Title

Context-dependent olfactory learning monitored by activities of salivary neurons in cockroaches

Authors and affiliations

Chihiro SATO MATSUMOTO [1, 2], Yukihisa MATSUMOTO [2], Hidehiro WATANABE[3], Hiroshi NISHINO[4], Makoto MIZUNAMI [2]

[1)] Graduate School of Life Sciences, Tohoku University, Sendai 980-8577, Japan

[2)] Graduate School of Life Science, Hokkaido University, Sapporo 060-0810, Japan

[3)] Faculty of Science, Fukuoka University, Fukuoka 814-0180, Japan

[4)] Research Institute for Electronic Science, Hokkaido University, Sapporo 060-0810, Japan

Corresponding Author

Dr. Makoto Mizunami

Graduate School of Life Science

Hokkaido University

Kita 10 Nishi 8, Kita-ku, Sapporo, 060-0810, Japan

Phone & Fax: +81-11-706-3446

e-mail: mizunami@sci.hokudai.ac.jp

Abstract

Context-dependent discrimination learning, a sophisticated form of nonelemental associative learning, has been found in many animals, including insects. The major purpose of this research is to establish a method for monitoring this form of nonelemental learning in rigidly restrained insects for investigation of underlying neural mechanisms. We report context-dependent olfactory learning (occasion-setting problem solving) of salivation, which can be monitored as activity changes of salivary neurons in immobilized cockroaches, *Periplaneta americana*. A group of cockroaches was trained to associate peppermint odor (conditioned stimulus, CS) with sucrose solution reward (unconditioned stimulus, US) while vanilla odor was presented alone without pairing with the US under a flickering light condition (1.0 Hz) and also trained to associate vanilla odor with sucrose reward while peppermint odor was presented alone under a steady light condition. After training, the responses of salivary neurons to the rewarded peppermint odor were significantly greater than those to the unrewarded vanilla odor under steady illumination and those to the rewarded vanilla odor was significantly greater than those to the unrewarded peppermint odor in the presence of flickering light. Similar context-dependent responses were observed in another group of cockroaches trained with the opposite stimulus arrangement. This study demonstrates context-dependent olfactory learning of salivation for the first time in any vertebrate and invertebrate species, which can be monitored by activity changes of salivary neurons in restrained cockroaches.

Keywords

context-dependent learning, nonelemental learning, olfaction, vision, insects, cockroaches

1. Introduction

The context, the static and diffuse environmental stimuli or background cues present during conditioning or testing (Maren & Holt, 2000), often influences learning acquisition and memory retrieval in animals. In context-dependent fear conditioning in rats, for example, animals learn different associations between conditioned stimulus (CS) and unconditioned stimulus (US) under different contexts (Holland & Bouton, 1999; Maren & Holt, 2000). In this learning, contextual stimuli serve as occasion setters to disambiguate the meaning of the CS and get appropriate behavioral responses to the CSs. This form of nonelemental associative learning critically depends on intact hippocampus, whereas elemental CS-US associative learning does not (Hirsh, 1974; Holland & Bouton, 1999; Yoon, Graham, & Kim, 2010). Sophisticated forms of nonelemental learning by use of contextual stimuli as occasion setters have also been demonstrated in some insects. We have reported that crickets and cockroaches learn to select one of a pair of odors in one visual context and to select the other in another visual context (Matsumoto & Mizunami, 2004; Sato, Matsumoto, Sakura, & Mizunami, 2006). The influence of circadian system on olfactory learning, which can be considered as a form of context-dependent learning, has been demonstrated in cockroaches (Decker, McConaughey & Page, 2007), and the capability of context-dependent visual pattern discrimination learning has been demonstrated in bumblebees (Colborn, Ahmad-Annuar, Fauria, & Collett, 1999; Fauria, Dale, Colborn, & Collett, 2002) and ants (Chameron, Schats, Pastergue-Ruiz, Beugnon, & Collett, 1998).

Insects have been used as model animals to study molecular and cellular mechanisms of learning and memory (Daly, Thomas, Christensen, Lei, Smith, & Hildebrand, 2004; Davis, 2005; Giurfa, 2003; Heisenberg, 2003; Menzel & Giurfa, 2006), but most previous studies on brain mechanisms of learning in insects have been restricted to elemental associative learning between CS and US. Electrophysiological and optophysiological studies on activity changes of brain neurons during learning are

ideal for elucidating brain mechanisms underlying context-dependent discrimination learning, but adequate preparation to achieve such learning in strictly restrained animals needs to be developed to carry out such studies.

