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Classical conditioning of activities of salivary neurones in the cockroach

Hidehiro Watanabe and Makoto Mizunami*

Graduate School of Life Sciences, Tohoku University, Katahira 2-1-1, Sendai 980-8577, Japan

*Author for correspondence (e-mail: makoto@biology.tohoku.ac.jp)

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Summary

Secretion of saliva to aid swallowing and digestion is a basic physiological function found in many vertebrates and invertebrates. For mammals, classical conditioning of salivation in dogs was reported by Pavlov a century ago. However, conditioning of salivation or of related neural activities in non-mammalian species has not been reported. In many species of insects, salivation is regulated by salivary neurones. In this study, we found that salivary neurones of the cockroach *Periplaneta americana* exhibited a strong response to sucrose solution applied to the mouth and a weak response to odours applied to an antenna, and we studied the effect of conditioning on the activities of salivary neurones. After three sets of differential conditioning trials in which an odour was presented just before the presentation of sucrose solution and the other odour was presented alone, the response of salivary neurones to sucrose-associated odour significantly increased but that to the odour presented alone was unchanged. Backward pairing trials in which an odour was presented after the presentation of sucrose solution were not effective in achieving conditioning. Our study of the change in the level of saliva secretion in response to electrical stimulation of salivary neurones suggested that the magnitude of increase in odour response of salivary neurones by conditioning is sufficient to lead to an increased level of salivation. This study suggests classical conditioning of salivation in an insect.

Key words: learning, memory, olfaction, taste, salivary neurones, insect.

Introduction

Pavlov reported classical conditioning of salivation in dogs in a congress held in 1903 (Pavlov, 1927). In studying the mechanisms of digestion, Pavlov discovered that when a bell was regularly sounded just before feeding, the sound of the bell would eventually trigger salivation. He also showed lesions in several regions of the cerebral cortex affect classical conditioning of salivation (Grimsley and Windholz, 2000). Later studies on lesions and electrical stimulations of various brain regions in dogs and rats have suggested that many regions of the brain, including the orbital cortex, caudate nucleus, hypothalamus and amygdala, are involved in classical conditioning of salivation (Lagowska and Fonberg, 1975; Danilova, 1981; Danilova, 1983; Matsuo and Kusano, 1984). However, the cellular mechanisms underlying classical conditioning of salivation remain obscure because of the complexity of information processing in the mammalian brain (Kandel et al., 2000).

It has been shown that classical conditioning by repeated pairing of a conditioning stimulus (CS), such as the sound of a bell, and an unconditioned stimulus (US), such as food, is very common among many vertebrates (Passe and Walker, 1985) and invertebrates (Menzel, 1999; Lechner et al., 2000). However, as far as we know, classical conditioning of salivation has so far been reported only in mammals. Since secretion of saliva to aid swallowing and digestion is a basic physiological function found in many animals, including flatworms (Orido et al., 1998) and nematodes (Zunke, 1990), the following question arises: is classical conditioning of salivation specific to mammals that are equipped with elaborated autonomous nervous systems?

The control of salivary secretion has been the subject of detailed study in insects such as cockroaches and locusts (Ali, 1997). In cockroaches, salivation is regulated by the salivary duct nerve (SDN) (Whitehead, 1971; Rietdorf et al., 2003). The SDN consists of two neurones with large-diameter (3-4 μm) axons (salivary neurones 1 and 2; SN1 and SN2) and several neurones with small-diameter (~1 μm) axons (Whitehead, 1971), the cell bodies of the former neurones being located in the suboesophageal ganglion (SOG) (Gifford et al., 1991; Ali, 1997); the latter neurones have been reported to belong to the stomatogastric nervous system (Davis, 1985; Ali, 1997).

Immunohistochemical studies suggest that SN1 is dopaminergic (Elia et al., 1994) and that small-diameter neurones are serotonergic (Davis, 1985), and *in vitro* application of dopamine and serotonin to salivary glands induces secretion of protein-free saliva and protein-rich saliva, respectively (Just and Walz, 1996). The neurotransmitter of SN2 has not yet been determined. In the locust, salivary neurones exhibit activity during feeding (Baines et al., 1989;

Schachtner and Bräunig, 1993) that is modulated by activity of the mouthpart motor pattern generator (Rast and Bräunig, 2001). However, responses of salivary neurones to food-associated sensory stimuli, such as taste or olfactory stimuli, have not been studied.

Cockroaches can be trained to associate olfactory CSs with gustatory USs by an operant (Sakura and Mizunami, 2001; Sakura et al., 2002) or a classical conditioning procedure (Watanabe et al., 2003). The latter procedure is effective for both freely moving and restrained cockroaches. Here we report that responses of salivary neurones to an odour significantly increased after repeated pairing of the odour with sucrose reward. Moreover, we suggest that the observed increase in odour response of salivary neurones after conditioning (5–10 Hz) is sufficient to lead to an increased level of saliva secretion. Our results provide a unique opportunity to study cellular mechanisms of conditioning of activities of salivary neurones in animals whose central nervous systems are accessible to detailed electrophysiological analysis.

Materials and methods

Insects

Adult male cockroaches, *Periplaneta americana* L., were obtained from a laboratory colony maintained under a 12 h:12 h light:dark cycle at 26–28°C. One week before the start of the experiment, a group of 10–20 cockroaches was placed in a chamber. The wall of the chamber was smeared with liquid paraffin to prevent the cockroaches from escaping, and the floor was covered with black cardboard. There was a wooden refuge and two small cups, one supplying water *ad libitum* and the other supplying sugar-free yeast extract, which enhanced the motivation of the cockroaches to take up sucrose.

Metal fillings of salivary neurones

Backfills and forwardfills of the SDN were made for each of 20 animals. Each animal was anaesthetized with ice for 1–2 h. After removal of its legs and wings, it was pinned ventral-side-up on a wax-coated dish and the cuticle of the ventral part of the neck was removed. One SDN was cut and its proximal or distal cut-stump was inserted into a plastic tube filled with a solution containing 0.16 mol l^{-1} $NiCl_2$ and 0.04 mol l^{-1} $CoCl_2$ (Okada et al., 2003). The preparations were kept in a moist chamber at 4°C for 4 days.

After backfilling, the ventral cuticle of the head was removed to expose the SOG. After forwardfilling, the ventral cuticle of the thorax was removed to expose the salivary gland. Then one or two droplets of rubeanic acid were applied onto the SOG or the salivary gland for 3–5 min to precipitate the metals (Okada et al., 2003). The SOG or the salivary gland was rinsed many times with cockroach saline (Yamasaki and Narahashi, 1959), dissected out, fixed in 3–4% paraformaldehyde in cockroach saline for 30–60 min, dehydrated in a graded series of ethanol, cleared in methyl salicylate, and observed as whole mounts under a light microscope. After observation of the specimens, they were rehydrated in an ascending series of ethanol. Then the specimens were intensified with silver (Bacon and Altman, 1977) and observed as whole mounts. Digital images were taken using a digital camera (Camedia C-3040 Zoom; Olympus, Tokyo, Japan) and were processed using Adobe Photoshop 7.0.

Extracellular recordings of activities of salivary neurones

We used two preparations for extracellular recordings from the SDN. In one preparation (called the semi-intact preparation), an animal was anaesthetized with ice for 0.5–1 h, its wings were removed, and it was restrained on a wax-coated dish ventral-side-up with thin plastic plates at the neck and between the thorax and abdomen. Then the legs and antenna were fixed with low-melting wax and staples, respectively. In another preparation (called the highly dissected preparation), the oesophagus was punctured to prevent its expansion during chronic recording, and the neck and the cerci were fixed with low-melting point wax. The advantage of the latter preparation is that the movement of the head and the oesophagus and also the resulting artefact in the recording were less frequent and this facilitated reliable segregation of unit activities. In both preparations, the restrained animal could move its mouthparts freely.

Semi-intact preparations and highly dissected preparations were kept in a moist chamber at 26–28°C overnight and for 1–2 h, respectively, and then a small incision was made in the ventrolateral sclerite of the neck to expose the salivary duct. Since the SDN runs along the surface of the salivary duct, one SDN, as well as the salivary duct, was hooked on a pair of tungsten electrodes (Fig. 1A). To prevent drying of the SDN, the salivary duct was covered with a mixture of white Vaseline and liquid paraffin saturated with cockroach saline.

The activity of the SDN was amplified with a differential AC 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 DAT tapes (PC208AX, Sony, Tokyo, Japan). Activities of individual units were segregated out using a window discriminator equipped with a spike counter (MET1100, Nihon Kohden, Tokyo, Japan).

Effects of surgical ablation of salivary neurone 1 or 2 on activities of one SDN

To determine which of the units of the SDN reflect activities of salivary neurones 1 and 2 (SN1 and SN2), the SOG was exposed by removing ventral parts of the neck and labia in highly-dissected preparations, and the part of the SOG where the cell body of SN1 or SN2 was located was surgically ablated using a fine needle or scissors, and the resulting change in activities of one SDN was studied. When one of the units of the SDN was removed by surgery, the SDN was cut and backfilled with metal to confirm which of the neurons, SN1 or SN2, had been ablated.

