을 조절한다는 것을 말해준다.

말초신경계인 더듬이 내부 전반에서 일어나는 후각 정보 전달 기작을 첫번째 연구에서 다루었다면 두번째 연구 (Chapter II)는 더듬이를 구성하는 화학감각 섬모의 구조를 동정 및 분류하고 기능을 밝히는 연구이다. 꿀벌 내 Apis. 속의 *Apis mellifera* 와 *Apis cerana* 간의 후각 민감도의 차이를 확인하기 위해 꿀벌의 더듬이 표면에 존재하는 섬모의 종류 및 분포 양상을 확인한 결과 냄새를 인지하는 특정 섬모의 갯수가 *A. cerana* 에서 훨씬 많이 존재한다는 것을 알 수 있었다. 실제로 주요 꽃 냄새 및 페로몬 성분에 대한 후각민감도 측정 결과는 *A. cerana* 가 *A. mellifera* 보다 냄새를 인지하는 능력이 높다는 것을 전기 신경생리학적 분석을 통해 알 수 있었다. 이는 두 종간의 생태적 환경의 상이함이 화학감각 정보처리 방식 및 감각행동의 차이를 불러온 것이라고 할 수 있다.

한편, 꿀벌의 화학감각기관 중의 하나인 더듬이는 후각을 감지하는 섬모 외에 미각 및 습도를 감지하는 기능을 가진 섬모들도 존재한다고 알려졌다. 따라서 본 연구는 꿀벌이 맛을 인지하는 과정을 알아보고 미각수용체의 동정 및 기능 분석을 위해 시도되었다 (Chapter III). 꿀벌의 더듬이에 존재하는 섬모들을 형태학적으로 분류하여 이 중 맛을 인지한다고 알려진 섬모가 더듬이 끝부분에 집중적으로 분포하고 있음을 확인하였다. 이는 꿀벌이 더듬이를 통해 맛을 인지하는 섬모를 이용하여 먹이의 성분을 인지한다고 여겨진다. 따라서 화밀이나 꿀

속에 존재하는 자당, 포도당, 과당을 인지하는 미각 수용체를 동정하기 위해 초파리에서 당을 인지하는 미각수용체군의 유전자서열을 꿀벌의 미각수용체 유전자서열과 매칭시켜보았더니 미각수용체 1 번, 2 번은 자당 및 포도당을 인지하는 미각 수용체와 상동성이 매우 높고, 미각수용체 3 번은 과당 인지를 담당하는 미각수용체와 상동성이 높다는 것을 알 수 있었다. 또한 미각수용체 1, 2, 3 번의 유전자 발현이 더듬이 끝에서 높다는 것을 관찰을 통해 꿀벌이 당을 인지하는 미각 수용체는 1, 2, 3 번으로 예상하여 HEK293T 세포 및 Xenopus oocyte 에 해당 미각 수용체를 발현시켜 Ca^{2+} imaging 및 Two-voltage clamp 실험을 진행하였다. 꿀벌 미각 수용체 1 번은 자당, 포도당, 맥아당 등에 반응하였으며 과당은 미각수용체 3 번에서 반응을 보인 반면, 미각수용체 2 번은 당에서 반응하지 않음을 알 수 있었다. 또한 미각수용체 1 번과 2 번을 동시에 발현시킨 데이터의 경우 각 물질의 반응이 미각수용체 1 번 단독처리시 나타나는 결과와 다른 결과를 보여주는데 이는 미각수용체 2 번이 곤충의 후각수용체 시스템과 구조적으로 유사한 heterodimer 로서의 역할을 하고 있을 수 있다는 것을 암시한다. 꿀벌에서 단맛을 느끼는 미각수용체 1, 2 번이 꿀벌 더듬이에 발현양상을 보기위해 면역염색법을 수행하였고 그 결과 미각수용체 1 번과 2 번은 homodimer 혹은 heterodimer 를 이루면서 단맛을 내는 물질에 대한 sensitivity 를 다이내믹하게 조절하고 있음을 알게 되었다. 꿀벌 더듬이의 S. chaetica 는 단맛을 인지하는 섬모로서 기능을 하고 있음을 본 전기생리실험을 통해 관찰하였고, 여러 곤충과 마찬가지로 자당에서 반응이 가장 높게 나왔다. 과당에 대한 반응은

자당 다음으로 높게 나왔는데 미각수용체 1 번과 2 번이 과당 수용체가 아닌, 미각수용체 3 번이 S. chaetica 에서 담당하고 있다고 확인할 수 있다. 본 연구는 또한 감칠맛을 인지 (umami receptor)하는 미각수용체 (미각수용체 10 번)를 위와 동일한 실험방식으로 진행하여 동정하였다. 미각수용체 10 번은 6 가지 아미노산에 반응하였고, 포유류에 있어서 감칠맛의 반응을 더 높여주는 물질인 inosine monophosphate (IMP)를 첨가하였을 아미노산에 대한 반응성이 매우 증가함을 확인하였다. 감칠맛을 인지하는 수용체의 동정은 곤충모델에서는 거의 유일한 경우이며 초파리에서도 현재 발견하지 못한 결과로서 이러한 아미노산에 반응하는 미각수용체의 동정 및 향후 여러 아미노산 수용체의 동정은 꿀벌의 화학감각정보 처리 및 internal physiology 를 연구하는데 아주 중요한 연구가 될 것으로 생각한다. 끝으로 감칠맛을 인지하는 꿀벌 미각수용체 10 번을 이용하여 생체유래 미각 센서를 제작 (Chapter IV), 조미료로 알려진 MSG 에 대한 반응성이 기존의 센서보다 약 100 배이상 높게 나타남을 확인하였다. 또한 기존의 센서는 감칠맛을 깊게 느끼게 만드는 촉진자인 IMP 의 synergic effect 를 측정하지 못했던 반면 본 연구로 제작한 꿀벌 유래 미각수용체를 이용한 센서는 강한 synergic effect 를 인지함으로서 인간의 혀가 느끼는 맛을 재현할 수 있음을 확인하였다. 이는 향후 인공 조미료를 감지하는 센서 개발에도 꿀벌 화학감각기 수용체를 활용할 수 있는 길을 제시하였다.