Recently, we reported classical conditioning of salivation and associated activity changes of salivary neurons in the restrained cockroach (Watanabe & Mizunami, 2006, 2007; Watanabe, Sato, Kuramochi, Nishino, & Mizunami, 2008). Salivation in cockroaches is controlled by two salivary neurons of the subesophageal ganglion and a few neurons of the stomatogastric nervous system, axons of which are contained in the salivary duct nerve (Ali, 1977; Davis, 1985; Rotte, White, Blenau, Baumann, & Watz, 2009; Whitehead, 1971). Cockroaches that had been trained to associate an odor with sucrose solution applied to the mouth or the antenna showed an increased level of salivation (Watanabe & Mizunami, 2007). A concordant increase in the activities of salivary neurons in response to the rewarded odor is demonstrated by extracellular recordings from strictly immobilized cockroaches (Watanabe & Mizunami, 2006). Electrical stimulation of the salivary neurons led to an increased level of saliva secretion, indicating a causal relationship between the change in activities of salivary neurons and that in the level of salivation (Watanabe & Mizunami, 2006). By use of local injection of mecamylamine, a type of acetylcholine receptor antagonist, into various regions of the brain, it has been suggested that the calyces or the lobes of the mushroom body are the sites in which olfactory CS and sucrose solution US converge for salivary conditioning (Watanabe, Matsumoto, Nishino, & Mizunami, 2011).

The major aim of this study was to establish a method for context-dependent discrimination learning in rigidly immobilized cockroaches in order to allow investigation of the neural basis of nonelemental (or configural) associative learning. We trained cockroaches to associate one of a pair of olfactory CS with sucrose US in one visual context and to associate another CS with the US in another visual context, and investigated whether salivary neurons exhibit activity changes associated with this

conditioning in order to pave the way for revealing the neural basis underlying a nonelemental form of learning. To our knowledge, this is the first report of a context-dependent form of salivary conditioning in any vertebrate or invertebrate species. Pavlov (1927) extensively examined salivary conditioning in dogs, but he did not report a context-dependent form of salivary conditioning.

2. Material and methods

2.1. Insects

Adult male cockroaches (*Periplaneta americana*) obtained from a colony that were maintained under a light-dark cycle of 12h: 12h (photophase 8:00~20:00) at 28±2℃ were used. More than 1 week prior to the training, 10-20 cockroaches were isolated from their colony and placed in a plastic container. The walls of the plastic container were smeared with liquid paraffin to prevent escape, and the floor was covered with a sheet of black construction paper. A wooden refuge and two small cups, one cup supplying water and the other supplying sugar-free yeast extract, were placed in the container. This diet enhances the motivation to ingest sucrose.

2.2. Extracellular recording of neural activities of salivary neurons

The preparation for extracellular recordings from salivary neurons was “highly dissected preparation” described previously (Watanabe & Mizunami, 2006). In this preparation, the animal was anesthetized by cooling with ice for 0.5-1 h and then a piece of cuticle at the dorsal part of the abdomen was removed and the esophagus was punctured. The puncture prevents expansion of the esophagus during a long period of recording. Subsequently, the cockroach was restrained ventral-side-up on a paraffin platform using two thin plastic plates, one at the neck and the other between the thorax and the abdomen. The neck region, legs and cerci were fixed on the platform with low-melting point wax, and each antenna was threaded through a small piece of acrylic

tube. The mouthparts were free to move. The restrained cockroaches were then kept in darkness for 1-2 h at room temperature. A small incision was made in the ventral region of the neck cuticle to expose the salivary duct. Then the salivary duct was hooked on a pair of tungsten electrodes (Fig. 1). Since the salivary duct nerve, which contains axons of two salivary neurons, runs along the surface of the salivary duct, hooking the salivary duct allows recording of activities of salivary neurons. To prevent salivary neurons from drying, the salivary duct was covered with a mixture of white Vaseline and liquid paraffin saturated with the cockroach saline.

The activity of salivary neurons was amplified with a differential AC amplifier (DAM80, World Precision Instruments, Sarasota, FL, USA) and displayed on an oscilloscope and a digital recorder (Omniace RT3100N, NEC, Tokyo, Japan). Data were stored on DAT tapes (PC208AX, Sony, Tokyo, Japan) and analyzed using a pulse counter (MET-1100, Nihon Kohden, Tokyo, Japan).

2.3. Olfactory, gustatory, and visual stimulations

Peppermint odor (P) or vanilla odor (V) was used as conditioned stimulus (CS), sucrose solution was used as appetitive unconditioned stimulus (US), and steady or flickering light was used as contextual stimulus. Delivery of odor to an antenna was achieved by the use of a continuous airflow system described previously (Nishino, Yamashita, Yamazaki, Nishikawa, Yokohari, & Mizunami, 2003). The use of this system enables odor presentation without changing the total air flow rate by operating a solenoid valve. A portion of airflow passed though one of the odor cartridges containing two pieces of filter paper (1×3 cm) that were each soaked with 20 µl of either peppermint (P), vanilla (V) or apple (A) essence (peppermint essence and apple essence from Mikoya Kosho Co., Ltd.; vanilla essence from Kyoritsu Foods Co., Inc., Tokyo, Japan). The air around the preparation was continuously sucked out of the experimental room by an exhaust system.

