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Title: Critical roles of mecamylamine-sensitive mushroom body neurons in insect olfactory learning

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Footnotes to the title: Roles of mushroom body neurons in learning

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**ABSTRACT**

In insects, cholinergic neurons are thought to transmit olfactory conditioned stimulus (CS) to the sites for associating the CS with unconditioned stimulus (US), but the types of acetylcholine (ACh) receptor used by neurons participating in the association have not been determined. In cockroaches, a type of nicotinic ACh receptor specifically antagonized by mecamylamine (MEC) has been characterized. Here we investigated the roles of neurons possessing MEC-sensitive ACh receptors (MEC-sensitive neurons) in olfactory conditioning of salivation, monitored by changes in activities of salivary neurons, in cockroaches. Local and bilateral microinjection of MEC into each of the three olfactory centers, antennal lobes, calyces of the mushroom bodies and lateral protocerebra, impaired olfactory responses of salivary neurons, indicating that MEC-sensitive neurons in all olfactory centers participate in pathways mediating olfactory responses of salivary neurons. Conditioning of olfactory CS with sucrose US was impaired by injection of MEC into the antennal lobes or calyces, i.e., conditioned responses were absent even after recovery from MEC injection, suggesting that the CS-US association occurs in MEC-sensitive neurons in calyces (Kenyon cells) or in neurons in downstream pathways. In contrast, conditioned responses were present after recovery from MEC injection into the lateral protocerebra, suggesting that MEC-sensitive neurons in the lateral protocerebra are downstream of the association sites. Since lateral protocerebra are major termination areas of mushroom body efferent neurons, we suggest that input synapses of MEC-sensitive Kenyon cells, or their output synapses upon mushroom body efferent neurons, are the sites for CS-US association for olfactory conditioning of salivation.

(250 words).

*Keywords:* acetylcholine; olfactory learning; mushroom bodies; conditioned stimulus; unconditioned stimulus.

## 1. Introduction

Acetylcholine (ACh) is a major excitatory neurotransmitter in vertebrates and invertebrates. In insects, many neurons in neural pathways mediating olfactory signals, including olfactory receptor neurons on antennae, are cholinergic (Frontali, Piazza, & Scopelli, 1971; Kreissl and Bicker, 1989; Sanes & Hildebrand, 1976). Axons of olfactory receptor neurons project to the antennal lobe (AL, primary olfactory center) and make synaptic connections with local interneurons and projection neurons (Barbara, Grünewald, Paute, Gauthier, & Raymond-Delpech, 2008; Bicker, 1999; Moreaux & Gilles, 2007; Watanabe, Nishino, Nishikawa, Mizunami & Yokohari, 2010). Most projection neurons are also cholinergic. Axons of projection neurons enter the protocerebrum and terminate in the calyces of the mushroom body (MB) and the lateral protocerebrum (LPR) (Yasuyama, Meinertzhagen, & Schümann, 2002; Yasuyama, Meinertzhagen, & Schümann, 2003; Yu, Baird, Tsien, & Davis, 2003). In the calyces, projection neurons make synaptic connections with MB intrinsic neurons, called Kenyon cells (Yasuyama et al., 2002). Axons of Kenyon cells project to the pedunculus and lobes and make synaptic connections with efferent (output) neurons of the MB. MB efferent neurons project to the LPR, in addition to several other areas of the protocerebrum (Li & Strausfeld, 1997; 1999).

ACh receptors are divided into muscarinic and nicotinic types. In insects, two types of nicotinic ACh receptors (nAChRs) are pharmacologically identified: one type is antagonized by  $\alpha$ -bungarotoxin and the other type is not (Goldberg, Grünewald, Rosenboom, & Menzel, 1999; Hermsen, Stetzer, Thees, Heiermann, Schrattenholz, Ebbinghaus, Kretschmer, Methfessel, Reinhardt, & Maelicke, 1998; Jones, Raymond-Delpech, Thany, Gauthier, & Sattelle, 2006; Thany, Lenaers, Raymond-Delpech, Sattelle, & Lapied, 2007). In studies using isolated dorsal unpaired median neurons of the cockroach,  $\alpha$ -bungarotoxin-insensitive nAChRs have been further divided into mecamylamine (MEC)-sensitive and d-tubocurarine-sensitive types, and the biophysical and pharmacological properties of both types have been characterized (Courjaret, Grolleau, & Lapied, 2003; Courjaret & Lapied,

2001; Thany, Courjaret, & Lapied, 2008).

Insects have been used as pertinent models for studying neural mechanisms of olfactory learning (Davis, 2005; Heisenberg, 2003; Menzel, 1999). Critical issues in the study of olfactory learning are in which neurons and by what molecular mechanisms the olfactory conditioning stimulus (CS) is associated with appetitive or aversive unconditioned stimulus (US) in the central olfactory pathways. It has been suggested, in insects, that neurons in the AL and MB play critical roles in the CS-US association (Cano Lozano, Armengaud, & Gauthier, 2001; Davis, 2005; Gerber, Tanimoto, & Heisenberg, 2004; Heisenberg, 2003; Menzel, 1999), and also that these neurons receive olfactory CS from cholinergic neurons (Gu & O'Dowd, 2006), in addition to appetitive and aversive US from octopaminergic and dopaminergic neurons, respectively (Hammer, 1993; Hammer & Menzel, 1998; Schwaerzel, Monastirioti, Scholz, Friggi-Grelin, Birman, & Heisenberg, 2003; Thum, Jenett, Ito, Heisenberg, & Tanimoto, 2007). However, the types of ACh receptors used by these neurons remained unknown, and this has hampered the progress of study of cellular and molecular mechanisms of olfactory conditioning.

In this study, we investigated the effects of local microinjection of MEC solution into each of the three olfactory centers, ALs, MB calyces and LPRs, on olfactory conditioning in cockroaches, *Periplaneta americana*. We have shown that cockroaches have excellent olfactory learning capabilities (Sakura & Mizunami, 2001; Sakura, Okada, & Mizunami, 2002; Watanabe, Kobayashi, Sakura, Matsumoto & Mizunami, 2003) and that cockroaches exhibit conditioning of salivation, i.e., they exhibit increased levels of salivation in response to an odor applied to an antenna after the odor (CS) is paired with sucrose solution (US) applied to the mouth or an antenna (Watanabe & Mizunami, 2006; 2007; Watanabe, Sato, Kuramochi, Nishino, & Mizunami, 2008). This conditioning can be monitored by changes in activities of salivary neurons in immobilized animals (Watanabe & Mizunami, 2006; Watanabe et al., 2008), thus allowing for studying the effects of local microinjections of drugs into specific brain areas on conditioning. Based on the obtained results, we suggest that input

synapses of MEC-sensitive Kenyon cells or their output synapses upon MB efferent neurons are the sites of CS-US association for olfactory conditioning.

## 2. Materials and Methods

### 2.1. Insects

Adult male cockroaches, *Periplaneta americana*, were obtained from a laboratory colony maintained under a light-dark cycle (LD 12:12) at 26-28 °C. More than one week before the start of the experiment, 10-20 cockroaches were placed in a chamber. The cockroaches were fed a diet of sugar-free yeast extract and drinking water *ad libitum*.

### 2.2. Extracellular recordings of activities of salivary neurons

Preparations for extracellular recording from a salivary duct nerve, which contains axons of two salivary neurons and of several neurons of the stomatogastric nervous system, were modified from those reported previously (Watanabe & Mizunami, 2006; Watanabe et al., 2008). A cockroach was restrained on a wax-coated stage ventral-side-up. Each antenna was immobilized by threading a plastic ring. The preparations were kept overnight in a moist chamber at room temperature, and then the cuticle in the neck was removed to expose salivary duct nerves. A salivary duct nerve was hooked on a pair of tungsten electrodes. To prevent drying the salivary duct nerve, the salivary duct was covered with a mixture of white Vaseline and liquid paraffin saturated with cockroach saline. Tungsten electrodes were fixed on a neck plastic plate with wax.

Activity of salivary neurons was recorded with a differential AC amplifier (DAM80, World Precision Instruments, Sarasota, FL) and displayed on an oscilloscope and a digital recorder. Data were stored on an MO disk. Activities of salivary neurons were segregated out from those of neurons of the stomatogastric nervous system using a window discriminator (Watanabe & Mizunami, 2006).

### 2.3. Injections and evaluation of drug restriction

The frontal surface of the brain was exposed by removing a small piece of cuticle. A borosilicate glass capillary pulled on a laser microelectrode puller (P-2000, Sutter Instruments,

Novato, CA) was filled with saline or saline containing MEC at the tip. The tapered glass capillary (tip diameter: 10  $\mu\text{m}$ ) was inserted into the brain with a micromanipulator after removal of the neural sheath. A volume of about 0.5 nl of saline or saline containing 1 mM, 10 mM, 20 mM, 40mM or 100 mM MEC (Sigma) was injected bilaterally into the ALs, median and lateral calyces, or LPRs by using a pressure microinjector (PV 820, World Precision Instruments, Sarasota, FL).

To evaluate the diffusion pattern of the injected solution, 0.1% Lucifer Yellow (Sigma) was added to the injected solution. Immediately after the experiments, brains were dissected out, and fixed in 4% formaldehyde in Millonig's buffer (pH 7.2) overnight at 4 °C. After the brains had been dehydrated and cleared, the distribution of Lucifer Yellow was examined by using a confocal laser microscope (LSM 510, Zeiss). Only animals with the dye localized in the neuropils of interest were used for data analysis.