Taste and olfactory stimulation

The continuous airflow system used to deliver odour stimulation to an antenna of the immobilized animal was described previously (Nishino et al., 2003). Briefly, air, which

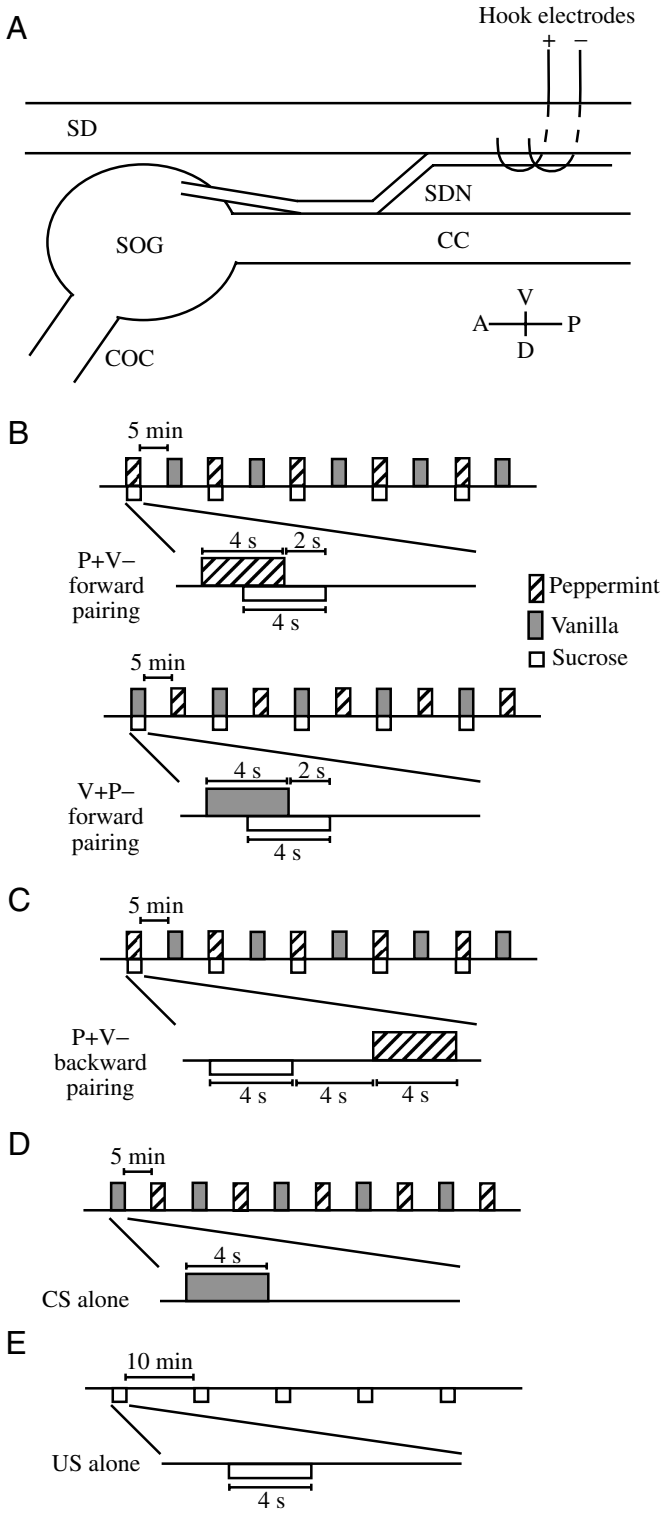

Fig. 1. Experimental procedure. (A) Arrangement of extracellular recording from an SDN, lateral view. The salivary duct nerve (SDN) originates from the suboesophageal ganglion (SOG) and runs along the surface of the salivary duct (SD) to innervate the salivary glands. The SD and SDN were hooked by a pair of tungsten electrodes. CC, cervical connective; COC, circumoesophageal connective; A, anterior; P, posterior; V, ventral; D, dorsal. (B,C) Stimulus schedules for forward-pairing (B) and backward-pairing (C) trials. Five sets of differential conditioning trials were carried out. For each set of P+V– and V+P– forward-pairing trials, peppermint (hatched squares) or vanilla (shaded squares) odour was presented 2 s before the presentation of sucrose solution (open squares) and then vanilla or peppermint odour was presented alone, respectively. For each set of P+V– backward-pairing trials, peppermint odour was presented 4 s after the presentation of sucrose solution and then vanilla odour was presented alone. (D) Stimulus schedule for unpaired presentation of peppermint and vanilla odours (CS alone). Peppermint and vanilla odours were alternately presented five times without pairing with sucrose solution. (E) Stimulus schedule for unpaired presentation of sucrose solution (US alone). Sucrose solution was presented five times without pairing with odour. The inter-stimulus intervals were 5 min in B–D and 10 min in E. The durations of odour and sucrose stimulations were 4 s in B–E.

was passed through a small chamber containing a piece of filter paper soaked with 40 μl of an extract of vanilla or peppermint, could be delivered without changing the flow rate by operating a solenoid valve. The air around the antenna was continuously sucked out of the room through a vacuum system. For gustatory stimulation, the mouth was gently touched with a wooden stick soaked with 10% sucrose solution, 20% sodium chloride solution or distilled water. To avoid sensory adaptation, odour or taste stimuli were applied with an interval of >30 s.

Classical conditioning procedures

The classical conditioning procedures used in this study were modified from those used for cockroaches (Watanabe et al., 2003) and crickets (Matsumoto and Mizunami, 2002; Matsumoto and Mizunami, 2004). Five sets of forward or backward CS/US pairing trials were performed on immobilized animals during recording of the activities of the SDN. One set of 'P+V–' or 'V+P–' forward-pairing trial consisted of a presentation of peppermint or vanilla odour 2 s prior to the presentation of sucrose solution and subsequent presentation of vanilla or peppermint odour without pairing with sucrose reward, respectively (Fig. 1B). One set of P+V– backward-pairing trial consisted of a presentation of peppermint odour 4 s after the presentation of sucrose solution and subsequent unpaired presentation of vanilla odour (Fig. 1C). The interval between trials was 5 min. In a control experiment, peppermint and vanilla odours were alternately presented five times without pairing with sucrose solution (CS alone, Fig. 1D). The interval between odour stimuli was 5 min. In another control experiment, sucrose solution was presented five times without pairing with odours (US alone, Fig. 1E). The interval between sucrose solution stimuli was 10 min. The duration of the odour or sucrose stimulation was 4 s.

In experiments to study short-term retention of the conditioning effect, responses to vanilla and peppermint odours presented 3–5 times >10 min prior to conditioning or control trials were compared with responses to these odours presented at 1 or 5 min and 30 min after conditioning trials or with responses to these odours presented at 6 and 35 min after

control trials (presentation of US alone). The duration of the stimulation was 2 s and the interval between stimulations was >10 s. The measurement was initiated >15 min after completing the set-up of electrophysiological recording to stabilize the preparation.

In an experiment to study 1-day retention of the conditioning effect, a group of immobilized cockroaches was subjected to P+V– forward- or P+V– backward-pairing trials. Cockroaches were kept in a moist chamber at 26–28°C for 1 day, and then the ventral cuticle of the neck was removed and activities of the salivary duct nerve were recorded to study their responses to peppermint or vanilla odour.

Measurements of salivation in response to electrical stimulation of the SDN

Secretion of saliva from a salivary duct in response to electrical stimulation of an SDN was measured in a highly dissected preparation. One SDN was hooked on two pairs of tungsten electrodes (Fig. 2), the distal pair of which was used to electrically stimulate the SDN and the proximal pair was used to monitor the resulting spikes of salivary neurones. The salivary duct was exposed and cut at the site where it enters the head capsule, and the distal cut-stump was inserted into a plastic chamber that had a hole in the upper part. The tip of the plastic chamber was covered with white Vaseline to prevent leakage of saliva (Fig. 2). Brief (0.2 ms in duration) square-wave pulses were delivered to the SDN by a stimulator equipped with an isolator (SEN-3301, Nihon Kohden, Tokyo, Japan). The SDN was stimulated at 5 Hz for 2, 5, 10, 20 or 40 s with intervals of 6 min.

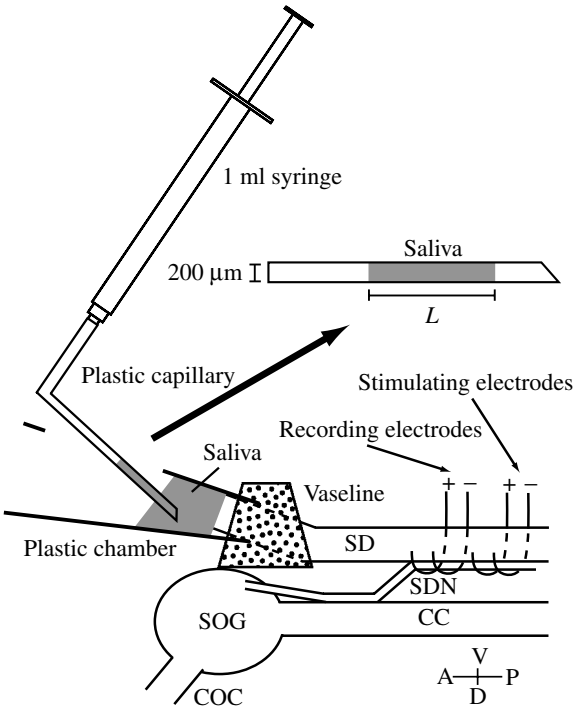

Fig. 2. Arrangement for the measurement of saliva secretion upon electrical stimulation of the salivary duct nerve (SDN), lateral view. The distal cut-stamp of the salivary duct (SD) was inserted into a small plastic chamber, and the tip of the plastic chamber was covered with white Vaseline (dotted area) to prevent leakage of saliva. Saliva (shaded area) secreted from the SD was collected at 1 min intervals using a plastic capillary attached to a syringe. The amount of secreted fluid was calculated from the length (*L*) of the fluid column. The SDN was electrically stimulated by a pair of hook electrodes, and the resulting spikes were monitored by another pair of hook electrodes. Bars, 100 μm. CC, cervical connective; COC, circumoesophageal connective. A, anterior; P, posterior; V, ventral; D, dorsal.