For gustatory stimulation, the mouth was gently touched with a bamboo skewer soaked with 500 mM sucrose solution. To avoid sensory adaptation, odor and taste stimulations were applied at intervals of >30 s.

A sinusoidally flickering or steady light was used as a contextual stimulus. A green light-emitting diode (LED, λ=525 nm, Φ=5.0, E1L513G, Toyoda Gosei, Aichi, Japan) connected to a function generator (FG-273A, Kenwood, Tokyo, Japan) was used to present sinusoidally flickering light (1.0 Hz; the intensity at the peak and the trough being 0.24 and 0.13 Wm^{-2} at the cockroach's head, respectively) and steady light (0.18 ~ 0.19 Wm^{-2} at the cockroach's head). A blank sheet of paper beneath the platform served as a screen for the LED light. The LED was positioned 15 cm above the screen. The distance from the screen to head of the cockroach was 4 cm. To reduce unwanted visual stimulations such as those caused by the experimenter, a partition was placed in front of the cockroach.

2.4. Training and testing procedures

For training, a flickering or steady light was presented to a restrained cockroach for 5 min, and then pairing trials with a CS and a US (CS1-US pairing trial) and unpairing trials to present another CS alone (CS2-unpairing trial) were performed 3 times each in alternate sequence (for group A) or in a pseudorandom order (for group B) (P+ or V-training shown in Fig. 2A, B and Fig. 3A). For a pairing trial, peppermint (P) or vanilla (V) odor was presented 2 s prior to the presentation of sucrose solution. For an unpairing trial, vanilla or peppermint odor was presented alone (V- or P- training shown in Fig. 2B). The durations of the odor and taste stimulations in pairing or unpairing trials were 4 s, and inter-trial intervals (ITI) were 2 min. At 15 min after completing one training session, the light condition was changed to start the next training session. Cockroaches were subjected to one training session consisting of 3 sets of CS1-US pairing trials and CS2-unpairing trials under one light condition and the opposite

training under another light condition (e.g., P+/V- training under flickering light and V+/P- training under steady light). Numbers of training sessions were 6 in group A and 8 in group B (Fig. 2A, Fig. 3A).

In testing sessions, responses of salivary neurons to vanilla, peppermint, and apple odors presented 3-5 times in a pseudorandom order were measured under flickering light and steady light conditions. The durations of odor stimulations were 2 s and the intervals between stimulations were >25 s. In a test session, odor stimulation was initiated 5 min after changing the light condition, and 2 min after the last odor stimulation, the light condition was changed to start the next test session (Fig. 2A, Fig. 3A). The first test session was started > 10 min after completion of the recording set-up. In most preparations, presentations of apple odors evoked strong responses in salivary neurons. Thus, the apple odor was used to test whether olfactory responses remain intact during recordings, and preparations with no significant level (<5%, see Data analysis) of apple responses were not used for data evaluation.

2.5. Data analysis

Responses of salivary neurons to peppermint or vanilla odor (R) were measured as R_s-R_o, where R_o is the spontaneous activity of salivary neurons during a 2-s period before odor stimulation and R_s is the spike frequency during 2 s odor stimulation for testing or the first 2 s of odor stimulation for training. These were averaged from 3-5 measurements in each testing session and from 3 measurements in each training session. For each testing session, the R values for peppermint odor (R_p) and for vanilla odor (R_v) were statistically compared using the Wilcoxon's signed-rank test (WCX-test). For each training session, the difference in responses to peppermint and vanilla odors (R_p-R_v) was also calculated, positive value of which indicates greater responses to peppermint odor over vanilla odor and negative value of which indicates the opposite. The R_p-R_v values for each set of training sessions with the two contexts (flickering vs. steady) were

statistically compared using the WCX-test for evaluation of the acquisition process. Statistical evaluations were performed by Microsoft Excel and Excel statistics software programs (Esumi, Tokyo, Japan).

3. Results

3.1. Experiment 1: The effect of P+/V- training under flickering light and V+/P- training under steady light in group A cockroaches

Group A cockroaches received P+/V- training under flickering light condition and V+/P- training under steady light condition (Fig. 2A,B). Before training, the magnitude of responses of salivary neurons to peppermint odor (R_p) did not significantly differ from that to vanilla odor (R_v) under the flickering light condition and under the steady light condition (WCX-test, flickering: $P>0.05$; steady: $P>0.05$) (Fig. 2C). At 20 minutes after three P+/V- training sessions under flickering light and three V+/P- training sessions under steady light, the magnitude of responses of salivary neurons to peppermint odor was significantly greater than that to vanilla odor under the flickering light condition (WCX-test, flickering: $P<0.05$) (Fig. 2C). On the other hand, the magnitude of responses to peppermint odor was significantly less than that to vanilla odor under the steady light condition (WCX-test, steady: $P<0.01$) (Fig. 2C).