#### *2.4. Measurement of olfactory and gustatory responses of salivary neurons in untrained cockroaches*

As a control for the study of the effects of MEC injection on olfactory conditioning, the effects of MEC injection on olfactory and gustatory responses of salivary neurons were studied in untrained cockroaches. The schedule of the measurements of olfactory responses is shown in Fig. 1A and B. In one experiment, cockroaches were injected with saline or saline containing MEC, and the responses of salivary neurons to apple (control) odor were measured at 10 min before (Test 1) and 10 min (Test 2), 30 min (Test 3) and 60 min (Test 4) after injection (Fig. 1A). In another experiment, the responses of salivary neurons to peppermint, vanilla or apple odor were measured at 10 min before (Test 1) and 10 min (Test 2), 30 min (Test 3), 60 min (Test 4) and 3 hours (Test 5) after injection of MEC solution (Fig. 1B). In the latter experiment, the initial test (Test 1) was performed at 15 min after exposure of the brain. In all tests, olfactory stimuli were delivered to an antenna on the ipsilateral side of the recorded salivary duct nerve using a continuous airflow system described previously

(Watanabe and Mizunami, 2006) and were presented 3 times for 2 sec. For the latter experiment, the 3 odorants were presented in a random sequence at intervals of >20 sec. Methods to study gustatory responses of salivary neurons were modified from those reported previously (Watanabe et al., 2008). Briefly, a droplet (10  $\mu$ l) of 500 mM sucrose solution or distilled water was applied to an antenna (at about 2 cm from the scape), and then carefully removed by a gentle touch of a piece of filter paper on the antenna at 10 sec after the onset of stimulation. The responses of salivary neurons to sucrose or distilled water were measured at 10 min before and 10 min and 30 min after injection, and gustatory stimuli were presented 2 times with a random sequence at intervals of >40 sec.

### *2.5. Conditioning procedure and evaluation of conditioning effects*

The procedure for classical conditioning of responses of salivary neurons to olfactory stimulus (CS) coupled to sucrose stimulus (US) was modified from Watanabe et al. (2008). Immobilized cockroaches were subjected to five sets of differential conditioning trials with inter-trial intervals of 2 min (Fig. 1C). One set of differential conditioning trials consisted of presentation of peppermint or vanilla odor (CS+) paired with sucrose solution and subsequent unpaired presentation of vanilla or peppermint odor (CS-), respectively. We previously demonstrated that there was no difference in conditioning effect for peppermint CS and vanilla CS (Watanabe & Mizunami, 2006; 2007; Watanabe et al., 2008). Both olfactory CS and sucrose US were presented to an antenna on the ipsilateral side of the recorded salivary duct nerve. For presentation of sucrose solution, the antenna was gently touched with a rectangular filter paper soaked with 10  $\mu$ l of 500 mM sucrose solution. The duration of CS and US was 4 sec, and the onset of CS+ preceded that of US by 3 sec (Fig. 1C). Conditioning trials were performed at 10 min after injection of saline or saline containing MEC, and apple (control) odor was presented at 1 min before the initial trial and at 1 min after the final trial. Memory induced by five sets of conditioning trials is retained for at least one day (Watanabe & Mizunami, 2006; Watanabe et al., 2008).

To evaluate the conditioning effects, responses of salivary neurons to odors were measured at 20 min before (10 min before injection: Test 1) and 30 min (60 min after injection: Test 2) and 2.5 hours (3 hours after injection: Test 3) after conditioning trials (Fig. 1C). In the test, peppermint, vanilla and apple (control) odors were each presented 3 times for 2 sec with a random sequence at intervals of >20 sec. The first test (Test 1) was initiated >30 min after completing the set-up of the recording from salivary neurons.

## 2.6. Data analysis

Salivary neurons exhibit spontaneous spike activities (Watanabe & Mizunami, 2006). Responses of salivary neurons to odor stimulation were measured as the increase of spike frequency from the spontaneous level, i.e.,  $R_{(\text{odor})} = R_{\text{during}} - R_{\text{before}}$ , where  $R_{\text{during}}$  or  $R_{\text{before}}$  is summed spike frequency of two salivary neurons during the first 2 sec of odor stimulation or that during a 2-sec period before the onset of odor stimulation, respectively. Similarly, responses to sucrose or water responses were defined as  $R_{(\text{taste})} = R_{\text{during}} - R_{\text{before}}$ , where  $R_{\text{during}}$  was measured as the summed spike frequencies during a 2-s period from 1 s to 3 s after the onset of antennal stimulation, because the antennal contact often induced large artifacts at the onset of the stimulation and this prevented precise spike counts. The conditioning effect was evaluated by comparing the responses to the odor paired with sucrose US (CS+) from those to the odor presented alone (CS-) at each conditioning trial and in each test. Because Kolmogorov-Smirnov test showed that the distribution of the olfactory or taste responses was deviated from normal distribution and Bartlett's test showed heterogeneous variances in many comparisons, the non-parametric Steel's many-one rank test (Steel-test) and Wilcoxon's test (WCX-test) were used for data evaluation. The former was used for multiple comparisons of responses to apple odor in tests after injection with those before injection, and the latter was used to compare spike frequency during odor stimulation ( $R_{\text{during}}$ ) with that before stimulation ( $R_{\text{before}}$ ) and to compare responses to peppermint odor with those to vanilla odor in each test. For the latter, we did not perform a two-way factorial analysis with repeated measures for

each individual, because no appropriate methods were available for non-parametric data to adjust for this kind of multiplicity. The results of statistical analysis, however, were highly consistent throughout the experiments (see Results), demonstrating reliability of our statistical results. Statistical evaluation was performed using Microsoft Excel and Excel statistics software programs (Esumi, Tokyo, Japan). In all figures, data are shown as box and whisker diagrams. The line in the box is the median and the box represents the 25 - 75 percentiles. Whiskers extend to the extreme values as long as they are within a range of  $1.5 \times$  box length. Outliers are not shown in the figures for simplicity, but they were included for data analysis.

### 3. Results

At first, we briefly describe the basic rationale of our study. We examined the effects of local microinjection of MEC solution into three olfactory centers, the ALs, MB calyces and LPRs, on olfactory responses and olfactory conditioning of salivation, which were monitored by changes in activities of salivary neurons (Watanabe & Mizunami, 2007; Watanabe et al., 2008). The results were used to estimate whether MEC-sensitive neurons in each center are upstream or downstream of (i.e., presynaptic or postsynaptic to) the sites for associating olfactory CS with sucrose US in central olfactory pathways. We first studied the effects of local injection of MEC into the ALs, MB calyces and LPRs on olfactory responses of salivary neurons in untrained (naïve) cockroaches, and the results suggested that MEC-sensitive neurons in all of these areas participate in pathways mediating olfactory responses of salivary neurons. On the basis of the results, we proceeded to the study of the effect of local injection of MEC into the ALs, MB calyces and LPRs on olfactory conditioning. If conditioning was impaired by injection of MEC into a particular brain area of the olfactory pathways, i.e., conditioned responses were absent even after recovery from MEC injection, we deduced that MEC-sensitive neurons in that area participate in the CS-US association or they are upstream of the CS-US association sites, conveying olfactory CS to the association sites, but not downstream of the CS-US association sites. On the other hand, if injection of MEC into a particular brain area of the olfactory pathways had no effect on conditioning, we deduced that MEC-sensitive neurons in that area are downstream of the association sites, conveying conditioned olfactory signals from the association sites to the salivary neurons. By combining the results of systematic pharmacological manipulation of all of the three olfactory centers of the insect brain, we aimed to estimate the CS-US association sites.

#### *3.1. Effects of local injection of MEC into the ALs on olfactory responses of salivary neurons in untrained cockroaches*

In order to determine adequate dose of MEC, four groups of cockroaches were injected

with 0.5 nl of saline or saline containing 1 mM, 10 mM or 100 mM MEC into the ALs of both sides of the brain, and olfactory responses of their salivary neurons were examined. The experimental schedule is illustrated in Fig. 1A. We used apple odor for this experiment, because this odor induces strong responses in salivary neurons (Watanabe et al., 2008). The injected solution contained Lucifer Yellow in order to evaluate the diffusion of injected solution. The distribution of dye, examined after completion of the experiment, was restricted to the ALs in most preparations (Fig. 2A). Data were discarded when Lucifer Yellow was diffused outside the ALs. Responses were measured as relative changes of spike frequency from the spontaneous level.

Responses of salivary neurons to apple odor were inhibited by injection of MEC into the ALs in a dose-dependent manner (Figs. 2B-E). In the control group injected with saline solution, responses to apple odor in all tests after injection were as great as those before injection (Fig. 2B, Steel-test, before - 10 min:  $P > 0.05$ ; before - 30 min:  $P > 0.05$ ; before - 60 min:  $P > 0.05$ ). Thus, exposure of the brain and injection of saline solution containing Lucifer Yellow did not affect olfactory responses. On the other hand, the group injected with 0.5 nl of saline containing 10 mM (Fig. 2D) or 100 mM (Fig. 2E) MEC exhibited no significant level of responses to apple odor in any of the tests after injection, i.e., the spike frequency during odor stimulation did not significantly differ from that before stimulation (Fig. 2D, WCX-test, 10 mM, 10 min:  $P > 0.05$ ; 30 min:  $P > 0.05$ ; 60 min:  $P > 0.05$ ; Fig. 2E, WCX-test, 100 mM, 10 min:  $P > 0.05$ ; 30 min:  $P > 0.05$ ; 60 min:  $P > 0.05$ ), indicating complete impairment of responses by MEC injection. This finding suggests that MEC-sensitive neurons in the ALs, which are most likely projection neurons (see Introduction), participate in pathways mediating olfactory responses of salivary neurons. The impairment was incomplete in the group injected with 0.5 nl of saline containing 1 mM MEC: the group exhibited a significant level of responses in any test after injection (Fig. 2C, WCX-test, before:  $P < 0.01$ ; 10 min:  $P < 0.01$ ; 30 min:  $P < 0.01$ ; 60 min:  $P < 0.01$ ). In subsequent experiments, we injected 0.5 nl saline containing 10 mM MEC into the ALs. The final concentration of the MEC estimated from the diffusion volume of

Lucifer Yellow, was 150 - 200  $\mu$ M.