Fluid secreted from the duct to the plastic chamber was drawn into a plastic capillary (inner diameter: 200 μm) every 1 min, and the length of the fluid column was measured to calculate the volume of the fluid (Fig. 2). The measurement was initiated >10 min after completing the set-up of preparation to stabilize the salivation.

Data analysis

Salivary neurones exhibited spontaneous spike discharges. The magnitude of responses of salivary neurones to odour stimulation was measured as relative increase in spike frequency from the spontaneous level, i.e. $100(R–R_o)/R_o$ (%), where R and R_o are spike frequency during the first 2 s of odour stimulation and that during a 2 s period before odour stimulation, respectively.

All statistical evaluation was performed using Microsoft Excel and Excel statistics software programs (Esumi, Tokyo, Japan). In most cases, odour response data fitted to the normal distribution, and the paired *t*-test was used to evaluate the data. However, the distribution of data for SN1 obtained from the highly dissected preparation deviated from the normal distribution, and thus non-parametric Wilcoxon's test (WCX-test) was used for statistical evaluation. The data for secreted volume of saliva for 1-min periods before and after the onset of electrical stimulation of the SDN also deviated from the normal distribution and were thus compared using Wilcoxon's test.

Results

Morphology

A brief description of the morphologies of the SDN salivary neurones follows to facilitate interpretation of their sensory responses. Backfills from the SDN revealed two neurones with large cell bodies in the SOG (Fig. 3A,B), the morphology of which matched salivary neurones 1 and 2 (SN1 and SN2) reported previously (Gifford et al., 1991; Elia et al., 1994). The cell body of SN1 is located at the anteroventral surface of the SOG and that of SN2 is located posterior to that of SN1. Dendrites of SN1 are located in dorsal and ventral parts of the mandibular and maxillary neuromere, and dendrites of SN2 are located in the ventral part of maxillary and labial neuromeres (Fig. 3B). Forwardfills of the SDN revealed the existence of two large-diameter neurones (Fig. 3C, black arrowhead) and at least one small-diameter neurone (Fig. 3C, white arrowhead) along the salivary duct. The former two neurones obviously correspond to SN1 and SN2, while no axons or cell bodies

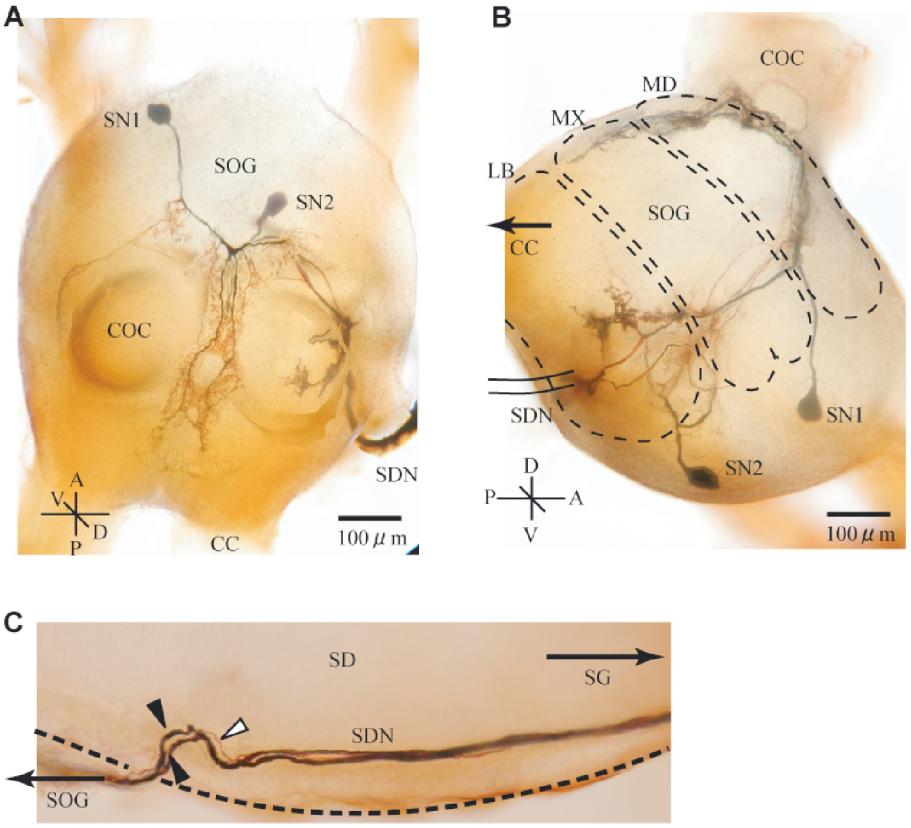

Fig. 3. Two large salivary neurones (SN1 and SN2) in the suboesophageal ganglion (SOG), stained by metal backfilling of one salivary duct nerve (SDN), viewed dorsally (A) and laterally (B). Areas surrounded by broken lines are mandibular (MD), maxillary (MX) and labial (LB) neuromeres, respectively. CC, cervical connective; COC, circumoesophageal connective. (C) An SDN at the surface of a salivary duct (SD), filled with metal. The broken line indicates the outline of the SDN. Two large-diameter axons (black arrowheads) and one small-diameter axon (white arrowhead) are visible. SG, salivary gland; A, anterior; P, posterior; V, ventral; D, dorsal.

corresponding to the latter were stained in the SOG, in agreement with previous reports that small-diameter neurones belong to the stomatogastric nervous system (Davis, 1985; Ali, 1997).

Spontaneous activity of the SDN

We performed chronic extracellular recordings from the SDN. The SDN generated spontaneous spike activities, from which we discriminated three to five units with different amplitudes (Fig 4A left, B left; Fig. 5), which were segregated into two large amplitude units and one to three small amplitude units. One large unit exhibited a spontaneous spike activity of a low frequency (0–10 Hz) and the other large unit fired at a higher frequency (10–30 Hz). In most (>80%) recordings, the low-frequency unit was the

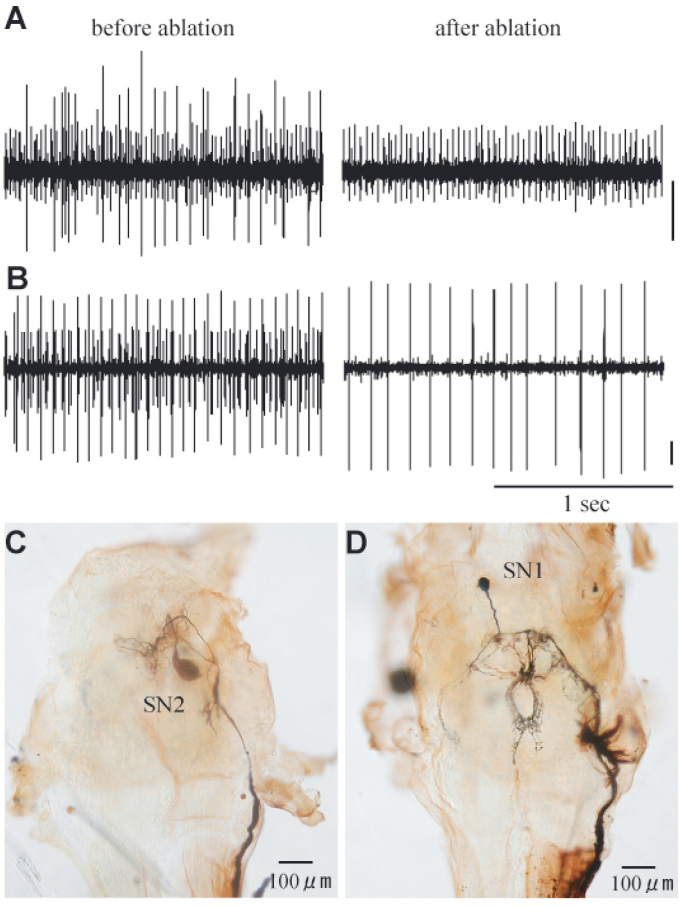

Fig. 4. Effects of surgical ablation of one of the salivary neurones SN1 or SN2 on unit activities of a salivary duct nerve (SDN). (A,B) Spontaneous spike activity of an SDN 10 min before (left) and 10 min after (right) surgical ablation of a part of the suboesophageal ganglion (SOG). In A, a lower-frequency unit with the largest amplitude disappeared after surgery, and post-mortal backfilling of the SDN revealed elimination of the cell body and some dendrites of SN1 (C). In B, a higher-frequency unit with the second-largest amplitude disappeared after surgery, and post-mortal histological examination revealed ablation of the cell body and some dendrites of SN2 (D). Vertical bars, 2 mV; horizontal bars, 1 s (A,B); 100 μm (C,D). The SOG is viewed dorsally in C and D.

largest in amplitude and the higher-frequency unit was the second largest (Fig. 4A left, B left; Fig. 5).