To evaluate the acquisition process, we calculated the difference between the responses to peppermint odor and those to vanilla odor (R_p-R_v) in each set of training sessions under flickering light condition and under the steady light condition (Fig. 2D). In the first set of training, R_p-R_v under the flickering light condition (FL 1) did not significantly differ from that steady light condition (ST 1) (WCX-test; FL 1 vs. ST 1: $P > 0.05$), while R_p-R_v in second and third sets of training sessions were greater under flickering light conditions than that under steady light conditions (WCX-test; FL 2 vs. ST 2: $P<0.01$; FL 3 vs. ST 3: $P<0.01$), indicating that one or two sets of training sessions are sufficient to achieve conditioning.

The results demonstrate that, in cockroaches that received P+/V- training under flickering light condition and V+/P- training under steady light condition, salivary neurons exhibit greater responses to reward-associated peppermint odor than to unpaired vanilla odors under the flickering light condition and exhibit the opposite responses under the steady light condition.

3.2. Experiment 2: The effect of V+/P- training under flickering light and P+/V- training under steady light in group B cockroaches

Group B cockroaches received conditioning with a stimulus arrangement different from that used in group A cockroaches in that: (1) peppermint odor was rewarded under the steady light condition and vanilla odor was rewarded under the flickering light condition, (2) 8 training sessions, rather than 6 training sessions, were carried out, (3) in training, odors were presented in a pseudorandom order, and (4) after training, tests under the flickering light condition were preceded by those under the steady light condition (Fig. 3A).

Before training, the magnitude of responses of salivary neurons to peppermint odor (R_p) was significantly greater than that to vanilla odor (R_v) under the flickering light condition but it did not significantly differ under the steady light condition (WCX-test; flickering: $P<0.05$; steady: $P>0.05$) (Fig. 3B). After eight training sessions, the magnitude of responses of salivary neurons to peppermint odor was significantly greater than that to vanilla odor under the steady light condition, but was significantly less than that to vanilla odor under the flickering light condition (WCX-test; flickering: $P<0.01$; steady: $P<0.01$) (Fig. 3B).

The difference in the responses to peppermint odor and vanilla odor (R_p-R_v) in each sets of the training sessions were significantly greater under steady light conditions compared to that under flickering light conditions (WCX-test; FL 1 vs. ST 1: $P<0.01$; FL 2 vs. ST 2: $P<0.01$; FL 3 vs. ST 3: $P<0.01$; FL 4 vs. ST 4: $P<0.01$) (Fig. 3C),

indicating that one set of training is sufficient to achieve conditioning. The results confirm context-dependent olfactory learning of activities of salivary neurons.

4. Discussion

We demonstrated context-dependent olfactory conditioning of salivation, monitored by activity changes of salivary neurons, in rigidly immobilized cockroaches. In this learning paradigm, animals are requested to select one of a pair of odors in one visual context and to select the other in another visual context, which can be referred to as biconditional discrimination procedure or transswitching paradigm (Menzel, Brembs, & Giurfa, 2007). This preparation is ideal for studies on the neural basis of configural associative learning by the use of various experimental techniques, including intracellular recording and optical recording, which demand strict immobilization of the animal. This preparation can also be used for studies on the capability of cockroaches to discriminate different flickering frequencies, in addition to their capability of learning and memory and of discriminating different odors. Moreover, we will be able to proceed to study the cockroaches' capability to use color or visual pattern stimulus as contextual stimulus.

Pavlov (1927) reported a huge amount of data on salivary conditioning in dogs, but he did not report a context-dependent form of salivary conditioning. To the best of our knowledge, this is the first study showing a context-dependent form of salivary conditioning in any vertebrate or invertebrate species. The results of this study demonstrate that there are sophisticated mechanisms for regulation of saliva secretion in cockroaches, and cockroaches thus provide an excellent model system to study the mechanisms of configural associative learning of an autonomic function. This is also the first study to show context-dependent conditioning of autonomic function in invertebrates. Previous studies on this subject in invertebrates have been confined to habituation of the heart rate (in the blowfly *Calliphora vomitoria*; Thon, 1980),

sensitization of the heart rate (in *Aplysia*; Krontiris-Litowitz, 1999), and conditioning of the heart rate according to context that can be characterized as a form of elemental associative learning between the context stimulus and aversive US (in crabs, *Chasmagnathus*; Hermitte & Maldonado, 2006).