Next, we tested whether responses to apple odor inhibited by MEC injection could be recovered (Fig. 2F, G). A group of cockroaches was injected with 0.5 nl saline containing 10 mM MEC solution in the ALs and responses of salivary neurons to odors were measured at 10 min before (Test 1) and 10 min (Test 2), 30 min (Test 3), 60 min (Test 4) and 3 hours (Test 5) after injection. The experimental schedule is illustrated in Fig. 1B. We used apple, peppermint and vanilla odors, the later two odors being used as CS in our previous studies (Watanabe and Mizunami, 2006, 2007; Watanabe et al., 2008).

We observed a full recovery of olfactory responses at 3 hours after MEC injection. At 10, 30 and 60 min after injection, responses of salivary neurons to apple odor were significantly less compared with those before injection (Fig. 2F, Steel-test, Test 1 - Test 2:  $P < 0.01$ ; Test 1 - Test 3:  $P < 0.01$ ; Test 1 - Test 4:  $P < 0.01$ ). At 3 hours after injection, on the other hand, responses to apple odor did not significantly differ from those before injection (Steel-test, Test 1 - Test 5:  $P > 0.05$ ). We observed similar tendency of inhibition and recovery for responses to peppermint and vanilla odors (Fig. 2G). However, statistical comparisons of these responses after injection with those before injection yielded less consistent results (data not shown), obviously because responses to these odors were much weaker than those to apple odor and thus a slight change of responses during long-term recordings of up to 3 hours affects statistical results. On the other hand, comparison of responses to peppermint and those to vanilla odors in a given test yielded consistent results: there was no significant difference in responses to these two odors in any tests before and after MEC injection (Fig. 2G, WCX-test, Test 1:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Test 3:  $P > 0.05$ ; Test 4:  $P > 0.05$ ; Test 5:  $P > 0.05$ ). This observation also indicates that MEC injection yielded no odor-specific effect. In conditioning experiments with MEC injection into the ALs, therefore, one of these two odors was paired with sucrose reward (CS+) and the other was used as a control odor presented alone (CS-), and responses to these two odors were compared in each test before and after conditioning, for evaluating the effect of conditioning, as in our previous studies (Watanabe & Mizunami,

2006; Watanabe et al., 2008). For simplicity, the difference of responses to peppermint odor and those to vanilla odor,  $R_{(p)}-R_{(v)}$ , are shown in most subsequent figures. Apple odor was used as control odor for testing whether the preparation remained intact during an experiment of an extended period: responses to apple odor,  $R_{(a)}$ , after injection were compared with those before injection.

### *3.2. Effects of local injection of MEC into the MB calyces or LPRs on olfactory responses of untrained cockroaches*

Next, we studied the effects of MEC injection into the MB calyces or LPRs of both sides of the brain on olfactory responses of salivary neurons in untrained cockroaches. When MEC was injected into the calyces, the distribution of co-injected Lucifer Yellow was restricted to median and lateral calyces in most preparations (Fig. 3A). Data were discarded when Lucifer Yellow was diffused outside the calyces. When MEC was injected into the LPRs, however, the distribution of co-injected dye was not restricted to the LPRs in most preparations; it was diffused in nearby protocerebral areas, including a part of the medial and dorsal protocerebra (Fig. 3B). This is probably because, in contrast to the ALs and the MB calyces, there are no glial sheets to compartmentalize the LPRs. However, there were no preparations in which dye was diffused into the ALs or the MBs. The volume of diffused area after injection into the LPRs was about two-times larger than that after injection into the AL or MB calyces. We thus used twice the larger dose of MEC for injection into the LPRs. Two groups of cockroaches were each injected with saline containing MEC solution into the calyces (Fig. 3C, D) or LPRs (Fig. 3E, F), and responses of salivary neurons to apple, peppermint and vanilla odor were measured. The doses were 10 mM x 0.5 nl for injection into the calyces and 20 mM x 0.5 nl for injection into the LPRs. In both groups, the final concentration of the drug could be estimated to 150 - 200  $\mu$ M.

Responses of salivary neurons to apple odors were greatly reduced by MEC injection into the calyces or LPRs. Responses to apple odor at 10, 30 and 60 min after injection were

significantly less compared with those before injection in both groups (Fig. 3C, MBs, Steel-test, Test 1 - Test 2:  $P < 0.01$ ; Test 1 - Test 3:  $P < 0.01$ ; Test 1 - Test 4:  $P < 0.01$ ; Fig. 3D, LPRs, Steel-test, Test 1 - Test 2:  $P < 0.01$ ; Test 1 - Test 3:  $P < 0.01$ ; Test 1 - Test 4:  $P < 0.01$ ). This reduction of responses was not due to damage, because we show later that groups injected with saline into the calyces or LPRs exhibited no significant decrement of responses of salivary neurons to apple odor (see Figs. 4F, 5A). Responses to apple odor at 3 hours after MEC injection into the calyces or LPRs did not significantly differ from those before injection (Steel-test, Test 1 - Test 5, MBs;  $P > 0.05$ ; LPRs;  $P > 0.05$ ), indicating that a complete recovery was achieved. The observed reduction of olfactory responses by MEC injection into the calyces or LPRs suggests that MEC-sensitive neurons in the calyces (no doubt Kenyon cells, see Introduction) and those in the LPRs or in neighbouring protocerebral areas participate in pathways mediating olfactory responses of salivary neurons.

The inhibition of olfactory responses by MEC injection into the calyces or the LPRs was incomplete. There were significant levels of responses to apple odor in any tests performed after injection (WCX-test, Test 2:  $P < 0.05$ ; Test 3:  $P < 0.01$ ; Test 4:  $P < 0.01$ ). This observation suggests that neurons other than MEC-sensitive Kenyon cells also participate in mediating odor responses of salivary neurons; these neurons may be MEC-insensitive Kenyon cells in the calyces or neurons in a neural pathway parallel to the MB, i.e., the pathway from the ALs to the LPRs mediated by projection neurons. There were also significant levels of responses to apple odor after MEC injection into the LPRs (WCX-test, Test 2:  $P < 0.01$ ; Test 3:  $P < 0.01$ ; Test 4:  $P < 0.01$ ). These observations suggest that MEC-insensitive neurons in the LPR or neurons in olfactory pathways not involving the LPR, such as the pathway involving MB efferent neurons projecting to the medial protocerebrum, also participate in mediating olfactory responses of salivary neurons.

There was a similar tendency of inhibition and recovery for responses to peppermint odor or those to vanilla odor after MEC injection into the calyces or LPRs (data not shown). The difference of responses to peppermint odor and vanilla odor did not significantly differ in each

test before and after injection (Fig. 3D, MBs, WCX-test, Test 1:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Test 3:  $P > 0.05$ ; Test 4:  $P > 0.05$ ; Test 5:  $P > 0.05$ ; Fig. 3F: LPRs, WCX-test, Test 1:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Test 3:  $P > 0.05$ ; Test 4:  $P > 0.05$ ; Test 5:  $P > 0.05$ ), indicating no odor-specific effect of MEC injection into the calyces of LPRs. Therefore, one of these two odors was used as a CS and the other as an unpaired control odor in conditioning experiments with MEC injection into the calyces or LPRs.

### 3.3. *Effects of local injection of MEC into the ALs on olfactory conditioning*

We then proceeded to the study of the effect of MEC injection into the ALs on olfactory conditioning (Fig. 4A-E). Two groups of cockroaches were each injected with saline or saline containing 10 mM MEC into the ALs. Animals were subjected to five sets of “P-sucrose/V-alone” conditioning trials, in which peppermint odor was paired with sucrose US and vanilla odor was presented alone without pairing with sucrose. We have shown that “P-sucrose/V-alone” trials and “V-sucrose/P-alone” trials are equally effective in inducing olfactory conditioning of activities of salivary neurons (Watanabe & Mizunami, 2006; Watanabe et al., 2008), as is evidenced by the results of an experiment with “V-sucrose/P-alone” trials (see Fig. 5E, F). Responses to apple (control), peppermint or vanilla odors were measured at 20 min before (Test1: 10 min before injection) and 30 min (Test 2: 60 min after injection) and 2.5 hours (Test 3: 3 hours after injection) after conditioning. The experimental schedule is illustrated in Fig. 1C.

Injection of saline into the ALs did not impair olfactory conditioning. In the group injected with saline into the ALs (Fig. 4A-C), responses to apple odor during conditioning trials and in all tests after conditioning were as great as those before conditioning (Fig. 4A, Steel-test, Test 1 - 1<sup>st</sup>:  $P > 0.05$ ; Test 1 - 5<sup>th</sup>:  $P > 0.05$ ; Test 1 - Test 2:  $P > 0.05$ ; Test 1 - Test 3:  $P > 0.05$ ), indicating that the preparation remained intact throughout the course of the experiment. Responses to sucrose-associated peppermint odor and those to unpaired apple odor are shown in Fig. 4B, and the differences between them in each test are shown in Fig. 4C.