Identification of unit activities corresponding to SN1 and SN2

In order to determine which units of the SDN reflect the activities of SN1 and SN2, we surgically ablated the part of the SOG where the cell body of SN1 or SN2 was located, and the resulting loss of unit activities of the SDN was studied. After recordings, the SDN was backfilled to examine which of the salivary neurones was ablated (Fig. 4C,D). In all preparations where the lower-frequency unit with the largest amplitude disappeared after surgery (N=10), *post-mortem* histological examination revealed that the cell body and some dendrites of SN1 had been eliminated (Fig. 4C). In contrast, in all preparations where the higher frequency unit with the second-largest amplitude disappeared after surgery (N=10), the cell body and some dendrites of SN2 had disappeared (Fig. 4D). In subsequent sections, we focus on two large units of the SDN and thus on two large salivary neurones (SN1 and SN2).

Responses of salivary neurones to taste or odour stimuli

Both SN1 and SN2 exhibited a prominent increase in spike frequency when 10% sucrose solution, 20% sodium chloride solution or distilled water was applied to the mouth (Fig. 5), although responses to distilled water were weaker than those to sucrose or saline solution. Taste stimulation often induced a movement of the mouthpart and the oesophagus, and salivary neurones exhibited an increase in spike frequency in response to the movement of the mouthpart. In most recordings, quantitative evaluation of taste responses of these units was difficult because of occasional large artefacts induced by vigorous movement of the mouth and the oesophagus (Fig. 5, small arrow). Both salivary neurones responded very weakly to peppermint or vanilla odour applied to an antenna (Examples of neural activities during odour responses are shown in Fig. 6A and averaged odour responses before training are shown in Figs 7, 8). Odour stimulation occasionally induced a slight movement of the mouth and oesophagus, but this usually did not prevent reliable discrimination of neural activities from artefacts; recordings of odour responses in which there was ambiguity in discriminating neural activities from artefacts (which represent <5% of the total number of recordings) were excluded from data evaluation.

Effects of conditioning on odour responses of salivary neurones

Studies on the effect of conditioning on odour responses of salivary neurones were carried out using two preparations, i.e. semi-intact preparations and highly dissected preparations. In the former preparations, occasional movement of the mouth or the oesophagus and resulting artefacts often prevented reliable segregation of SN1 and SN2. Thus, the responses were evaluated as the sum of activities of SN1 and SN2. In the latter preparations, reliable segregation of activities of SN1 and SN2 was achieved. Results from the former preparations are shown in Figs 4–7 and those from the latter preparations are shown in Fig. 8. Two groups of cockroaches used for semi-intact preparations were each subjected to five sets

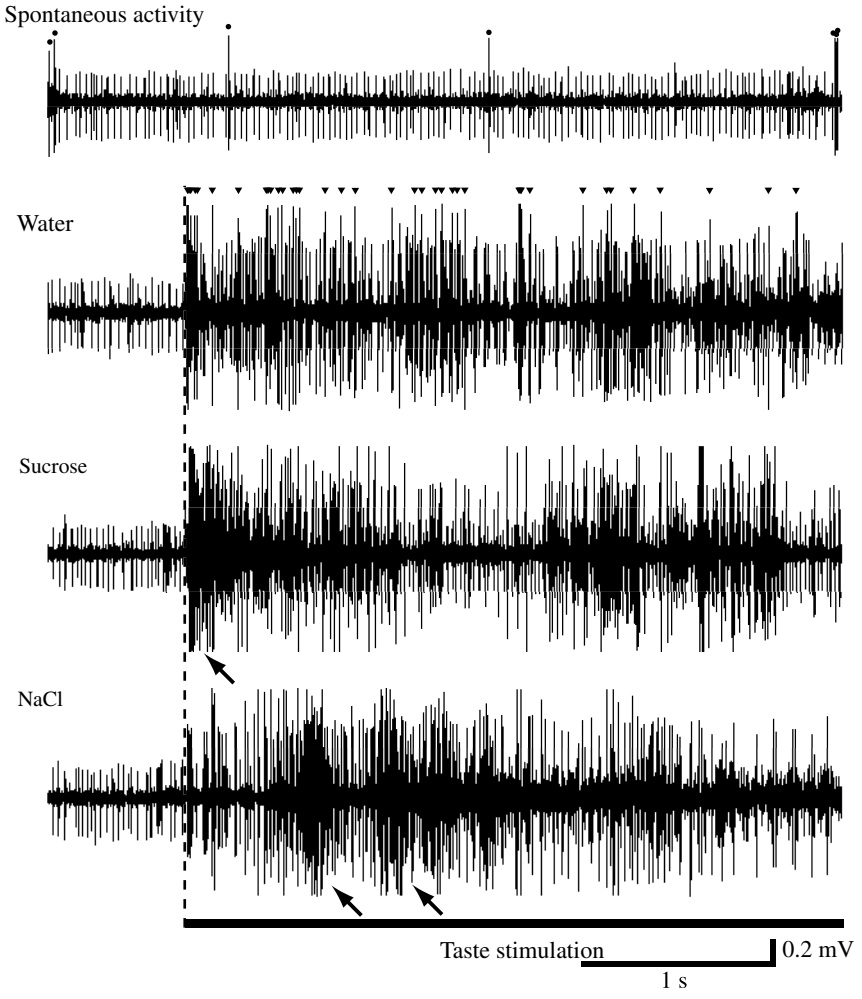

Fig. 5. Responses of a salivary duct nerve (SDN) to distilled water, 10% sucrose solution and 20% sodium chloride (NaCl) solution applied to the mouth. Both of the two large units, a low spontaneous frequency unit with the largest amplitude (black circle) and a higher frequency unit with the second-largest amplitude, exhibited strong responses to water, sucrose and NaCl solution. Coincident occurrence of the two large units resulted in larger-amplitude potential (triangles). The short arrows indicate artefacts caused by movement of the mouth or the oesophagus. The broken line indicates the onset of taste stimulation. All four recordings were from the same preparation. Vertical bar, 0.2 mV; horizontal bar, 1 s.

of P+V– or V+P– forward-pairing trials during chronic recording of the activities of their salivary neurones. Each set of P+V– or V+P– forward-pairing trials consisted of presentation of peppermint or vanilla odour 2 s prior to the onset of presentation of sucrose reward to the mouth and subsequent presentation of vanilla or peppermint odour without pairing with sucrose reward, respectively (Fig. 1B). Responses were measured as relative increase in spike frequency for the first 2 s of odour stimulation to that for a 2 s period just before odour stimulation.

The effect of conditioning was evaluated, at first, by comparing summed responses of SN1 and SN2 to sucrose-associated odours after conditioning with those before conditioning (Fig. 7). In the P+V– conditioning group (Fig. 7A), the magnitudes of responses to peppermint odour after the first, third and fourth sets of conditioning trials were significantly greater than the magnitude of response before conditioning (*t*-test, *N*=20; trial 0 *vs* trial 1: *P*=0.035, d.f.=19, *t*=2.272; trial 0 *vs* trial 3: *P*=0.043, d.f.=19, *t*=2.167; trial 0 *vs* trial 4: *P*=0.02, d.f.=19, *t*=2.549), although the magnitude of responses after the second trial did not significantly differ from that before conditioning (*t*-test, *N*=20, *P*=0.142, d.f.=19, *t*=1.533). In the V+P– conditioning group (Fig. 7B), the magnitude of response to vanilla odour after the first, second, third and fourth sets of conditioning trials were significantly greater than the magnitudes of responses before conditioning (*t*-test, *N*=20; trial 0 *vs* trial 1: *P*=0.026, d.f.=19, *t*=2.418; trial 0 *vs* trial 2: *P*=0.004, d.f.=19, *t*=3.289; trial 0 *vs* trial 3: *P*=0.0002, d.f.=19, *t*=4.526; trial 0 *vs* trial 4: *P*=0.009, d.f.=19, *t*=2.905). In contrast, the magnitudes of responses to the odour presented alone after the first, second, third and fourth unpaired presentations did not significantly differ from the magnitude of initial response for both the P+V– group (Fig. 7A, *t*-test, *N*=20; trial 0 *vs* trial 1: *P*=0.619, d.f.=19, *t*=0.506; trial 0 *vs* trial 2: *P*=0.576, d.f.=19, *t*=0.572; trial 0 *vs* trial 3: *P*=0.282, d.f.=19, *t*=1.108; trial 0 *vs* trial 4: *P*=0.093, d.f.=19, *t*=1.77) and the V+P– group (Fig. 7B, *t*-test, *N*=20; trial 0 *vs* trial 1: *P*=0.288, d.f.=19, *t*=1.304; trial 0 *vs* trial 2: *P*=0.098, d.f.=18, *t*=1.743; trial 0 *vs* trial 3: *P*=0.953, d.f.=18, *t*=0.06; trial 0 *vs* trial 4: *P*=0.957, d.f.=18, *t*=0.05).

In one control group of cockroaches (CS alone group, Fig. 1D), peppermint and vanilla odours were alternately presented five times without pairing with sucrose reward (Fig. 7C). The magnitudes of responses to peppermint and vanilla odours after the first, second, third and fourth unpaired presentations did not significantly differ from the magnitude of the initial response (*t*-test, *N*=21; peppermint, trial 0 *vs* trial 1: *P*=0.419, d.f.=20, *t*=0.825; trial 0 *vs* trial 2: *P*=0.485, d.f.=19, *t*=0.711; trial 0 *vs* trial 3: *P*=0.2, d.f.=17, *t*=1.334; trial 0 *vs* trial 4: *P*=0.837, d.f.=19, *t*=0.208; vanilla, trial 0 *vs* trial 1: *P*=0794, d.f.=17, *t*=0.265; trial 0 *vs* trial 2: *P*=0.832, d.f.=17, *t*=0.215; trial 0 *vs* trial 3: *P*=0.636, d.f.=18, *t*=0.482; trial 0 *vs* trial 4: *P*=0.497, d.f.=18, *t*=0.693). Thus, presentations of odour (CS) alone had no significant effect on subsequent responses to that odour.

