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Salivary conditioning with antennal gustatory unconditioned stimulus in an insect ☆

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ABSTRACT

Classical conditioning of olfactory conditioning stimulus (CS) with gustatory unconditioned stimulus (US) in insects has been used as a pertinent model for elucidation of neural mechanisms underlying learning and memory. However, a conditioning system in which stable intracellular recordings from brain neurons are feasibly obtained while monitoring the conditioning effect has remained to be established. Recently, we found classical conditioning of salivation in cockroaches *Periplaneta americana*, in which an odor was associated with sucrose solution applied to the mouth, and this conditioning could be monitored by activities of salivary neurons. Application of gustatory US to the mouth, however, leads to feeding movement accompanying a movement of the brain that prevents stable recordings from brain neurons. Here we investigated whether a gustatory stimulus presented to an antenna could serve as an effective US for producing salivary conditioning. Presentation of sucrose or sodium chloride solution to an antenna induced salivation and also increased activities of salivary neurons. A single pairing trial of an odor with antennal presentation of sucrose or sodium chloride solution produced conditioning of salivation or of activities of salivary neurons. Five pairing trials led to a conditioning effect that lasted for one day. Water or tactile stimulus presented to an antenna was not effective for producing conditioning. The results demonstrate that gustatory US presented to an antenna is as effective as that presented to the mouth for producing salivary conditioning. This conditioning system provides a useful model for studying the neural basis of learning at the level of singly identifiable neurons.

1. Introduction

Conditioning of salivation was discovered by Pavlov about a century ago in dogs (Pavlov, 1927). After repeated presentation of auditory stimulus (conditioning stimulus, CS) prior to the presentation of food (unconditioned stimulus, US), the dog salivated in response to auditory CS. Pavlov and his successors energetically studied neural mechanisms underlying salivary conditioning in dogs. Pavlov (1927) reported that a lesion of the cerebral cortex decreased salivation in response to auditory CS, and subsequent studies suggested that the orbital cortex played a role in modulation of salivation in response to auditory CS (Baranov, 1983; Zernicki & Santibanez-H, 1961). However, further details of neural mechanisms underlying salivary conditioning remain unknown.

Conditioning of salivation has also been demonstrated in humans (Feather, Delse, & Bryson, 1967; Wells & Feather, 1968), but, to our knowledge, it has not been demonstrated in any other animals until we recently demonstrated it in cockroaches (Watanabe & Mizunami, 2006, 2007). After differential conditioning trials in which an odor was paired with sucrose solution presented to the mouth and another odor was presented without pairing with sucrose solution, cockroaches exhibited increased levels of salivation in response to the sucrose-associated odor but not to the unpaired odor (Watanabe & Mizunami, 2007). This conditioning can be easily monitored by changes in activities of salivary neurons (Watanabe & Mizunami, 2006). In cockroaches, salivation is regulated by two large salivary neurons of the subesophageal ganglion (salivary neuron 1 and salivary neuron 2; SN1 and SN2) and several small neurons belonging to the stomatogastric nervous system, both being contained in the salivary duct nerve (Baumann, Kühnel, Dames, & Walz, 2004; House & Ginsborg, 1976). Extracellular recordings from the salivary duct nerve showed that the activities of SN1 and SN2 in response to an odor increased after pairing of the odor with sucrose solution applied to the mouth (Watanabe & Mizunami, 2006).

Because many of brain neurons are readily identifiable by intracellular recordings and stainings in cockroaches (Mizunami, 1990, 1996; Li & Strausfeld, 1997, 1999; Nishino et al., 2003), the salivary conditioning enables to study neural mechanisms underlying learning on the level of singly identifiable neurons. A major obstacle for intracellular recording from brain neurons during salivary conditioning, however, is that presentation of gustatory US to the mouth induces vigorous movements of the mandibles (Watanabe & Mizunami, 2006) and this results in movement of the brain and prevents stable recordings from

☆ Salivary conditioning with antennal gustatory US.