During conditioning trials, responses to sucrose-associated peppermint odor at third and subsequent conditioning trials were significantly greater than those at first conditioning trial (Fig. 4B, Steel-test, 1<sup>st</sup> - 2<sup>nd</sup>:  $P > 0.05$ ; 1<sup>st</sup> - 3<sup>rd</sup>:  $P < 0.05$ ; 1<sup>st</sup> - 4<sup>th</sup>:  $P < 0.05$ ; 1<sup>st</sup> - 5<sup>th</sup>:  $P < 0.01$ ), but responses to unpaired vanilla odor did not significantly differ between the first trial and any of subsequent trials (Steel-test, 1<sup>st</sup> - 2<sup>nd</sup>:  $P > 0.05$ ; 1<sup>st</sup> - 3<sup>rd</sup>:  $P > 0.05$ ; 1<sup>st</sup> - 4<sup>th</sup>:  $P > 0.05$ ; 1<sup>st</sup> - 5<sup>th</sup>:  $P > 0.05$ ), indicating that conditioning is successful. Indeed, responses to sucrose-associated peppermint odor were significantly greater than those to unpaired vanilla odor at the second, fourth and fifth conditioning trials and in tests at 30 min and 2.5 hours after conditioning (Fig. 4C, WCX-test, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P > 0.05$ ; 2<sup>nd</sup>:  $P < 0.05$ ; 3<sup>rd</sup>:  $P > 0.05$ ; 4<sup>th</sup>:  $P < 0.05$ ; 5<sup>th</sup>:  $P < 0.01$ ; Test 2:  $P < 0.01$ ; Test 3:  $P < 0.01$ ), indicating that conditioning is successful. In subsequent figures, the results of conditioning experiments are shown as the difference of the responses to conditioned odor and control odor, to simplify the description.

Injection of MEC into the ALs induced an inhibition and recovery of responses to apple odors (Fig. 4D). Responses to apple odor immediately before the first conditioning trial and immediately after the fifth conditioning trial and at 30 min after conditioning were significantly less than those before conditioning (Fig. 4D, Steel-test, Test 1 - 1<sup>st</sup>:  $P < 0.01$ ; Test 1 - 5<sup>th</sup>:  $P < 0.01$ ; Test 1 - Test 2:  $P < 0.01$ ), but the responses at 2.5 hours after conditioning did not significantly differ from those before conditioning (Steel test, Test 1 - Test 3:  $P > 0.05$ ), indicating that the preparations remained intact.

Injection of MEC into the ALs completely impaired olfactory conditioning. Differences of responses to sucrose-associated peppermint odor and those to unpaired vanilla odors are shown in Fig. 4E, in which no effect of conditioning is observable. Indeed, there was no significant differences between responses to sucrose-associated odor and those to unpaired odor during conditioning trials in all tests after conditioning, including that at 2.5 hours after conditioning (Fig. 4E, WCX-test, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P > 0.05$ ; 2<sup>nd</sup>:  $P > 0.05$ ; 3<sup>rd</sup>:  $P > 0.05$ ; 4<sup>th</sup>:  $P > 0.05$ ; 5<sup>th</sup>:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Test 3:  $P > 0.05$ ). The absence of conditioning effect at 2.5 hours after conditioning was not due to that the responses were not recovered, as is evidenced

by full recovery of responses to apple odors (Fig. 4D). We thus conclude that conditioning is fully impaired by MEC injection into the ALs. This finding suggests that MEC-sensitive AL neurons participate in association of olfactory CS with sucrose US or in conveying CS to the association sites.

### *3.4. Effects of local injection of MEC into the MB calyces on olfactory conditioning*

Next, we tested the effects of injection of MEC into the calyces on olfactory conditioning (Fig. 4F-I). Two groups of animals were each injected with saline (Fig. 4F, G) or saline containing 10 mM MEC (Fig. 4H, I) into the calyces and were subjected to five sets of “P-sucrose/V-alone” conditioning trials, as illustrated in Fig. 1C. Responses of salivary neurons to apple, peppermint or vanilla odor were measured before and 30 min and 2.5 hours after conditioning.

Injection of saline into the calyces did not affect conditioning. In the group injected with saline into the calyces, responses to apple odor during conditioning trials and in all tests after conditioning did not significantly differ from those in the initial test (Fig. 4F, Steel-test, Test 1 - 1<sup>st</sup>:  $P>0.05$ ; Test 1 - 5<sup>th</sup>:  $P>0.05$ ; Test 1 - Test 2:  $P>0.05$ ; Test 1 - Test 3:  $P>0.05$ ), indicating that the preparations remained intact. Responses to sucrose-associated peppermint odor were significantly greater than those to unpaired vanilla odor at the second and subsequent sets of conditioning trials and in all tests after conditioning (Fig. 4G: WCX-test, Test 1:  $P>0.05$ ; 1<sup>st</sup>:  $P>0.05$ ; 2<sup>nd</sup>:  $P<0.01$ ; 3<sup>rd</sup>:  $P<0.01$ ; 4<sup>th</sup>:  $P<0.01$ ; 5<sup>th</sup>:  $P<0.01$ ; Test 2:  $P<0.01$ ; Test 3:  $P<0.01$ ), indicating that saline injection into the calyces does not impair conditioning.

In contrast, injection of MEC into the calyces fully impaired conditioning. In the group injected with 10 mM MEC into the calyces (Fig. 4H, I), responses to apple odor immediately before and after conditioning and at 30 min after conditioning were significantly less than those in the initial test (Fig. 4H, Steel-test, Test 1 - 1<sup>st</sup>:  $P<0.05$ ; Test 1 - 5<sup>th</sup>:  $P<0.01$ ; Test 1 - Test 2:  $P<0.05$ ), but at 2.5 hours after conditioning, responses to apple odor did not significantly differ from those before conditioning (Steel-test, Test 1 - Test 3:  $P>0.05$ ),

indicating that the preparations remained intact. Responses to sucrose-associated peppermint odor did not significantly differ from those to unpaired vanilla odor during conditioning trials and in all tests before and after conditioning (Fig. 4I, WCX-test, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P > 0.05$ ; 2<sup>nd</sup>:  $P > 0.05$ ; 3<sup>rd</sup>:  $P > 0.05$ ; 4<sup>th</sup>:  $P > 0.05$ ; 5<sup>th</sup>:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Test 3:  $P > 0.05$ ), indicating that MEC injection into the calyces fully impairs conditioning. This observation suggests that association of olfactory CS with sucrose US occurs in MEC-sensitive Kenyon cells or in neurons downstream of MEC-sensitive Kenyon cells, not in upstream areas, and thus suggests that the AL is not the CS-US association site for conditioning of salivation.

### *3.5. Effects of local injection of MEC into the LPRs on olfactory conditioning*

Next, we studied the effect of MEC injection into the LPRs on conditioning. Two groups of animals were each injected with saline or saline containing 20 mM MEC into the LPRs and were subjected to five sets of “P-sucrose/V-alone” conditioning trials. Another group was injected with 40 mM MEC into the LPRs and subjected to “V-sucrose/P-alone” conditioning trials. Responses to apple, peppermint and vanilla odors were measured before and 30 min and 2.5 hours after conditioning.

Injection of saline into the LPRs did not impair conditioning (Fig. 5A, B). Responses to apple odor immediately before and after conditioning and at 30 min and 2.5 hours after conditioning did not significantly differ from those in the initial test (Fig. 5A, Steel-test, Test 1 - 1<sup>st</sup>:  $P > 0.05$ ; Test 1 - 5<sup>th</sup>:  $P > 0.05$ ; Test 1 - Test 2:  $P > 0.05$ ; Test 1 - Test 3:  $P > 0.05$ ), indicating that the preparations remained intact. Responses to sucrose-associated peppermint odor were significantly greater than those to unpaired vanilla odor at the second and subsequent sets of conditioning trials and in all tests after conditioning (Fig. 5B, WCX-test, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P < 0.05$ ; 2<sup>nd</sup>:  $P < 0.01$ ; 3<sup>rd</sup>:  $P < 0.01$ ; 4<sup>th</sup>:  $P < 0.05$ ; 5<sup>th</sup>:  $P < 0.01$ ; Test 2:  $P < 0.01$ ; Test 3:  $P < 0.01$ ), indicating that conditioning was unaffected.