The conditioning effect was also evaluated by comparing the responses to sucrose-associated odour with those to the odour presented alone. Before the first set of conditioning trials, the magnitude of responses to peppermint odour did not significantly differ from that to vanilla odour in both the P+V– group (*t*-test, *N*=20, *P*=0.678, d.f.=19, *t*=0.422) and the V+P– group (*t*-test, *N*=20, *P*=0.157, d.f.=19, *t*=1.475). However, after the first, second, third, and fourth sets of P+V– conditioning

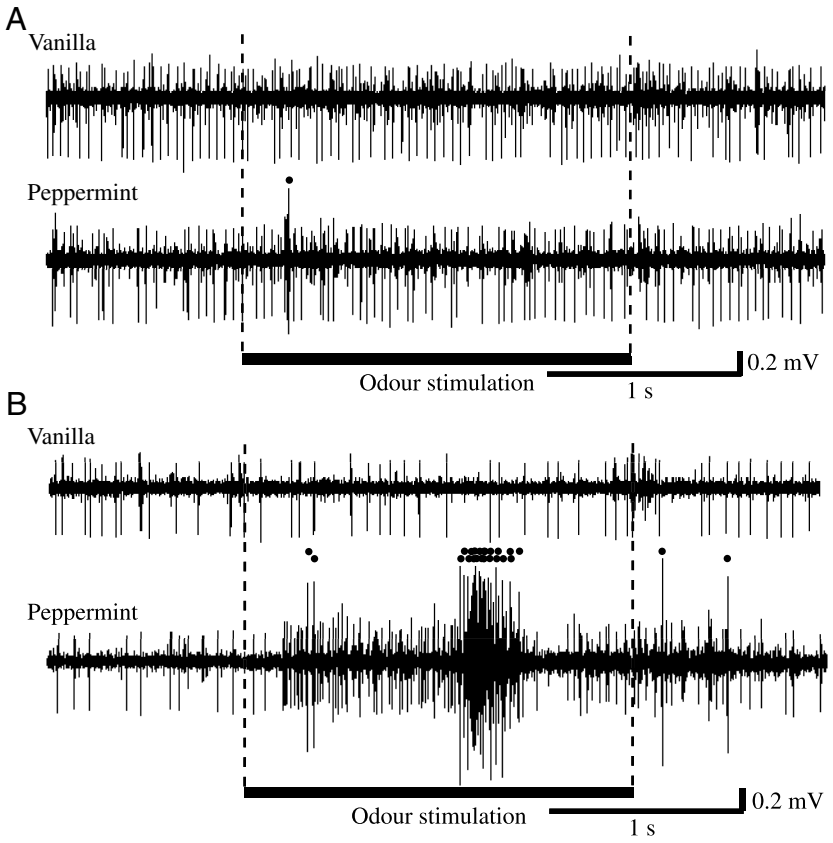

Fig. 6. Responses of a salivary duct nerve (SDN) to vanilla or peppermint odour presented to an antenna. (A) Activities of an SDN during 2 s vanilla or peppermint odour stimulations. The responses of the salivary neurones, SN1 (black circle, largest unit) and SN2 (second-largest unit), to vanilla or peppermint odour were very weak and barely detectable in these recordings. (B) Responses of the SDN to vanilla or peppermint odour 30 min after five sets of P+V– differential conditioning trials recorded in the same preparation. SN1 and SN2 exhibited prominent responses to conditioned odour (peppermint), but their responses to control odour (vanilla) were barely detectable. Broken lines indicate the onset and outset of odour stimulation. Vertical bars, 0.2 mV; horizontal bars, 1 s.

trials, the magnitudes of responses to sucrose-associated peppermint odour were significantly greater than the magnitudes of responses to vanilla odour presented alone (*t*-test, *N*=20; trial 1: *P*=0.008, d.f.=19, *t*=2.988; trial 2: *P*=0.019, d.f.=19, *t*=2.571; trial 3: *P*=0.006 d.f.=19, *t*=3.091; trial 4: *P*=0.004, d.f.=19, *t*=3.307). Similarly, after the second, third and fourth sets of V+P– conditioning trials, the magnitudes of responses to sucrose-associated vanilla odour were significantly greater than the magnitudes of responses to peppermint odour presented alone (*t*-test, *N*=20; trial 2: *P*=0.01, d.f.=18, *t*=2.869; trial 3: *P*=0.024 d.f.=18, *t*=2.471; trial 4: *P*=0.049, d.f.=18, *t*=2.11). In the CS alone group, the magnitude of responses to peppermint odour did not significantly differ from that to vanilla odour (*t*-test, *N*=21; trial 0: *P*=0.269, d.f.=18, *t*=1.14; trial 1: *P*=0.913, d.f.=18, *t*=0.11; trial 2: *P*=0.509, d.f.=17, *t*=0.675; trial 3: *P*=0.509, d.f.=16, *t*=0.224; trial 4: *P*=0.548, d.f.=19, *t*=0.611). We conclude that three sets of conditioning trials are sufficient to achieve a significant level of conditioning.

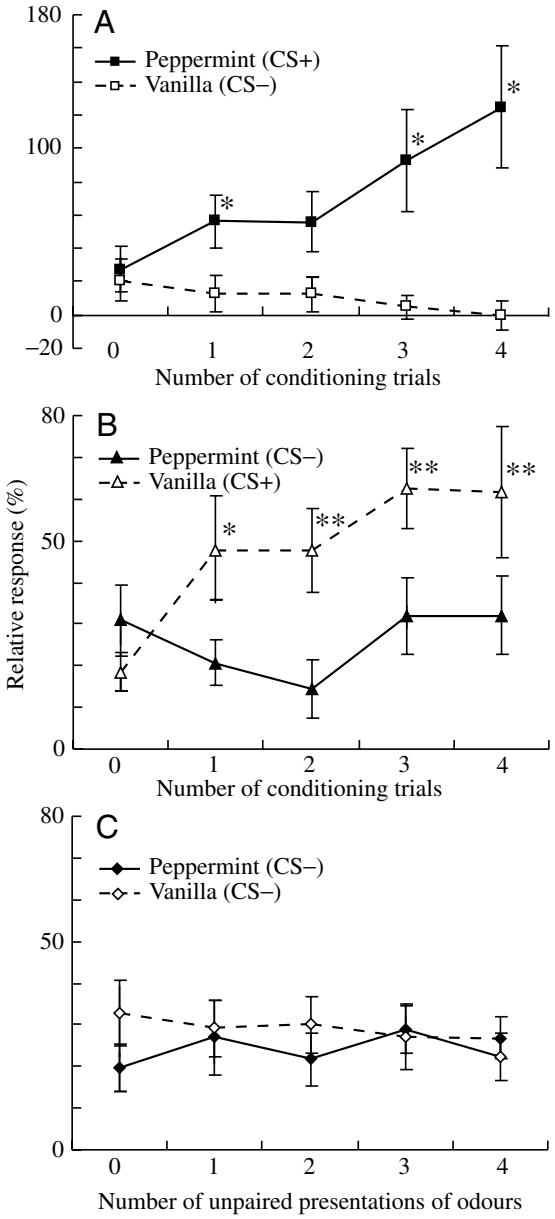

Fig. 7. Effects of forward-pairing trials (A,B) and unpaired presentation of odours (C) on responses of the salivary neurones (SN1 and SN2). Summed responses of SN1 and SN2 to peppermint or vanilla odour before and at 5 min after the first, second, third and fourth sets of P+V– (A) or P–V+ (B) conditioning trials or unpaired presentations of odours (C) are shown. Relative responses, measured as the relative increase in spike frequency for the first 2 s of odour stimulation compared to that during a 2 s period before odour stimulation, are shown as means ± s.e.m.; *N*=20 (A,B), *N*=21 (C). Asterisks indicate the results of statistical comparison with responses to peppermint or vanilla odour before conditioning (NS, *P*>0.05; **P*<0.05; ***P*<0.01; *t*-test).

Short-term retention and effects of backward pairing

Retention of the conditioning effect was tested at 1 min and 30 min after five sets of conditioning trials in the P+V– and V+P– forward-pairing groups. Examples of responses of salivary neurones to sucrose-associated odour (peppermint odour) and to the odour presented alone (vanilla odour) at 30 min after five sets of differential conditioning trials are shown in Fig. 6. Both SN1 and SN2 exhibited responses to sucrose-associated peppermint odour, while they exhibited much less prominent responses to the vanilla odour presented alone.

The magnitudes of summed responses of SN1 and SN2 to sucrose-associated odour at 1 min or 30 min after conditioning were significantly greater than those before conditioning in both the P+V– (Fig. 8A; *t*-test, *N*=20; before *vs* 1 min after training: *P*=0.0003, d.f.=19, *t*=4.489; before *vs* 30 min after training: *P*=0.009, d.f.=19, *t*=2.887) and V+P– forward-conditioning groups (Fig. 8B; *t*-test, *N*=20; before *vs* 1 min after training: *P*=0.002, d.f.=19, *t*=3.515; before *vs* 30 min after training: *P*=0.025, d.f.=19, *t*=2.43). Retention of the conditioning effect was also evaluated by comparing the responses to sucrose-associated odours with those to odours presented alone. Before conditioning, the magnitude of responses to peppermint odour did not significantly differ from the magnitude of responses to vanilla odour in both the P+V– group (Fig. 8A; *t*-test, *N*=20, *P*=0.992, d.f.=19, *t*=0.01) and the V+P– group (Fig. 8B, *t*-test, *N*=20, *P*=0.102, d.f.=19, *t*=1.72). At 1 min and 30 min after conditioning, the magnitude of the responses to sucrose-associated odour were significantly greater than the magnitude of responses to the odour presented alone in the P+V– group (Fig. 8A; *t*-test, *N*=20; 1 min after training: *P*=0.00005, d.f.=19, *t*=5.2; 30 min after training: *P*=0.000002, d.f.=19, *t*=6.752) and the V+P– group (Fig. 8B; *t*-test, *N*=20; 1 min after training: *P*=0.0005, d.f.=19, *t*=4.207; 30 min after training: *P*=0.0003, d.f.=19, *t*=4.362). The results indicate that the effect of conditioning is retained for 30 min after conditioning.