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brain neurons. To overcome this problem, we examined whether gustatory stimulation to an antenna can serve as an effective US for producing salivary conditioning. Cockroaches use their long, whip-like antennae to probe chemical and tactile compositions of the environment (Hansen-Delkeskamp, 1992; Nojima, Schal, Webster, Santangelo, & Roelofs, 2005; Pintér, Lent, & Strausfeld, 2005). The third segment of the antenna (flagellum) is covered with about 6500 bimodal bristles, each of which houses one tactile and several gustatory receptor neurons (Nishino, Nishikawa, Yokohari, & Mizunami, 2005). It has been reported that the two of three or four gustatory neurons housed in the single bristle respond specifically either to sugar solution or salt solution stimuli (Hansen-Delkeskamp, 1992; Hansen-Delkeskamp & Hansen, 1995). In addition, about 90 hygro-sensitive neurons that respond to increased humidity or water are known to be orderly distributed in the flagellum (Yokohari, 1999). We studied the effects of gustatory (sugar and salt), water and tactile stimulus applied to an antenna for producing olfactory conditioning of activities of salivary neurons and of salivation.

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. Cockroaches were fed a diet of sugar-free yeast extract and drinking water *ad libitum*.

2.2. Extracellular recordings of activities of salivary neurons

The preparations for extracellular recording of activities of SN1 and SN2 were modified from those in our previous study (Watanabe & Mizunami, 2006). The cockroach was anaesthetized on ice, the wings were removed, and the esophagus was punctured to prevent its movement and expansion during chronic recording. Then, the cockroach was restrained on a wax-coated stage ventral-side-up with thin plastic plates at the neck and abdomen (Fig. 1A). The legs, neck, cerci and mouthparts were fixed with low-melting point wax. Each antenna was fixed by a plastic ring placed at about 1 cm from its base.

Preparations were kept for 1–2 h at room temperature, and then the cuticle in the neck was removed to expose the salivary duct. Since the salivary duct nerve runs along the surface of the salivary duct, one salivary duct nerve, as well as the salivary duct, was hooked on a pair of tungsten electrodes (Fig. 1B). 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 the neck plastic plate with low-melting point wax (Fig. 1A). The preparation was mounted on the tilting stage, and then it was raised head-side-up.

The activity of the salivary duct nerve was amplified with a differential amplifier (DAM80, World Precision Instruments, Sarasota, FL USA) and displayed on an oscilloscope and a digital recorder (Omniace, NEC, Tokyo, Japan). Data were stored on an MO disk (LX-10, TEAC, Tokyo, Japan). Activities of salivary neurons were segregated out from those of neurons of the stomatogastric nervous system using a window discriminator equipped with a spike counter (MET1100, NIHON KOHDEN, Tokyo, Japan).

2.3. Sensory stimulation to an antenna

The continuous airflow system used to deliver olfactory stimuli to an antenna of the immobilized animal was described previously (Nishino et al., 2003). In short, air passed through a small chamber containing a piece of filter paper soaked with 40 μl of vanilla, peppermint or apple essence could be delivered by operating a solenoid valve, without changing the flow rate. The air around the antenna was continuously sucked out of the room through a vacuum system. To avoid sensory adaptation, olfactory stimuli were applied with an interval of >20 s. Vanilla essence was purchased from Kyoritsu Foods (Tokyo, Japan) and peppermint and apple essences were purchased from Mikoya Kosho (Tokyo, Japan).

In experiments to study gustatory or tactile responses of salivary neurons, gustatory stimulation was presented as follows: a droplet (10 μl) of sucrose solution or sodium chloride solution of various concentrations or of distilled water was applied to an antenna (at about 2 cm from the scape). The droplet was carefully removed by a gentle touch of a piece of filter paper on the antenna after 10 s from the onset of stimulation. For tactile stimulation, a similar region used for gustatory stimulation on the flagellum was gently touched for 4 s with a dry filter paper. The interval between stimuli was 2 min. For conditioning trials, an antenna was gently touched with rectangular filter paper (0.5 × 1 cm) soaked with 10 μl of 500 mM sucrose solution, 5 M sodium chloride solution or distilled water for 4 s or with dry filter paper.