Notably, MEC injection into the LPRs had no effects on conditioning. This was demonstrated in two groups, one group being injected with 20 mM MEC and subjected to

P-sucrose/V-alone conditioning trials (Fig. 5C, D) and another group being injected with 40 mM MEC and subjected with V-sucrose/P-alone conditioning trials (Fig. 5E, F). In both groups, responses to apple odor immediately before and after conditioning and 30 min after conditioning were significantly less compared with those in the initial test (Fig. 5C, Steel-test, 20 mM group, Test 1 - 1<sup>st</sup>:  $P < 0.01$ ; Test 1 - 5<sup>th</sup>:  $P < 0.01$ ; Test 1 - Test 2:  $P < 0.01$ ; Fig. 5E, 40 mM group, Test 1 - 1<sup>st</sup>:  $P < 0.01$ ; Test 1 - 5<sup>th</sup>:  $P < 0.01$ ; Test 1 - Test 2:  $P < 0.01$ ; Test 1 - Test 3:  $P > 0.05$ ), whereas at 2.5 hours after conditioning, they did not significantly differ from those before conditioning (Steel-test, Test 1 - Test 3:  $P > 0.05$ ), indicating that the preparation remained intact. In both groups, responses to sucrose-associated odor did not significantly differ from those to unpaired odor during conditioning trials and at 30 min after conditioning (Fig. 5D, WCX-test, 20 mM group, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P > 0.05$ ; 2<sup>nd</sup>:  $P > 0.05$ ; 3<sup>rd</sup>:  $P > 0.05$ ; 4<sup>th</sup>:  $P > 0.05$ ; 5<sup>th</sup>:  $P > 0.05$ ; Test 2:  $P > 0.05$ ; Fig. 5F, WCX-test, 40 mM group, Test 1:  $P > 0.05$ ; 1<sup>st</sup>:  $P > 0.05$ ; 2<sup>nd</sup>:  $P > 0.05$ ; 3<sup>rd</sup>:  $P > 0.05$ ; 5<sup>th</sup>:  $P > 0.05$ ; Test 2:  $P > 0.05$ ), but at 2.5 hours after conditioning, responses to sucrose-associated odor were significantly greater than those to unpaired odor (WCX-test,  $P < 0.01$  in both groups). An exception is that in the 40 mM group, responses to vanilla odor at fourth conditioning trial were significantly greater than those to peppermint odor (WCX-test,  $P < 0.01$ ). The reason for this exceptional observation is unknown. Findings in these experiments indicate that injection of MEC into the LPRs does not impair conditioning, even at a higher dose of MEC. We thus conclude that MEC-sensitive neurons in the LPRs or nearby areas are downstream of the CS-US association sites and convey conditioned olfactory signals from the association sites to the salivary neurons.

### *3.6. Effects of local injection of MEC into the MB calyces on responses to sucrose US*

Finally, we tested whether injection of MEC in the calyces affects responses of salivary neurons to sucrose solution applied to an antenna. This was to confirm that impairment of conditioning by MEC-injection into the calyces is due to blockade of olfactory CS pathway, not sucrose US pathway. We did not perform similar experiments on ALs and LPRs, because

our results suggest that these areas are not likely the sites for CS-US association and thus, such experiments are less important. In a group of cockroaches, a drop of 500 mM sucrose solution or distilled water (control) was applied to an antenna, and the resulting responses of salivary neurons were measured at 10 min before and 10 min and 30 min after MEC injection into the calyces (Fig. 6).

We found no impairment of responses to sucrose by MEC injection into the calyces. Before injection, there was a small but significant level of responses to water (WCX-test,  $P < 0.01$ ), indicating that salivary neurons respond to water and/or mechanical stimulation. Responses to sucrose solution were significantly greater than those to water (WCX-test,  $P < 0.01$ ), indicating that salivary neurons respond to sucrose, as we have reported (Watanabe et al., 2008). Responses to sucrose solution 10 min and 30 min after MEC-injection were also significantly greater than those to water (WCX-test, 10 min:  $P < 0.01$ ; 30 min:  $P < 0.01$ ), indicating that responses to sucrose were intact. We thus conclude that impairment of conditioning by MEC injection into the calyces is due to the blockade of olfactory CS pathway, not sucrose US pathway.

## 4. Discussion

### 4.1. Major findings

We investigated the effects of injection of MEC, an antagonist of a type of nAChRs, into three distinct olfactory centers, ALs, MB calyces or LPRs, on olfactory conditioning of activities of salivary neurons, which is a good measure of conditioning of salivation in cockroaches (Watanabe & Mizunami, 2006; 2007; Watanabe et al., 2008). The results are summarized in Table 1. First, olfactory responses of salivary neurons were completely or partially inhibited after injection of MEC into the ALs, MB calyces or LPRs. We thus concluded that MEC-sensitive neurons in all of these olfactory centers participate in pathways mediating olfactory responses in salivary neurons. We then studied the effects of injection of MEC into each of the three olfactory centers on olfactory conditioning. First, injection of MEC into the ALs before conditioning completely impaired conditioning, i.e., conditioned responses were absent even after recovery from the MEC injection, indicating that the CS-US association occurs in neurons possessing MEC-sensitive nAChRs (MEC-sensitive neurons) in the AL or in neurons downstream of the olfactory pathways. Second, injection of MEC into the calyces also completely impaired conditioning. Since Kenyon cells receive cholinergic inputs from projection neurons in the calyces (Yasuyama et al., 2002), the neurons affected were no doubt Kenyon cells. We thus suggest that the association occurs in MEC-sensitive Kenyon cells or in neurons downstream of the Kenyon cells possessing MEC-sensitive nAChRs (MEC-sensitive Kenyon cells). Third, when MEC was injected into the LPRs before conditioning, conditioning effect was not seen under the influence of MEC but was evident at 2.5 hours after conditioning, indicating no impairment of conditioning by MEC injection into the LPRs. This is in sharp contrast with complete impairment of conditioning by injection of MEC into the ALs or the calyces. These observations suggest that MEC-sensitive neurons in the LPR (or nearby protocerebral areas) are downstream of the association sites, mediating conditioned olfactory signals from the association sites to the salivary neurons. Since the LPR receives olfactory signals from neurons of the AL and MB (Li & Strausfeld, 1997; 1999), and

the AL is not likely the association site (see above), we reasoned that MEC-sensitive neurons in the LPR (or nearby protocerebral areas) receive conditioned olfactory signals from MB efferent neurons that receive synaptic inputs from MEC-sensitive Kenyon cells. Taken together, the results suggest that MEC-sensitive Kenyon cells or their postsynaptic neurons, i.e., MB efferent neurons that are presynaptic to MEC-sensitive neurons of the LPR (or nearby PR areas), are the sites for the CS-US association for olfactory conditioning of salivation in cockroaches (Fig. 7). In other words, MEC-sensitive Kenyon cells are either the CS-US association neurons or sending CS input to postsynaptic association neurons. Injection of MEC into the calyces had no effect on responses to sucrose US, thus suggesting that it impaired transmission of olfactory CS, not that of sucrose US.

There is abundant evidence suggesting that Kenyon cells participate in insect olfactory learning in fruit-flies (Akmal, Wilson, Zong, Tanaka, Ito, & Davis, 2006; Davis, 2005; Schwaerzel et al., 2003) and honey bees (Cano Lozano et al., 2001), but the types of ACh receptors used by Kenyon cells participating in olfactory learning have not been specified in any species of insects. This study is the first to suggest that Kenyon cells possessing MEC-sensitive nAChR or their postsynaptic neurons in the MB play critical roles in olfactory conditioning, providing a basis for extending our pharmacological and electrophysiological studies of molecular and neural circuit mechanisms of olfactory learning.

In this study, we revealed that MEC-sensitive Kenyon cells play critical roles in association of olfactory CS with gustatory US. The CS-US association can be dissected into acquisition and subsequent consolidation processes in many species of insects. (Gauthier, Dacher, Thany, Niggebrugge, Deglise, Kljucovic, Armengaud, & Grünewald, 2006; Keene, Krashes, Leung, Bernard, & Waddell, 2006; Krashes, M. J., Keene, Leung, Armstrong, & Waddell, 2007; Menzel., 1999). Since the effect of MEC injection last at least 60 min after conditioning trials in this study, the MEC-sensitive receptor may be blocked not only acquisition, but also consolidation process. Thus, it is unknown whether the injection abolishes acquisition or consolidation. Indeed, a specific type of Kenyon cells is reported to

be required for memory consolidation in fruit-flies (Keene et al., 2006; Krashes et al. 2007). However, because the time spent on the formation of sufficient levels of conditioning effects in the cockroach is significantly longer than those in the fruit-flies or honey bees, acquisition and consolidation of CS-US association might proceed in parallel during the conditioning trials. Thus, it is difficult to distinguish effects of MEC injection on acquisition process from those on consolidation in our conditioning paradigm. To clarify temporal significance of CS-US association, we have to establish the shorter conditioning paradigm.

The effects of MEC injection on olfactory conditioning have been studied in honey bees using olfactory conditioning of proboscis extension response, but the results were difficult to interpret. A global injection of MEC into the brain before one-trial olfactory conditioning resulted in an impairment of acquisition and retrieval of the conditioned response (Cano Lozano, Bonnard, Gauthier, & Richard, 1996). When MEC was injected into the calyces before a single conditioning trial, conditioning was completely impaired (Cano Lozano et al., 2001), suggesting that MEC-sensitive Kenyon cells participate in conditioning. However, when MEC was injected into the entire brain, including the MBs and ALs, before three conditioning trials, no impairment of conditioning was observed, i.e., there were intact conditioned responses after recovery from MEC injection (Gauthier et al., 2006). The reason for the trial-number-specific effect of MEC is unknown, but a hypothesis to explain these results is that multiple-trial learning activates MEC-insensitive (namely,  $\alpha$ -bungarotoxin-sensitive) nAChRs and this overcomes the effect of MEC.

#### *4.2. Localization of injected solution*

We evaluated localization of the injected drug by observing the distribution of co-injected Lucifer Yellow. When the drug was injected into the ALs or the MB calyces, Lucifer Yellow was confined within the injected neuropil in most preparations. When the drug was injected into the LPRs, however, Lucifer Yellow was diffused into nearby protocerebral areas, including a part of the medial and dorsal protocerebrum. This is probably because the AL and

the MB calyces are wrapped by glial sheets serving as a diffusion barrier. It can be argued that evaluation of diffusion areas of MEC by Lucifer Yellow is inaccurate, because MEC may not spread at the same rate as Lucifer Yellow. We observed, however, that conditioning was completely impaired when MEC was injected into the ALs or calyces, while there was no impairment of conditioning when MEC solution was injected into the LPRs even at a higher concentration, indicating that the effects of MEC were localized. Therefore, our conclusion that MEC-sensitive Kenyon cells or its postsynaptic neurons are the sites of CS-US association is valid even though there might be a degree of inaccuracy in evaluating diffusion areas.