This study shows that context-dependent learning with the use of visual context, olfactory CS and gustatory US can be achieved not only in freely behaving cockroaches (Sato et al., 2006), but also in highly restrained cockroaches. It should be noted that there are slight differences in conditioning procedures in these two studies, except that the cockroaches were immobilized in this study but they were freely behaving in the previous study. At first, a flickering light was used for contextual stimulus in this study but a steady illumination was used in our previous study. It needs to be clarified whether a steady illumination is effective as contextual stimulus for immobilized cockroaches. Second, one of a pair of odors was paired with sucrose solution and the other odor was presented alone in the present study, but one odor was paired with sucrose reward and the other odor was paired with a high concentration of sodium chloride solution in our previous study. This was because cockroaches exhibit preferences for sucrose solution or an odor associated with it and avoidance of sodium chloride solution or an odor associated with it (Sakura & Mizunami, 2001; Watanabe, Kobayashi, Sakura, Matsumoto, & Mizunami, 2003), but they exhibit an increase of the level of salivation in response to sucrose solution or sodium chloride solution or to an odor associated with either of them (Watanabe & Mizunami, 2006). This is analogous to that dogs exhibit salivation in response to food or acid solution or a sensory stimulus associated with either of them (Pavlov, 1927). Third, tests were performed 20 min after completing the last training session in the present study (measuring mid-term memory), whereas tests were performed one day after completing the training session in our previous study (measuring long-term memory). Considering these differences, more studies are needed to examine and compare the properties of context-dependent learning measured as

responses of salivary neurons to odors and those measured as the animals' behavioral responses.

The context-dependent learning described here requests the animals to use a visual context stimulus as "occasion setters" to specify the meaning of olfactory CS and thus is analogous to occasion setting problem solving reported in honey bees, in which animals are requested to use color stimulus, which is acutely presented prior to the presentation of olfactory CS, to specify the meaning of the CS (Mota, Giurfa, & Sandoz, 2011). It would be interesting to examine and compare neural mechanisms underlying these two different forms of occasion setting problem solving in insects. In rats, the role of the dorsal hippocampus in occasion setting with diffuse contextual and discrete light stimulus serving as occasion setters has been studied in classical fear conditioning, and it has been concluded that the dorsal hippocampus is important in processing of information about the signaling value of contextual, but not discrete stimuli (Yoon, et al., 2011).

The ultimate goal of our study is to clarify neural mechanisms of context-dependent olfactory learning, namely, the mechanisms by which different associations between olfactory CS and sucrose US are selected according to visual context stimulus for salivary conditioning in cockroaches. In a recent study, we have suggested that association between olfactory CS and sucrose US in elementary olfactory learning of salivation in cockroaches takes place in the calyces or the lobes of the mushroom body (Watanabe, et al., 2011). This is in accordance with reports that the mushroom bodies participate in elemental associating olfactory learning in honey bees (Menzel, 2001) and fruit-flies *Drosophila* (Berry, Krause, & Davis, 2008; Davis, 2005; Heisenberg, 2003; Waddell, 2010), although contribution of the antennal lobe (the primary olfactory center) has also been demonstrated (in honey bees: Giurfa, 2007; Menzel, 2001; in fruit-flies: Thum, Jenett, Ito, Heisenberg, & Tanimoto, 2007; in moths: Daly, et al., 2004). In cockroaches, the mushroom bodies have been shown to receive visual stimuli,

as well as olfactory, gustatory and mechanosensory stimuli (Li & Strausfeld, 1997; 1999; Mizunami, Okada, Li, & Strausfeld, 1998; Nishikawa, Nishino, Mizunami, & Yokohari, 1998), and thus the mushroom bodies are candidate site for the configural association for context-dependent olfactory conditioning of salivation. In elemental appetitive olfactory learning of insects, octopamine is suggested to convey reward signal (in honey bees: Farooqui, Robinson, Vaessin, & Smith, 2003; Hammer & Menzel, 1998; in crickets: Unoki, Matsumoto, & Mizunami, 2005; in fruit-flies: Honjo & Furukubo-Tokunaga, 2009; Schroll, Riemensperger, Bucher, Ehmer, Voller, Erbguth, Gerber, Hendel, Nagel, Buchner, & Fiala, 2006; Schwaerzel, Monastirioti, Scholz, Friggi-Grelin, Birman, & Heisenberg, 2003; but see Kim, Lee, & Han, 2007). It has also been demonstrated that octopaminergic neurons play critical roles in the recall of appetitive olfactory memory (in honey bees: Farooqui et al., 2003; in crickets: Mizunami, Unoki, Mori, Hirashima, Hatano, & Matsumoto, 2009; reviewed by Mizunami & Matsumoto, 2010). Moreover, it has been reported that the mushroom bodies receive terminations of octopamine-immunoreactive neurons (cockroaches: Sinakevitch, Niwa, & Strausfeld, 2005; honey bees: Kreissl, Eichmüller, Bicker, & Rapus, Eckert, 1994; fruit-flies: Sinakevitch & Strausfeld, 2006; moths: Dacks, Christensen, Agricola, Wollweber, & Hildebrand, 2005). Therefore, a study on the roles of octopaminergic neurons supplying the mushroom bodies in formation and recall of elemental olfactory conditioning of salivation in cockroaches will provide basis for the study of the mechanisms of context-dependent olfactory learning.