The magnitude of responses to sucrose-associated peppermint odour at 30 min after conditioning was significantly less than that 1 min after conditioning (Fig. 8A;

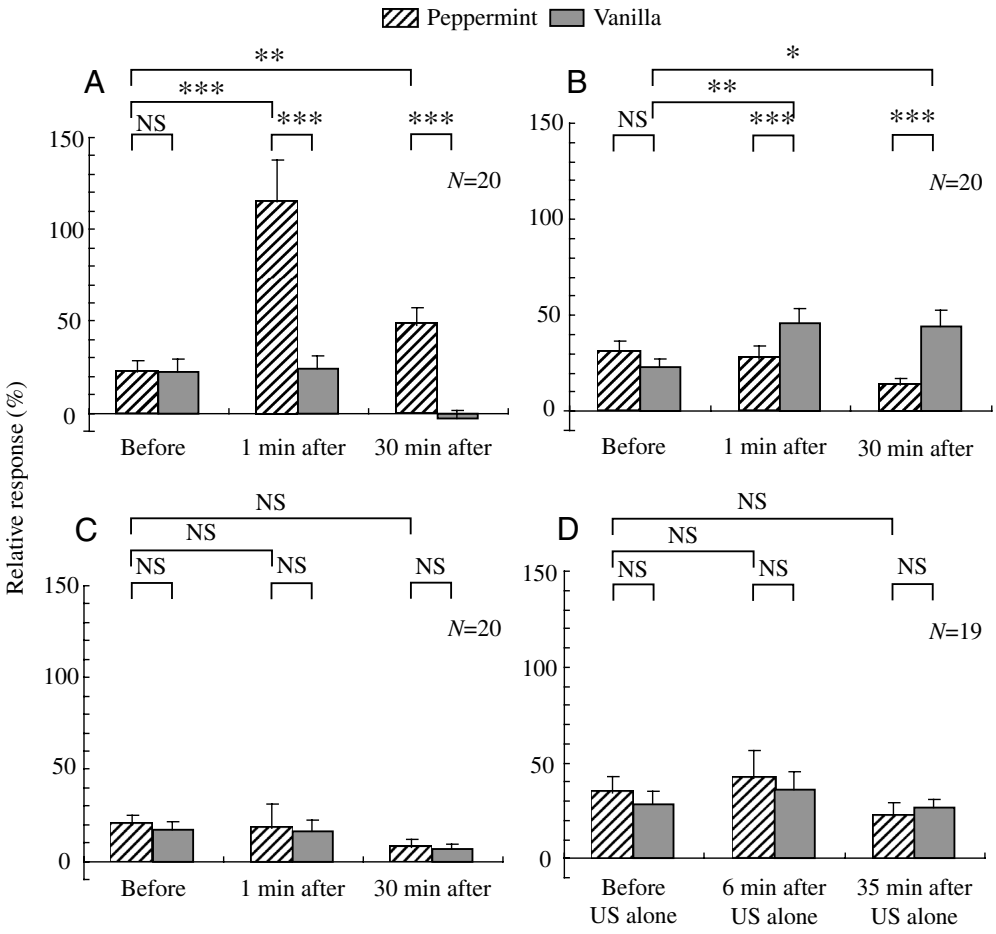

Fig. 8. Effects of forward and backward pairing trials and of non-associative control. (A,B) Summed responses of salivary neurones (SN1 and SN2) to peppermint (hatched bars) or vanilla (shaded bars) odour before and at 1 min and 30 min after five sets of P+V– (A) or V+P– (B) forward-pairing trials. (C) Summed responses of SN1 and SN2 to odours before and at 1 min and 30 min after five sets of P+V– backward-pairing trials. (D) Summed responses of SN1 and SN2 to odours before and at 6 min and 35 min after five presentations of sucrose solution without pairing with odour (US alone). The responses are shown as means ± s.e.m. The results of statistical comparison are shown above the bars (NS, $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$; *t*-test).

t-test, *N*=20, *P*=0.002, d.f.=19, *t*=3.673). By contrast, the magnitude of the responses to sucrose-associated vanilla odour at 30 min after conditioning did not significantly differ from that 1 min after conditioning (Fig. 8B; *t*-test, *N*=20, *P*=0.885, d.f.=19, *t*=0.146). It was, however, uncertain whether or not this was due to the odour-specific decay of memory, since the magnitude of responses to the odour presented alone at 30 min after conditioning was also significantly less than that before, or 1 min after, conditioning in both the P+V– group (Fig. 8A; *t*-test, *N*=20; before *vs* 30 min after training: *P*=0.004, d.f.=19, *t*=3.313; 1 min *vs* 30 min after training: *P*=0.007, d.f.=19, *t*=3.029) and the V+P– group (Fig. 8B; *t*-test, *N*=20; before *vs* 30 min after training: *P*=0.017, d.f.=19, *t*=2.608; 1 min *vs* 30 min after training: *P*=0.015, d.f.=19, *t*=2.662), while the magnitude of the responses at 1 min after conditioning did not significantly differ from that before conditioning in the P+V– group (Fig. 8A; *t*-test, *N*=20, *P*=0.12, d.f.=19, *t*=0.12) and the V+P– group (Fig. 8B; *t*-test, *N*=20, *P*=0.686, d.f.=19, *t*=0.411). Therefore, the possibility cannot be excluded that the decay of odour responses between 1 min and 30 min after conditioning is due to deterioration of the preparation.

We next studied the effect of five sets of backward CS/US pairing trials in another group of animals (Fig. 8C). One backward-pairing trial consisted of presentation of peppermint odour 4 s after the onset of presentation of sucrose reward and subsequent unpaired presentation of vanilla odour (Fig. 1C, backward pairing). The magnitude of summed responses of SN1 and SN2 to peppermint odour at 1 min or 30 min after backward-pairing trials did not significantly differ from that before trials (*t*-test, *N*=23; before *vs* 1 min after training: *P*=0.906, d.f.=22, *t*=0.119; before *vs* 30 min after training: *P*=0.074, d.f.=22, *t*=1.879; 1 min *vs* 30 min after training: *P*=0.332, d.f.=22, *t*=0.992). The magnitude of responses to unpaired vanilla odour at 1 min and 30 min after training did not significantly differ from that before trials (*t*-test, *N*=23; before *vs* 1 min after training: *P*=0.92, d.f.=22, *t*=0.102; before *vs* 30 min after training: *P*=0.055, d.f.=22, *t*=2.024; 1 min *vs* 30 min after training: *P*=0.143, d.f.=22, *t*=1.52).

The effect of backward pairing was also evaluated by comparing the responses to backward-paired odours and those to odours presented alone. The magnitudes of responses to backward-paired peppermint odour did not significantly differ from that to unpaired vanilla odours before and at 1 min and 30 min after conditioning (Fig. 8C; *t*-test, *N*=23; before training: *P*=0.689, d.f.=22, *t*=0.405; 1 min after training: *P*=0.866 d.f.=22, *t*=0.17; 30 min after training: *P*=0.809, d.f.=22, *t*=0.244). The results indicate that backward pairing is not effective in achieving conditioning of odour responses of salivary neurones.

In another control experiment, sucrose solution (US) was

presented five times without pairing with odour (Fig. 8D; see also Fig. 1E). The magnitudes of summed responses of SN1 and SN2 to odour stimulation measured at 6 and 35 min after presentations of US alone did not significantly differ from those before presentations of US alone for both peppermint odour (*t*-test, *N*=19; before *vs* 6 min after US alone trials: *P*=0.504, d.f.=18, *t*=0.682; before *vs* 35 min after US alone trials: *P*=0.222, d.f.=18, *t*=1.265; 6 min *vs* 35 min after US alone trials: *P*=0.176, d.f.=18, *t*=1.408) and vanilla odour (*t*-test, *N*=19; before *vs* 6 min after US alone trials: *P*=0.34, d.f.=18, *t*=0.98; before *vs* 35 min after US alone trials: *P*=0.717, d.f.=18, *t*=0.368; 6 min *vs* 35 min after US alone trials: *P*=0.238, d.f.=18, *t*=1.221). Moreover, the magnitudes of responses to peppermint and those to vanilla did not significantly differ before and at 6 min and 35 min after presentations of sucrose solution alone (*t*-test, *N*=19; before trials: *P*=0.482, d.f.=18, *t*=0.81; 6 min after US alone trials: *P*=0.707 d.f.=18, *t*=0.381; 35 min after US alone trials: *P*=0.609, d.f.=18, *t*=0.521). Thus, presentations of sucrose solution alone had no effects on odour responses of salivary neurones.