When MEC was injected into the calyces, we regarded that Kenyon cells are the most likely target of MEC from the distribution of co-injected Lucifer Yellow. This hypothesis supported by electron microscopic observation of the fruits-fly calyces, where Kenyon cells receive cholinergic inputs from projection neurons in the calyces (Yasuyama et al., 2002). However, we cannot exclude the possibility that other MEC responsive neurons in the calyx or nearby region have an essential role in the salivary conditioning. More study is needed to fully rule out the possibility that MEC responsive neurons in the calyx other than Kenyon cells.

#### *4.3. Neural pathways mediating conditioned olfactory responses of salivary neurons*

Fig. 7 shows presumptive neural pathways underlying olfactory conditioning of salivation (monitored by changes in activities of salivary neurons) in cockroaches. For the olfactory CS pathway, we suggest that MEC-sensitive projection neurons and MEC-sensitive Kenyon cells participate in mediating olfactory responses of salivary neurons. Because we observed partial, but not complete, impairment of olfactory responses of salivary neurons after MEC injection into the calyces, olfactory information may also be mediated, in part, by a direct pathway from the AL to the LPR mediated by projection neurons or by MEC-insensitive Kenyon cells. The former possibility is in accordance with findings in fruit-flies *Drosophila* that mutants

with defects in the MB exhibited normal odor recognition but impaired olfactory learning (Heisenberg, 2003).

For the gustatory US pathway, we observed no effects of MEC injection into the MB calyces on responses of salivary neurons to sucrose US. Contact chemoreceptor neurons on antennae, including sugar receptor neurons, extend their axons into the dorsal lobe of the deutocerebrum and subesophageal ganglion (Nishino, Nishikawa, Yokohari, & Mizunami, 2005), and their termination area in the subesophageal ganglion overlaps, in part, with dendritic fields of salivary neurons (Watanabe & Mizunami, 2006). Thus, salivary neurons are likely to receive synaptic inputs from antennal sucrose receptor neurons monosynaptically and/or polysynaptically via interneurons of the subesophageal ganglion. In honey bees, a putative octopaminergic neuron called VUMmx1 neuron in the subesophageal ganglion mediates reinforcing properties of sucrose US (Hammer, 1993). The axons of this neuron terminate extensively in the ALs, MB calyces and LPRs. In the cockroach, octopaminergic neurons with more or less similar morphology have been found (Sinakevitch, Niwa, & Strausfeld, 2005) and we speculate that some of these neurons convey sucrose US to various protocerebral areas. Roles of octopaminergic neurons for acquisition (Farooqui, Vaessin, & Smith, 2004; Nakatani, Matsumoto, Mori, Hirashima, Nishino, Arikawa, & Mizunami, 2009; Schroll, Riemensperger, Bucher, Ehmer, Voller, Erbguth, Gerber, Hendel, Nagel, Buchner, & Fiala, 2006; Schwaerzel et al., 2003; Unoki, Matsumoto, & Mizunami, 2005; Unoki, Matsumoto, & Mizunami, 2006) and retrieval (Mizunami, Unoki, Mori, Hirashima, Hatano, & Matsumoto, 2009) of appetitive memory have been demonstrated.

In honey bees and fruit-flies, it has been suggested that both the MB and AL are the sites of association of olfactory CS and sucrose US for olfactory conditioning (Davis, 2005; Hammer & Menzel, 1998; Thum et al., 2007). For example, pharmacological inactivation of the MB during the differential conditioning trials was not impaired olfactory memory acquisition, suggesting that output region of MB do not involve in the olfactory differential conditioning (Devaud, Blunk, Podufall, Giurfa, & Grünewald, 2007). On the other hand, the

responses of  $\alpha$ -lobe output neurons to sucrose associated odor clearly change during differential conditioning trials in honey bees (Mauelshagen, 1993; Okada, Rybak, Manz, & Menzel, 2007). Thus, the necessity of output region of Kenyon cells during olfactory conditioning in insects is still controversial. Our finding that conditioning was completely impaired by injection of MEC into the MB calyces does not support the possibility that the AL is also the site of CS-US association for olfactory conditioning of salivation in cockroaches. If association of olfactory CS with sucrose US occurs in the AL in conditioning of salivation, instructive signals from the MB, mediated by MEC-sensitive KCs, should be necessary for achieving the association in the AL (i.e., the AL should be downstream as well as upstream of MEC-sensitive Kenyon cells).

The LPR is one of termination areas of PNs from the AL and efferent neurons from the MB pedunculus or lobes that are postsynaptic to Kenyon cells (Li & Strausfeld, 1997; 1999). Olfactory conditioning was not affected when MEC was injected into the LPRs. This matches findings in honey bees that pairing of olfactory stimulus with local injection of octopamine, a presumable neurotransmitter mediating sucrose US, into the LPR did not yield conditioning effects (Hammer & Menzel, 1998). We suggest that MEC-sensitive neurons in the LPRs, or neurons in nearby protocerebral areas, participate in conveying conditioned olfactory signals from the CS-US association sites to the salivary neurons. Finally, we suggest that MEC-sensitive and MEC-insensitive LPR neurons project to the subesophageal ganglion and some of them make synaptic connections with salivary neurons. Because intracellular recording and staining from brain neurons are feasible in cockroaches (Li & Strausfeld, 1997; 1999; Mizunami, 1996; Nishino, Yamashita, Yamazaki, Nishikawa, Yokohari, & Mizunami, 2003), this model should provide a basis for future electrophysiological and pharmacological analysis of the roles of individual brain neurons in olfactory conditioning.

#### *4.4. Future perspective*

Our findings suggest that MEC-sensitive Kenyon cells play critical roles in the CS-US

association, but whether their input synapses or their output synapses upon efferent neurons of the pedunculus or lobes are the sites of the association remains to be determined. This question cannot be addressed by local injection of MEC into the pedunculus or lobes, because evidence suggests that Kenyon cells are not cholinergic (Johard, Enell, Gustafsson, Trifilieff, Veenstra, & Nässel, 2008; Yasuyama et al., 2002). Since the neurotransmitter of Kenyon cells is not known, one of our next steps is to study the effect of blockade of the US pathway, by locally injecting an octopamine receptor antagonist into the calyces or pedunculus/lobes of the MBs.

Another important subject is to examine pharmacological properties of MEC-sensitive nAChRs of Kenyon cells of the cockroach mushroom bodies. In dorsal unpaired median neurons of cockroaches, a nAChR type that is sensitive to MEC and insensitive to  $\alpha$ -bungarotoxin has been found, in addition to two MEC-insensitive nicotinic ACh receptors types (see Introduction: Courjaret et al., 2003; Courjaret & Lapied, 2001). It needs to be confirmed whether MEC-sensitive nAChRs of cockroach Kenyon cells have the same pharmacological properties. This is particularly important because previous studies on isolated Kenyon cells of crickets (Cayre, Buckingham, Yagodin, & Sattelle, 1999) and honey bees (Wüstenberg & Grünewald, 2004) have shown that they possess MEC-sensitive nAChRs, but no evidence has been reported to show that it is insensitive to  $\alpha$ -bungarotoxin. In honey bees, for example, it has been reported that some isolated Kenyon cells possess nAChRs that are highly sensitive to  $\alpha$ -bungarotoxin and also slightly sensitive to MEC (Wüstenberg & Grünewald, 2004). Studies of the effects of  $\alpha$ -bungarotoxin injection into the MB calyces of cockroaches will help to clarify pharmacological type of nAChRs possessed by Kenyon cells.

MEC-sensitive nAChR of dorsal unpaired median neurons of cockroaches exhibit unusual features (Thany et al., 2008). It is opened at rest and mediates outward  $K^+$  currents. Upon binding of the ACh, it is closed and induces a depolarization of the cell. Interestingly, outward  $K^+$  currents mediated via MEC-sensitive nAChRs are enhanced by an increase of intracellular  $Ca^{2+}$  concentration (Thany et al., 2008). If cockroach Kenyon cells have the same nAChR

type and the CS-US association occurs in input synapses of Kenyon cells and sucrose US increases intracellular  $\text{Ca}^{2+}$  concentration in Kenyon cells, simultaneous presentation of the olfactory CS and sucrose US may result in an enhancement of responses to olfactory CS in Kenyon cells. Thus, MEC-sensitive nAChRs in Kenyon cells may act as a molecular coincident detector for the occurrence of CS and US and may trigger intracellular biochemical events that lead to memory formation. Alternatively, if CS-US association occurs in MB efferent neurons postsynaptic to Kenyon cells and Kenyon cells receive depolarizing input from neurons other than projection neurons conveying olfactory CS, nAChRs in Kenyon cells may play roles in an enhancement of olfactory CS input to CS-US association neurons and thus facilitate olfactory learning. Characterization of pharmacological properties of isolated Kenyon cells of cockroaches will help to clarify the type of nAChRs blocked by injection of MEC into the calyces and thus to clarify molecular processes underlying olfactory learning.