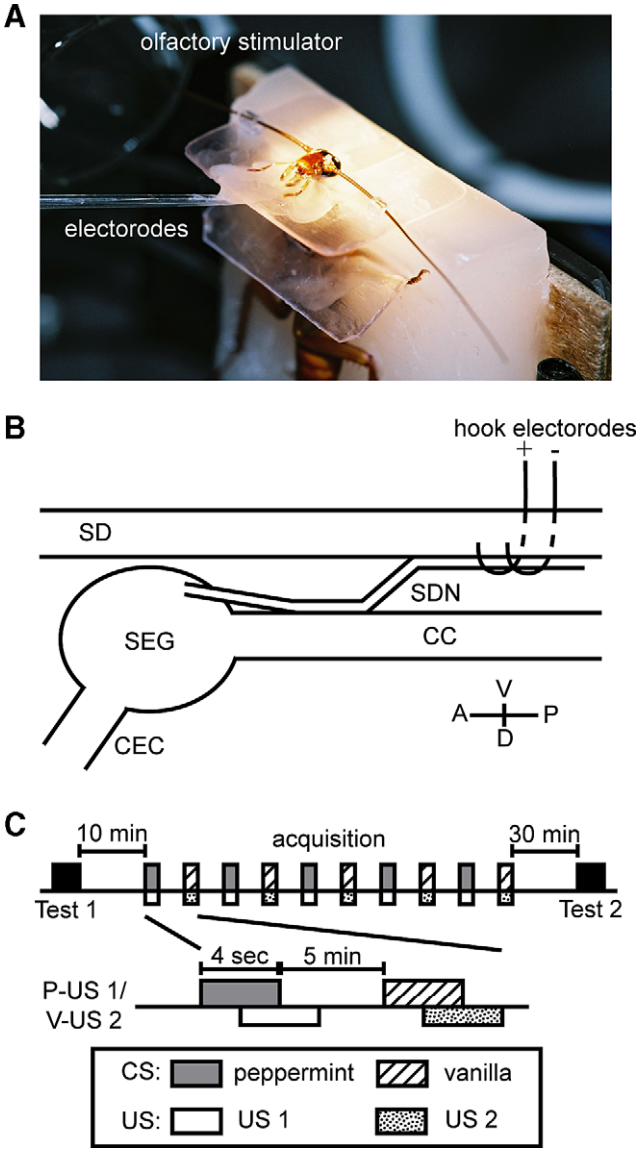

Fig. 1. Experimental procedures. (A) A cockroach is restrained for extracellular recording of activities from a salivary duct nerve (SDN). Olfactory, gustatory, tactile or water stimulus was presented to the antenna ipsilateral to the SDN under the recording. (B) Arrangement of extracellular recording from a SDN, a lateral view. The SDN originates from the subesophageal ganglion (SEG) and runs along the surface of the salivary duct (SD) to innervate the salivary glands. The SD and SDN were hooked by a pair of tungsten electrodes. CC, cervical connective; CEC, circumesophageal connective. A, anterior; P, posterior; V, ventral; D, dorsal. (C) Stimulus schedules for differential conditioning trials. Five sets of differential conditioning trials were carried out during recording from a SDN. One set of "P-US1/V-US2" differential conditioning trial set consisted of the presentation of peppermint odor (shaded squares) 2 s before the presentation of an unconditioned stimulus (US 1; open squares) and subsequent presentation of vanilla odor (hatched squares) 2 s before the presentation of another unconditioned stimulus (US 2; dotted squares). The durations of CS and US were 4 s. The inter-trial intervals were 5 min. To evaluate the conditioning effects, responses of salivary neurons to 2-s presentation of peppermint, vanilla or apple (control) odor were measured at 10 min before (Test 1; black square) and 30 min after (Test 2; black square) conditioning.

2.4. Conditioning procedure and evaluation of the conditioning effects

The procedure for classical conditioning was modified from that reported previously (Watanabe, Kobayashi, Sakura, Matsumoto, & Mizunami, 2003; Watanabe & Mizunami, 2006, 2007). Immobilized cockroaches were subjected to five sets of