One-day retention of the conditioning effect

Retention of the conditioning effect was tested 1 day after training. Immobilized animals were subjected to five sets of P+V– forward-pairing or backward-pairing trials. The preparations were kept in a moist chamber, and at ~24 h after conditioning, the ventral cuticle of the neck was removed and the activity of the SDN was recorded. In the P+V– forward-pairing group, the magnitude of summed responses of SN1 and SN2 to peppermint odour was significantly greater than that to vanilla odour (Fig. 9; *t*-test, *N*=18, *P*=0.005, d.f.=17, *t*=3.211). In the backward-pairing group, the magnitude of responses to peppermint odour did not significantly differ from that to vanilla odour (Fig. 9; *t*-test, *N*=23, *P*=0.948, d.f.=22, *t*=0.066). The results indicate that the effect of forward-pairing is retained 1 day after conditioning.

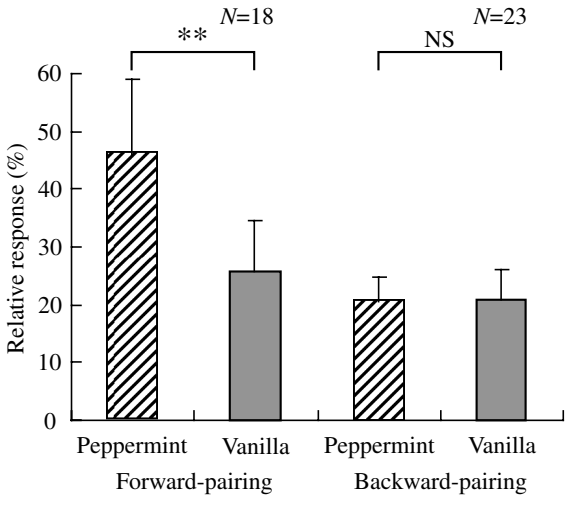

Fig. 9. Summed responses of salivary neurones (SN1 and SN2) to peppermint (hatched bars) or vanilla (shaded bars) odour 1 day after five sets of P+V– forward-pairing or backward-pairing trials. The responses are shown as means ± s.e.m. The results of statistical comparisons are shown above the bars (NS, *P*>0.05, ***P*<0.01; *t*-test).

Effects of conditioning on individual salivary neurones

We studied the effect of conditioning for each of the salivary neurones, SN1 and SN2. In order to achieve reliable segregation of SN1 and SN2, recordings were made in highly dissected preparations (see Materials and methods), in which movement of the mouth or the oesophagus and the resulting artefactual response occurred only very rarely. Recordings of odour responses in which there was ambiguity in discriminating activities of SN1 and SN2 (which represent <5% of the total number of recordings) were excluded from data evaluations.

Five sets of P+V– forward-pairing trials were performed. We noted that the distribution of data for SN1 deviated from the normal distribution. This was because in many, but not all cases, SN1 fired somewhat irregularly, with a spike frequency of 0–10 Hz (Fig. 4C,D left, Fig. 5). Thus, we used a non-parametric Wilcoxson's test for statistical evaluation of data for SN1.

The magnitude of responses of both SN1 (Fig. 10A) and SN2 (Fig. 10B) to sucrose-associated peppermint odour at 5 min or 30 min after P+V– conditioning was significantly greater than the magnitude of responses before conditioning (SN1, WCX-test, *N*=22; before *vs* 5 min after training: *P*<0.01, *T*=45; before *vs* 30 min after training: *P*<0.05, *T*=62; SN2; *t*-test, *N*=22; before *vs* 5 min after training: *P*=0.003, d.f.=21, *t*=3.301; before *vs* 30 min after training: *P*=0.002, d.f.=21, *t*=3.577). Typically, the increase of the response to sucrose-associated peppermint odour at 5 min after conditioning, compared to that before conditioning, was 5-10 Hz for both units. The magnitude of the response to sucrose-associated peppermint odour at 30 min after conditioning did not significantly differ from that at 5 min after conditioning (SN1, WCX-test, *N*=22, *P*>0.05, *T*=87; SN2; *t*-test, *N*=22, *P*=0.095, d.f.=21, *t*=1.749). The magnitude of the response to the odour presented alone (vanilla odour) after 5 min and 30 min did not significantly differ from that before conditioning (SN1, WCX-test, *N*=22; before *vs* 5 min after training: *P*>0.05, *T*=96; before *vs* 30 min after training: *P*>0.05, *T*=89; 5 min *vs* 30 min after training: *P*>0.05, *T*=118; SN2; *t*-test, *N*=22; before *vs* 5 min after training: *P*=0.36, d.f.=21, *t*=0.937; before *vs* 30 min after training: *P*=0.92, d.f.=21, *t*=0.102; 5 min *vs* 30 min after training: *P*=0.194, d.f.=21, *t*=1.342).

The effect of conditioning on the responses of SN1 or SN2 was also evaluated by comparing the responses to sucrose-associated peppermint odour and those to vanilla odour presented alone. Before conditioning, the magnitudes of responses of SN1 (Fig. 10A) and SN2 (Fig. 10B) to peppermint odour did not significantly differ from the magnitudes of responses to vanilla odour (SN1, WCX-test, *N*=22, *P*>0.05, *T*=65; SN2; *t*-test, *N*=22, *P*=0.542, d.f.=21, *t*=0.62). At 5 or 30 min after conditioning, however, the magnitude of the responses to sucrose-associated peppermint odour was

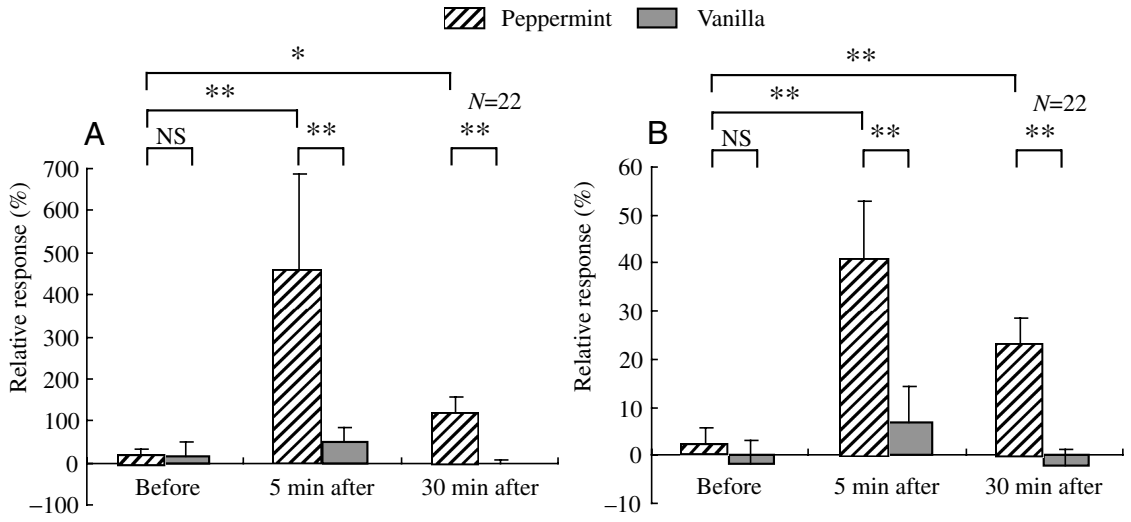

Fig. 10. Responses of salivary neurones, SN1 (A) and SN2 (B), to peppermint (hatched bars) and vanilla (shaded bars) odours before and at 5 min and 30 min after five sets of P+V– forward-pairing trials. The responses are shown as means ± s.e.m. The results of statistical comparison are shown above the bars (NS, $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$; WCX-test in Fig. 8B; *t*-test in Fig. 8B).

significantly greater than the magnitude of responses to unpaired vanilla odour for SN1 (WCX-test, $N=22$; 5 min after training: $P<0.01$, $T=48$; 30 min after training: $P<0.01$, $T=22$) and SN2 (*t*-test, $N=22$, 5 min after training: $P=0.001$, d.f.=21, $t=3.815$; 30 min after training: $P=0.00003$, d.f.=21, $t=5.342$). Therefore, conditioning is successful for both SN1 and SN2.

Saliva secretion upon electrical stimulation of one SDN

We noted that both SN1 and SN2 exhibited an increase in the response of 5–10 spikes s^{-1} for the first 2 s of odour stimulation after five sets of forward-pairing trials of the association of the odour with sucrose solution. We wondered whether or not the increase in responses of salivary neurones by conditioning was sufficient to induce an increased level of saliva secretion. We therefore measured the change in the level of saliva secretion from one salivary duct in response to electrical stimulation of one SDN in highly dissected preparations. Brief (0.2 msec) square-wave pulses were delivered to the SDN by a pair of hook electrodes at 5 Hz for 2, 5, 10, 20 and 40 s with intervals of 6 min, and the evoked compound action potentials were monitored by another pair of hook electrodes, so that the intensity of the stimulus could be adjusted at just above the threshold of spikes of large salivary neurones (~5 V). We deduced that spikes were not evoked in smaller-diameter neurones of the SDN, since they should have higher threshold for spike generation.

We found that the level of saliva secretion is continuously maintained and that the level increased in response to electric stimulation of the SDN (Fig. 11). The increase was statistically significant for all 2-, 5-, 10-, 20- and 40-sec stimulations (WCX-test, $N=12$; 2 s stimulation: $P<0.01$, $T=5$; 5 s stimulation: $P<0.05$, $T=8$; 10 s stimulation: $P<0.01$, $T=3$; 20 s stimulation: $P<0.05$, $T=13$; 40 s stimulation: $P<0.01$, $T=5$). The results suggest that increased response of salivary neurones after conditioning is sufficient to lead to increased levels of salivation.