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## Figure legends

**Fig. 1.** Experimental schedules. (A, B) Schedules for experiments to study the effects of MEC injection into local brain areas on olfactory responses of salivary neurons in untrained cockroaches. In (A), responses to 2-sec presentation of apple odor were measured at 10 min before (Test 1; black square) and 10 min (Test 2), 30 min (Test 3) and 60 min (Test 4) after injection of saline or saline containing MEC into the ALs. Injection was carried out immediately after exposure of the brain. In (B), responses to 2-sec presentation of peppermint, vanilla or apple odors were measured at 10 min before (Test 1) and 10 min (Test 2), 30 min (Test 3), 60 min (Test 4) and 3 hours (Test 5) after injection of saline containing MEC into the ALs, calyces or LPRs in untrained cockroaches. The initial test (Test 1) was carried out at 15 min after exposure of the brain. (C) Schedules for experiments to study the effects of injection of saline or saline containing MEC into ALs, calyces or LPRs on olfactory conditioning. At 10 min after injection, five sets of “P-sucrose/V-alone” or “V-sucrose/P-alone” differential conditioning trials were carried out. One set of conditioning trial consisted of 4-sec presentation of peppermint or vanilla odor (CS+, shaded squares) paired with 4-sec presentation of 500 mM sucrose solution to an antenna (US; open squares), which was initiated at 3 sec after the onset of CS+ presentation, and subsequent presentation of vanilla or peppermint odor (CS-, hatched squares) without pairing with sucrose solution, respectively. The intervals between trials were 2 min. Apple (control) odor (vertical striped bar) was presented at 1 min before the initial conditioning trial and at 1 min after the final conditioning trial. Responses to 2-sec presentation of peppermint, vanilla or apple (control) odor were measured at 20 min before (10 min before injection: Test 1; black square) and 30 min (60 min after injection: Test 2) and 2.5 hours (3 hours after injection: Test 3) after conditioning.

**Fig. 2.** Effects of local injection of MEC solution into the ALs on olfactory responses of salivary neurons in untrained cockroaches. (A) Confocal image of the distribution of Lucifer Yellow contained in test solution injected into the ALs; frontal view. (B-E) Four groups of

cockroaches were each injected with 0.5 nl of saline (B) or saline containing 1 mM (C), 10 mM (D) or 100 mM MEC (E) into their ALs. Responses to apple odor were measured at 10 min before and 10 min, 30 min and 60 min after injection. (F, G) Responses to apple (white boxes), peppermint (hatched boxes) or vanilla odor (black boxes) were measured at 10 min before and 10 min, 30 min, 60 min and 3 hours after injection of 0.5 nl of saline containing 10 mM MEC, and are shown as box and whisker diagrams. The line in the box is the median and the box represents the 25 - 75 percentiles. Whiskers extend to the extreme values as long as they are within a range of 1.5× box length. Outliers are not shown in diagrams, but are included for data analysis. In B and F, responses to apple odor after injection were compared with those in the initial test and the results of statistical comparison are shown as asterisks (Steel-test, \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; NS  $P > 0.05$ ). In C-E, spike frequencies during apple odor stimulation were compared with the spontaneous level and are shown as *Italic format* (WCX-test, ††  $P < 0.01$ ; NS  $P > 0.05$ ). In D, responses to peppermint and vanilla odor were compared at each test, and the results of statistical comparison are shown as *Italic format* (WCX-test, ††  $P < 0.01$ , †  $P < 0.05$ ; NS  $P > 0.05$ ).

**Fig. 3.** Effects of local injection of MEC solution into the MB calyces or LPRs on olfactory responses of salivary neurons in untrained cockroaches. (A, B) Confocal image of the distribution of Lucifer Yellow (0.1%) contained in test solution injected into the calyces (A) and LPRs (B); frontal view. In (B), Lucifer Yellow was observed in the LPR and nearby protocerebral areas but not in the AL or the MB. Broken line indicates the MB calyces and peduncle. (C-F) Responses to apple odor (white boxes) and differences of responses to peppermint odor and those to vanilla odor (gray boxes) at 10 min before and 10 min, 30 min, 60 min and 3 hours after injection of 0.5 nl of saline containing 10 mM MEC into the calyces (C, D) or the LPRs (E, F) are shown as box and whisker diagrams. Outliers are not shown in diagrams, but are included for data analysis. In C and E, responses to apple odors after injection were compared with those in initial test. In D and F, responses to peppermint and

vanilla odors were compared at each test. The results of statistical comparisons are shown as asterisks as in Fig. 2.

**Fig. 4.** Effects of local injection of MEC solution into the ALs or MB calyces on olfactory conditioning of activities of salivary neurons. Four groups of cockroaches were injected with 0.5 nl of saline or saline containing 10 mM MEC solution into the ALs (A-E) or calyces (F-I) and 10 min later, they were subjected to 5 sets of “P-sucrose/V-alone” conditioning trials. Responses to apple (white boxes), peppermint (hatched boxes) and vanilla odor (black boxes) were measured at 20 min before (Test 1), during (1st - 5th), and at 30 min (Test 2) and 2.5 hours after (Test 3) conditioning trials. In A, D, F and H, responses to apple odors in tests after conditioning were compared with those in the initial test. In B, responses to peppermint and vanilla odors at each test are shown. To help evaluation of conditioning effect by visual inspection, differences of responses to sucrose-associated peppermint odor and those to unpaired vanilla odor (gray boxes) are shown in C, E, G and I. Responses to peppermint odors were compared to those to vanilla at each test. All data are shown as box and whisker diagrams: outliers are not shown in diagrams, but are included for data analysis. The results of statistical comparisons are shown as asterisks (see legends in Fig. 2).

**Fig. 5.** Effects of local injection of MEC solution into the LPRs on olfactory conditioning of activities of salivary neurons. Three groups of cockroaches were injected with 0.5 nl of saline (A, B) or saline containing 20 mM (C, D) or 40 mM MEC (E, F) into the LPRs. At 10 min after injection, the former two groups were subjected to 5 sets of “P-sucrose/V-alone” conditioning trials (A-D) and the latter group was subjected to “V-sucrose/P-alone” conditioning trials (E, F). Responses to apple odor (white boxes) and differences of responses to sucrose-associated odor and those to unpaired odor (gray and black boxes) at 20 min before (Test 1), during (1st - 5th), and at 30 min (Test 2) and 2.5 hours after (Test 3) conditioning trials are shown as box and whisker diagrams. Outliers are not shown in diagrams, but are

included for data analysis. Responses to apple odor were compared before and after conditioning (A, C, E). Responses to peppermint odor were compared with those to vanilla odor at each test (B, D, F). The results of statistical comparisons are shown as asterisks as in Fig. 2.

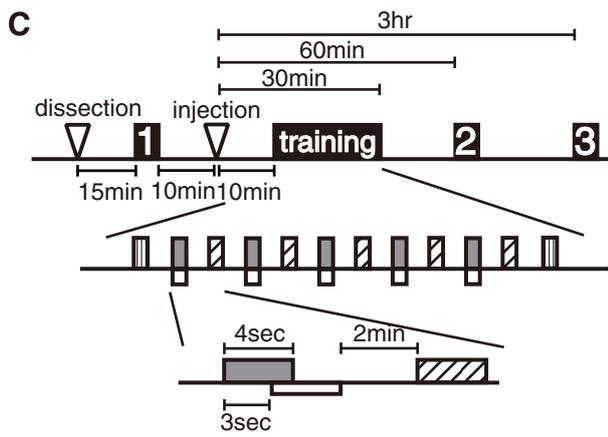
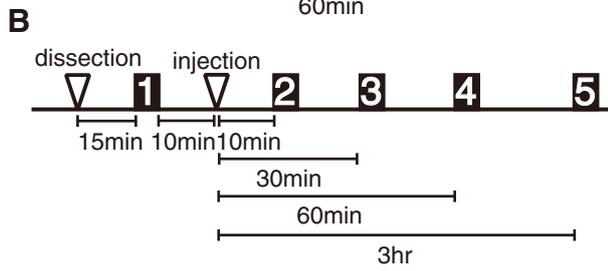
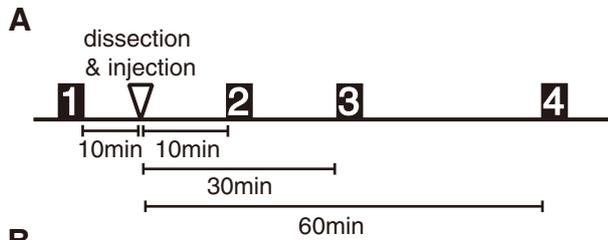
**Fig. 6.** Effects of local injection of MEC solution into the MB calyces on gustatory responses of salivary neurons in untrained cockroaches. Responses of salivary neurons to distilled water (white boxes) or 500 mM sucrose solution (black boxes) presented to an antenna were measured at 10 min before and 10 min and 30 min after injection. Responses are shown as box and whisker diagrams: outliers are not shown in diagrams, but are included for data analysis. Responses to sucrose solution were compared with those to water, and the results of statistical comparison are shown as asterisks (WCX-test, \*\*  $P < 0.01$ ; NS  $P > 0.05$ ).