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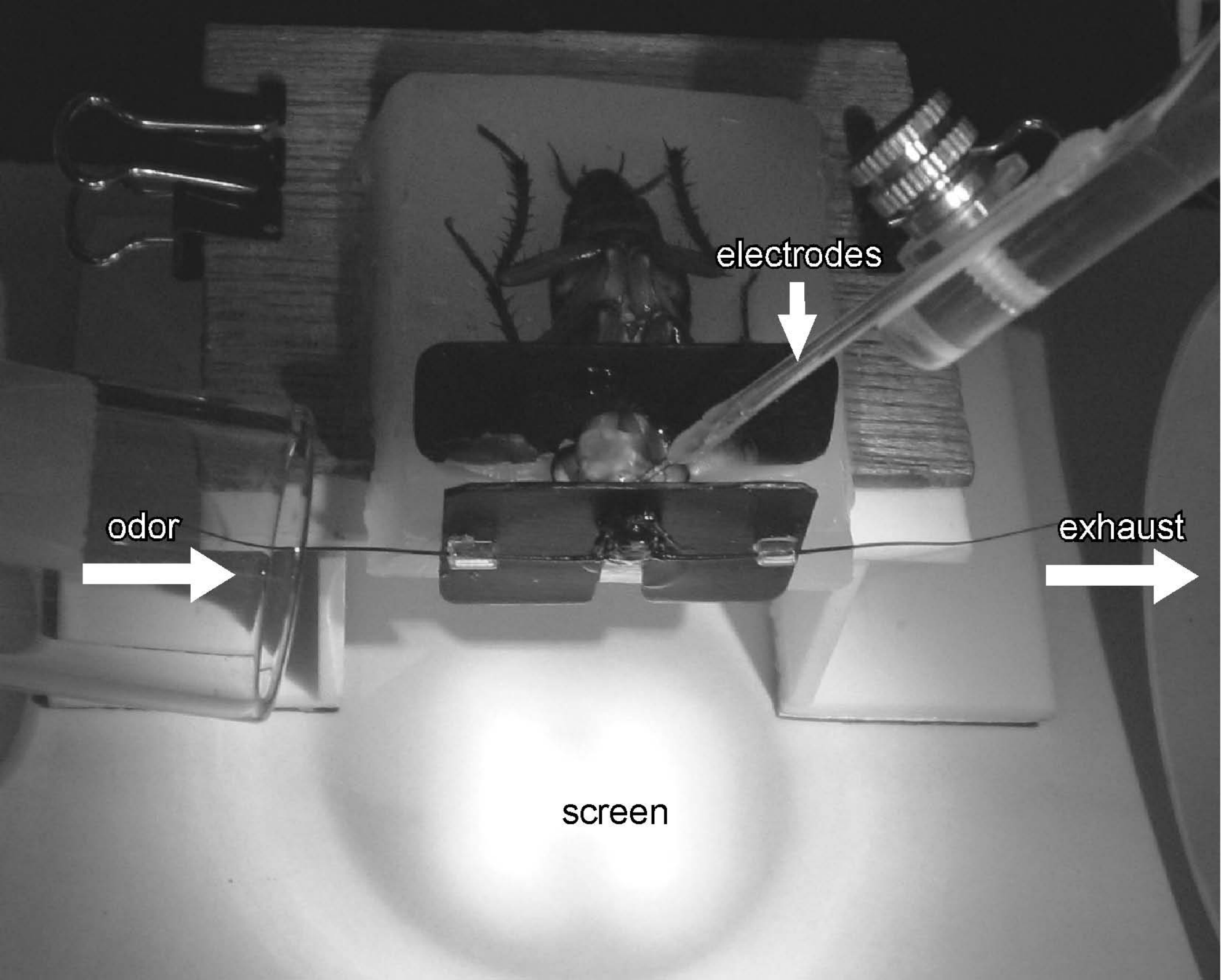
Figure captions

Fig. 1. Experimental setup for context-dependent conditioning of olfactory responses of salivary neurons in the cockroach. Extracellular recording was made from a salivary duct nerve, which contains salivary neurons, by using hook electrodes. Olfactory stimulus was presented to the antenna ipsilateral to the recorded salivary duct nerve by using a continuous airflow system. Sucrose solution was presented to the mouth. For illumination, an LED was set above the screen set in front of the head of the cockroach.