Discussion

Major findings

Classical conditioning of salivation has been extensively studied in mammals, especially in dogs (Pavlov, 1927; Miller, 1969; Harris and Brady, 1974), but, as far as we know, it has

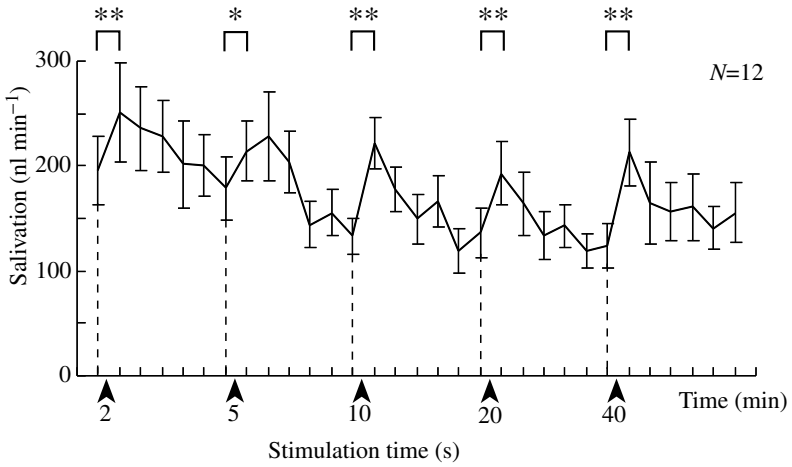

Fig. 11. Changes in the level of salivation upon electrical stimulations of one salivary duct nerve (SDN). The amount of saliva secreted from a salivary duct was measured every minute while brief (0.2 ms) electric pulses were delivered to the SDN at 5 Hz for 2, 5, 10, 20 and 40 s with intervals of 6 min. Averaged data from 12 preparations are shown as means ± s.e.m. The amounts of secretion (broken lines) before and after the onset of electrical stimulation were statistically compared, and asterisks indicate the level of significance (*$P<0.05$; **$P<0.01$; WCX-test).

not been reported in any non-mammalian species. In insects such as cockroaches and locusts, secretion of saliva is controlled by salivary neurones of the SOG (Whitehead, 1973; Smith and House, 1977; House and Smith, 1978; Baines and Tyrer, 1989). Here we reported that in cockroaches the responses of two large salivary neurones (SN1 and SN2) to an odour significantly increases after repeated pairing of the odour with sucrose solution. The increase in the response of both SN1 and SN2 was 5–10 spikes s^{-1} for the first 2 s of odour stimulation after conditioning, and electrical stimulation of the SDN at 5 Hz for 2 s or longer led to significantly increased saliva secretion. The latter finding is in accordance with results of previous reports on secretory response of the salivary gland to electrical stimulation of the SDN, measured for salivary glands isolated from cockroaches, *Nauphoeta cinerea* (House and Smith, 1978) and locusts (Baines and Tyrer, 1989). The results suggest that the increase in odour response of salivary neurones as a result of conditioning is sufficient to lead to an increase in the level of salivation.

Findings in this study suggest classical conditioning of salivation in the cockroach, but direct behavioural evidence needs to be provided to prove this speculation. We are currently performing experiments to compare the amount of salivation in response to odour stimulation before and after conditioning.

Taste and odour responses of salivary neurones

Both of the two large salivary neurones exhibited spontaneous activity and this should lead to a spontaneous level of saliva secretion. Salivary neurones exhibited a prominent increase in spike frequency in response to sucrose or saline solution applied to the mouth and also exhibited a very weak response to peppermint or vanilla odour applied to an antenna. Activation of salivary neurones in response to food-predicting odour and food-associated taste stimulation is no doubt functionally significant for effective feeding.

We also observed that both SN1 and SN2 were active during movement of the mouthpart. This is in accordance with an observation that activities of salivary neurones were modulated by activity of the mouthpart motor pattern generator in locusts (Rast and Bräunig, 2001). The present finding, that salivary neurones receive signals related to feeding motor activity as well as food-predicting olfactory signals and food-associated gustatory signals, may be reflected in the morphologies of their dendrites. The ventral part of the SOG is thought to participate mainly in sensory processing and the dorsal part of the SOG is thought to participate mainly in motor function (Rehder, 1988; Tyrer and Gregory, 1982), and salivary neurones have dendrites in both dorsal and ventral parts of the SOG. Notably, dendrites of SN1 are mainly located in the dorsal and ventral parts of mandibular and maxillary neuromeres, and dendrites of SN2 are mainly located in the ventral part of maxillary and labial neuromeres (Fig. 3B, Fig. 4C,D). How this different dendritic morphology reflects different functions of SN1 and SN2 remains a subject of future study.

Effects of conditioning on odour response of salivary neurones

We have shown that appetitive conditioning trials to associate an odour with sucrose reward lead to an increased preference for that odour in a dual-choice test (Watanabe et al., 2003), and we found in the present study that the same classical conditioning leads to an increase in response of salivary neurones to the odour associated with sucrose reward. It should be noted, however, that salivary neurones are activated in response to both appetitive (sucrose) and aversive (saline) taste stimuli (Fig. 5). Moreover, the magnitude of responses of salivary neurones to vanilla odour did not differ from that to peppermint odour before training (Figs 7, 8, 10), although cockroaches innately prefer vanilla odour over peppermint odour in a dual-choice test (Sakura and Mizunami, 2001; Watanabe et al., 2003). These results indicate that an increase in response of salivary neurones to an odour might not necessarily correlate with an increase in the preference for that odour. It would be interesting to determine whether or not classical conditioning trials to associate an odour with saline solution lead to an increase in response of salivary neurones to that odour, although such aversive conditioning trials have been shown to lead to a decrease in preference for that odour in crickets (Matsumoto and Mizunami, 2002).

Backward-pairing trials were not effective for achieving conditioning of odour responses of salivary neurones (Fig. 8C, Fig. 9). This is in accordance with previous findings that backward-pairing of olfactory CS with gustatory US was not effective in achieving olfactory conditioning in insects and mammals (honeybees: Hellstern et al., 1997; crickets: Matsumoto and Mizunami, 2002; rats: Maier et al., 1976), although backward-pairing of visual CS with olfactory US was found to be effective for achieving conditioning in cockroaches (Lent and Kwon, 2004).

There was a significant level of memory retention 1 day after conditioning. This is comparable to our previous finding that altered odour preference after three sets of classical conditioning trials was retained for 4 days after conditioning (Watanabe et al., 2003). The time course of memory retention after conditioning of activities of salivary neurones was not determined in detail in this study. The responses of salivary neurones to sucrose-associated vanilla odour did not significantly decay from 1 to 30 min after conditioning (Fig. 8B). The response of salivary neurones to sucrose-associated peppermint odour, however, significantly decayed from 1 to 30 min after conditioning in semi-intact preparations (Fig. 8A), but it did not significantly decay from 5 to 30 min after conditioning in highly dissected preparations (Fig. 10). In the former experiments, the response to the odour presented alone (vanilla) also decayed from 1 min to 30 min (Fig. 8A). Thus, the possibility that the observed decay of odour response was due to deterioration of the preparation cannot be ruled out. Further improvement of preparations is necessary to determine in detail the time course of memory retention.

Future perspective

Cockroaches may provide model systems in which to study cellular mechanisms of classical conditioning of activities of salivary neurones. In mammals, many studies have suggested that various brain regions participate in classical conditioning of salivation. For example, electrical stimulations of the orbital cortex (Danilova, 1983) or dorsal part of the caudate nucleus (Danilova, 1981) in dogs and the lateral hypothalamus (Matsuo and Kusano, 1984) in rats modulate salivation to conditioning stimulus. Lesions of the cerebral cortex (Grimsley and Windholz, 2000) and dorsomedial part of the amygdala (Lagowska and Fonberg, 1975) decreased salivation to conditioning stimulus in dogs. The exact cellular mechanisms of conditioning of salivation, however, remain elusive. Cockroaches are suitable materials for the study of neural mechanisms of conditioning of activities of salivary neurones at the level of individual neurones, since intracellular recordings from brain neurones are feasible (Mizunami, 1990; Mizunami, 1996; Li and Strausfeld, 1997; Li and Strausfeld, 1999; Strausfeld and Li, 1999; Nishino et al., 2003).

Olfactory learning in insects has been used as a pertinent model in which to study neural mechanisms underlying learning and memory (Menzel, 1999; Heisenberg, 2003; Daly et al., 2004). In honeybees, the antennal lobe (a primary olfactory centre) and the mushroom body (a higher olfactory centre that processes multisensory signals) have been implicated in olfactory memory processing (Menzel, 1999). In the fruit fly, *Drosophila melanogaster*, mutants with defects in structure and function of the mushroom body exhibited impairments in olfactory learning (Heisenberg, 2003). In moths, *Manduca sexta*, olfactory conditioning produced a modulation of the ensemble representations for odours in antennal lobe neurones (Daly et al., 2004). Conditioning of activities of salivary neurones should provide an excellent model for the study of the neural basis of olfactory conditioning, since chronic extracellular recordings from salivary neurones can be easily combined with intracellular recordings from brain neurones, thereby allowing for the study of activity changes in brain neurones during conditioning. One of our next steps is to investigate whether neurones in the antennal lobe and the mushroom body are involved in olfactory conditioning of activities of salivary neurones and whether there is an association of olfactory CS and gustatory US for conditioning of activities of salivary neurones in the SOG.

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