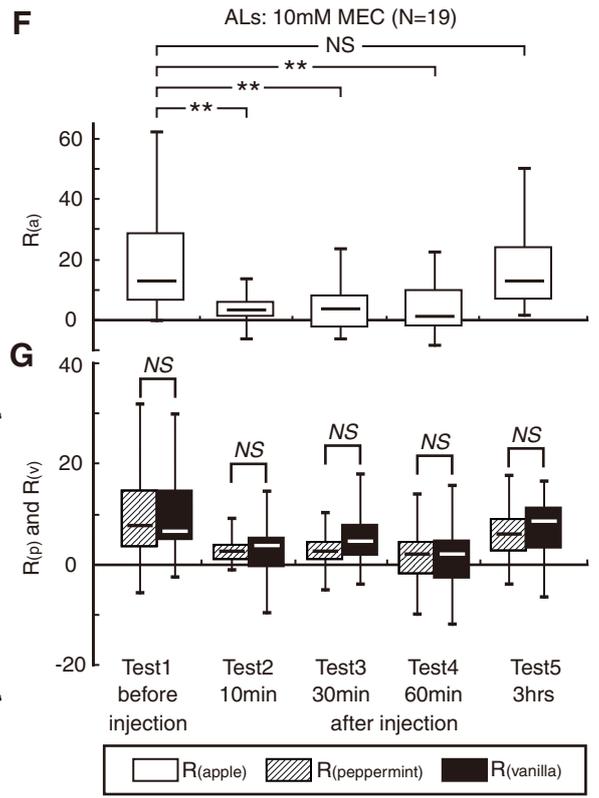
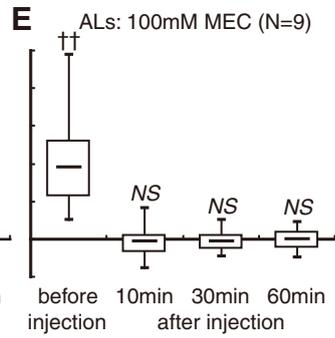
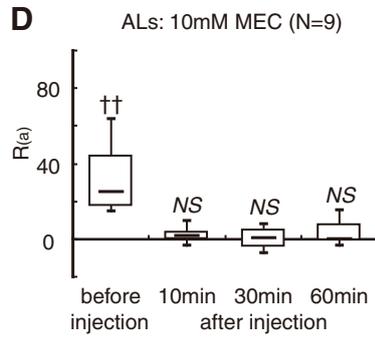
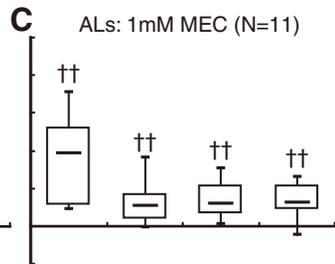
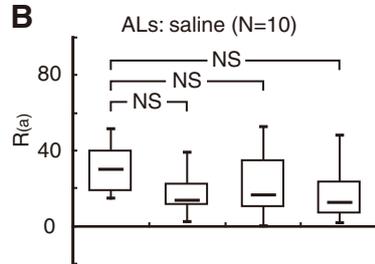
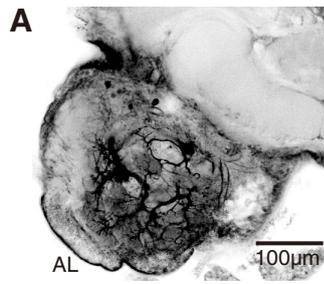
**Fig. 7.** A model of neural pathways mediating conditioned olfactory responses of salivary neurons (SNs), proposed to account for the results of this study. Axons of olfactory receptor neurons (ORNs), which are known to be cholinergic, project to the AL and make synaptic connections with projection neurons (PNs), which are equipped with MEC-sensitive nAChRs. Other types of AL neurons, such as MEC-insensitive PNs, are not drawn for simplicity: our results can be explained without assuming possible involvement of such neurons. At the MB calyces, MEC-sensitive PNs make synaptic connections with MB intrinsic neurons (Kenyon cells, KCs), some of which have MEC-sensitive nAChRs but others of which may not. MEC-sensitive PNs also make synaptic connections with neurons in the LPR, which may or may not have MEC-sensitive nAChRs. In the pedunculus and/or lobes, KCs make synaptic connections with MB efferent neurons, some of which are likely to be cholinergic and others not. Axons of MB efferent neurons terminate in the LPR, in addition to some other areas of the protocerebrum (Li and Strausfeld, 1997; 1999). We assume that MEC-sensitive and MEC-insensitive LPR neurons project to the subesophageal ganglion (SEG) and some of

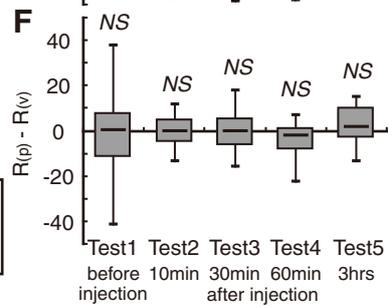
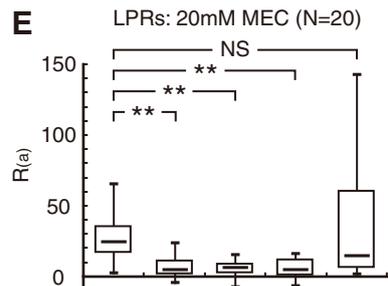
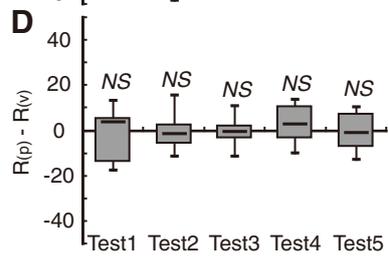
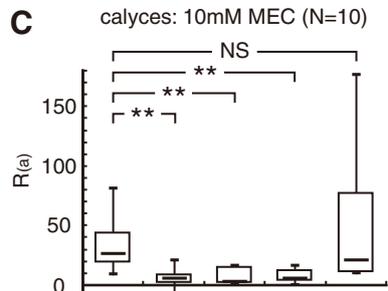
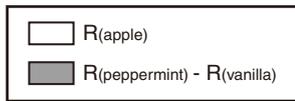
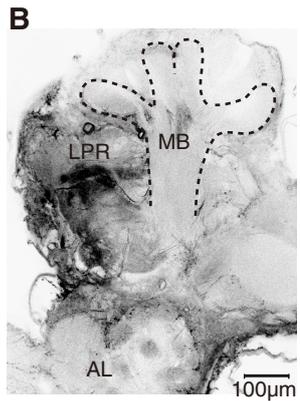
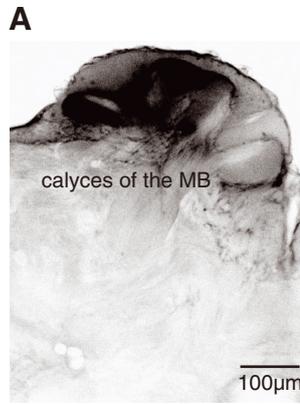
these make mono- or polysynaptic connections with SNs, which innervate salivary glands. Axons of antennal gustatory receptor neurons (GRNs) project to the SEG and make mono- or polysynaptic connections with SNs. We suggest that the association of olfactory CS and sucrose US underlying conditioning of salivation, monitored as conditioning of activities of SNs, occurs at input synapses (in the calyces) or output synapses (in the pedunculus and/or lobes) of MEC-sensitive KCs (shaded area). Broken line and black line indicate cholinergic and non-cholinergic neurons, respectively. Red and green lines indicate MEC-sensitive and MEC-insensitive neurons, respectively. The blue line indicates a putative pathway mediating sucrose US information.

Table 1: Effects of MEC injection on conditioned olfactory responses of salivary neurons.

Injected areas	ALs	MB calyces	LPRs
<i>Effects on olfactory responses:</i>			
<i>Immediately after injection</i>	fully impaired	partially impaired	partially impaired
<i>3 hours after injection</i>	fully recovered	fully recovered	fully recovered
<i>Effects on olfactory conditioning:</i>			
<i>Immediately after injection</i>	fully impaired	fully impaired	fully impaired
<i>3 hours after injection</i>	fully impaired	fully impaired	fully recovered
<i>Possible sites for the CS-US association</i>	ALs or downstream areas	calyces or downstream areas	areas upstream of the LPRs

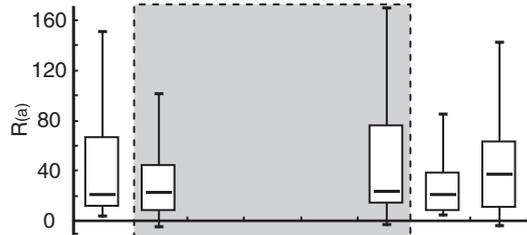








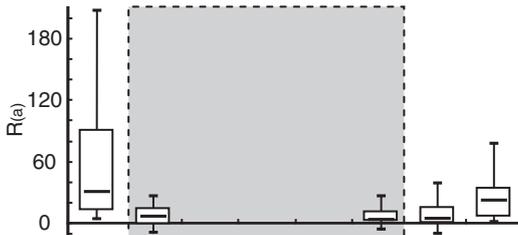
**A** LPRs: saline (N=19)



**B** "P-sucrose/V-alone"



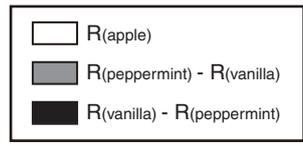
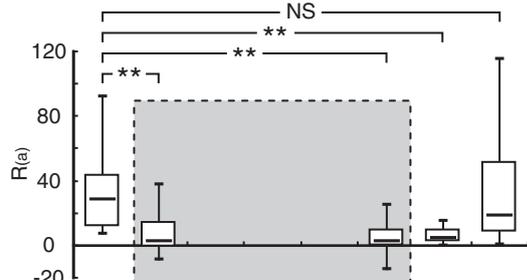
**C** LPRs: 20mM MEC (N=19)



**D** "P-sucrose/V-alone"



**E** LPRs: 40mM MEC (N=16)



**F** "V-sucrose/P-alone"