Fig. 2. Effects of context-dependent olfactory conditioning trials on the responses of salivary neurons in group A cockroaches (experiment 1). (**A**) The time schedules for the odor preference test (black) and training (white). The shaded and light gray boxes in a row indicate the flickering and steady light conditions, respectively. Cockroaches were subjected to P+/V- conditioning trials under flickering light and V+/P- conditioning trials under steady light. (**B**) The time course for one session of training in each light condition. (**C**) The responses of salivary neurons to peppermint odor (dark gray, R_p) and to vanilla odor (white, R_v) under flickering light and steady light conditions before and after training shown as box and whisker diagrams. (**D**) For evaluation of the acquisition process, R_p-R_v under the flickering light condition (lined) and steady light condition (light gray) in each of the six sessions of training (FL 1- ST 3) are shown as box and whisker diagrams. Session names correspond to those in the top diagram. 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 to simplify the figures, but are included for data analysis. The results of statistical comparisons are shown by asterisks (WCX-test, $^{**}P < 0.01$; $^{*}P < 0.05$; NS $P > 0.05$). The number (n) indicates the number of animals used for data analysis.

Fig. 3. Effects of context-dependent olfactory conditioning trials with reversed stimulus

arrangement on the responses of salivary neurons in group B cockroaches (experiment 2). (**A**) The time schedule. The legend symbols are the same as those in Fig. 2. During training, the trials were arranged in a pseudorandom sequence. Cockroaches were subjected to P-/V+ conditioning trials under flickering light and P+/V- conditioning trials under steady light. (**C**) The responses of salivary neurons to peppermint odor (dark gray, R_p) and to vanilla odor (white, R_v) under flickering light and under steady light. Data are shown as box and whisker diagrams. (**D**) For evaluation of the acquisition process, R_p-R_v under flickering light (lined) and steady light (light gray) in each of the eight training sessions are shown as box and whisker diagrams. The results of statistical comparisons are shown by asterisks (WCX-test, $^{**}P < 0.01$; $^{*}P < 0.05$; NS $P > 0.05$). The number (n) indicates the number of animals used for data analysis.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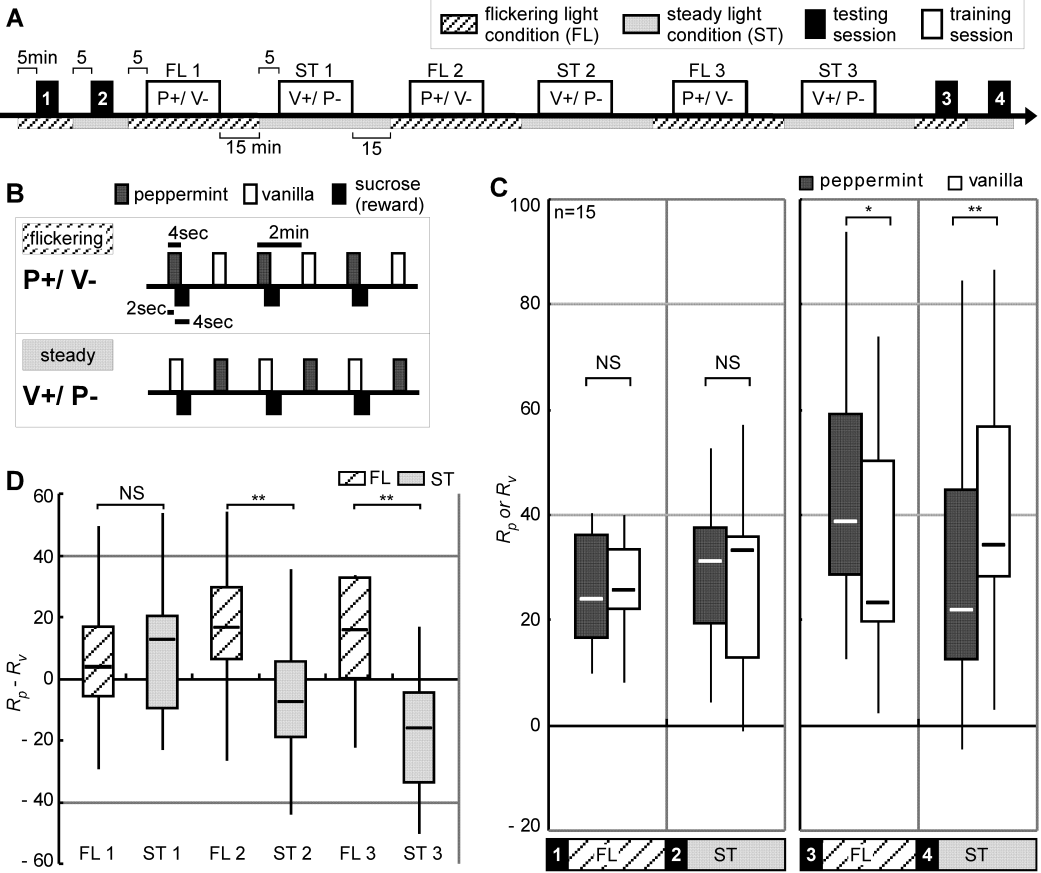
A
flickering light condition (FL)
steady light condition (ST)
testing session
training session
5min
5
5
5
FL 1
ST 1
FL 2
ST 2
FL 3
ST 3
P+/ V-
V+/ P-
P+/ V-
V+/ P-
P+/ V-
V+/ P-
1
2
3
4
15 min
15
B
peppermint
vanilla
sucrose (reward)
flickering
4sec
2min
P+/ V-
2sec
4sec
steady
V+/ P-
C
peppermint
vanilla
n=15
100
80
60
40
20
0
- 20
NS
NS
*
**
R_p or R_v
1
FL
2
ST
3
FL
4
ST
D
FL
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60
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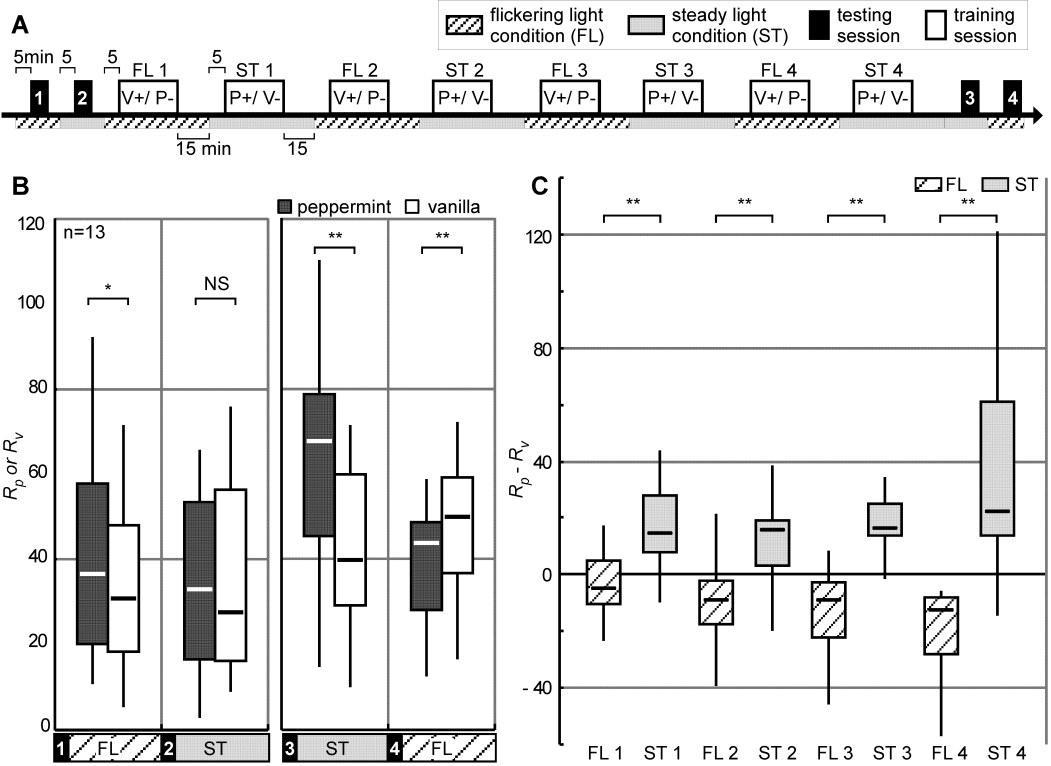
R_p - R_v
FL 1
ST 1
FL 2
ST 2
FL 3
ST 3


A
flickering light condition (FL)
steady light condition (ST)
testing session
training session
5min
5
5
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FL 1
ST 1
FL 2
ST 2
FL 3
ST 3
FL 4
ST 4
V+/ P-
P+/ V-
1
2
3
4
15 min
15
B
peppermint
vanilla
n=13
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NS
**
**
R_p or R_v
120
100
80
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1 FL 2 ST
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R_p - R_v
120
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- 40
FL 1
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FL 2
ST 2
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ST 3
FL 4
ST 4