

Antennal movements reveal associative learning in the American cockroach *Periplaneta americana*

David D. Lent^{1,*} and Hyung-Wook Kwon²

¹Arizona Research Laboratories, Division of Neurobiology, 611 Gould-Simpson Building, PO Box 210077, The University of Arizona, Tucson, AZ 85721, USA and ²Dept of Biological Sciences, 6270 Medical Research Building III, Vanderbilt University, 465 21st Ave. South, Nashville, TN 37235, USA

*Author for correspondence (e-mail: dlent@u.arizona.edu)

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Summary

Using antennal movements as an indicator of learning and retention, an associative learning paradigm has been developed to investigate associative memory between visual and olfactory stimuli. Experiments were performed on the restrained cockroach *Periplaneta americana*, which normally moves its antennae towards a localized odor source. Such 'antennal projection responses' (APRs) are exploited to demonstrate long-term memory, where an APR is elicited by a conditioned stimulus (CS; green light point source) paired with a spatially coincident odor [the unconditioned stimulus (US)]. Association of the CS with

the US is established after five trials. Before training, a visual cue alone does not elicit an APR. This behavior is elicited by a visual cue only after pairing it with an odor stimulus. The acquired APR to the green light cue persists for up to 72 h, indicative of long-term memory. This paradigm is thus suitable for future studies of neural correlates of learning and memory on restrained animals.

Key words: behavior, insect, cockroach, *Periplaneta americana*, memory, multimodal integration, antennal movement.

Introduction

Although *Drosophila* has provided valuable insights into the genetics of learning and memory (Tully and Quinn, 1985; Dubnau et al., 2001), it has so far been practically intractable for physiological studies of identified neurons and neuronal circuits. Even the honey bee (*Apis mellifera*), which has yielded much information about behaviors associated with olfactory learning and memory, has yielded only three studies suggesting learning-associated changes in neuronal physiology (Hammer, 1993; Mauelshagen, 1993; Grunewald, 1999). In comparison, the cockroach *Periplaneta americana* is demonstrably resilient to long-term intra- and extracellular studies of identified neurons and circuits (Mizunami et al., 1998; Li and Strausfeld, 1997; Strausfeld and Li, 1999) and has been shown to possess mammalian-like place memory mediated by its mushroom bodies (Mizunami et al., 1998).

While behavioral studies on cockroaches have demonstrated their suitability for learning and memory studies (Balderrama, 1980; Gadd and Raubenheimer, 2000), a valid argument against using this taxon is that the behavioral paradigms have been designed for free-moving animals and are thus unacceptable for studies at the cellular level. In this and the succeeding paper (Kwon et al., 2004), we describe learning paradigms that have been developed for use on restrained animals so that, as in the case of the honey bee's proboscis extension reflex, these can be employed for intracellular and biochemical studies.

Experiments described here rely on a stereotyped foraging behavior. This is the antennal projection response (APR), which is reminiscent of sniffing in mammals (Gray and Skinner, 1988) or antennular flicking in crayfish and spiny lobsters (Mellon, 1997; Derby, 2000). Such actions are used to assess a continuously changing olfactory milieu and provide the brain with data for locating smells. In lobsters, the frequency and directional control of antennular flicking behaviors increase as mixtures of odor components increase (Mellon, 1997). Other modalities can also trigger antennal projection responses. For example, in honey bees, antennal scanning can be elicited by visual, olfactory and mechanical cues (Erber et al., 1993), and antennal movements can be operantly conditioned (Kisch and Erber, 1999). When crickets track moving objects, their antennae move in the same direction as the object (Honegger, 1981).

Here, we describe experiments that demonstrate a plastic behavior that can be driven in immobilized cockroaches. The behavior, which is expressed by APRs towards an olfactory stimulus source, can be classically conditioned and can be used for studying spatial context in learning and memory. We describe classical conditioning of APRs towards a neutral stimulus [a green light cue (conditioned stimulus, CS)] coupled with an odor source (unconditioned stimulus, US). The classical conditioning results in an APR towards the green light cue (CS), mimicking the response towards an odor source

(US). The study explores whether an APR is indicative of recognition by the visual system of a stimulus location. The paradigm used here demonstrates a simple form of association between visual and olfactory information and shows that APRs can be used to test learning performance in immobilized cockroaches.

Materials and methods

Experiments were performed using adult male American cockroaches (*Periplaneta americana* L.), raised in a laboratory colony maintained on water and cat food (IAMS, Dayton, OH, USA). The cockroaches were kept at $25\pm 1^\circ\text{C}$ on a 12 h:12 h light:dark cycle. Animals with any external damage (e.g. missing antennal segments) were discarded. Each test cockroach was isolated from its colony and kept in a small plastic cage in which it was starved for 24 h before behavioral experiments. The cockroach was then cooled to 4°C for 6 min and restrained in a small plastic tube, holding the head in place but allowing the antenna to move freely. The head was immobilized using modeling wax and a 1:1 mixture of bee's wax and pine resin. The tube holding the restrained cockroach was positioned horizontally in the middle of an arena, supported by modeling wax. This allowed the cockroach to move its antennae freely but without contacting the arena (Fig. 1A). The attitude held by the body was the same as that during walking on a flat surface. After restraining, the cockroach usually required 10–20 min until it began to show spontaneous antennal movements and its body movements

subsided. Individuals showing no antennal movements to odor stimulation during training trials were discarded.

Arena and stimuli

All behavioral experiments were conducted inside a $1.5\text{ m}\times 1\text{ m}\times 1\text{ m}$ chamber surrounded uniformly by black curtains. An infrared heat lamp (Supreme Co., Mullins, SC, USA) was positioned above the behavioral chamber to provide warmth and red light illumination, a non-visible wavelength for cockroaches, for video recording. The 30 cm-diameter arena was made of polyethylene with 10 cm-high walls. Green light-emitting diodes (LEDs; peak wavelength 565 nm; diameter 3 mm; E166; Gilway Technical Lamp Co., Woburn, MA, USA) were positioned at regular intervals on the wall of the arena, to the right of the cockroach's midline. These provided stationary light flashes. Green light was presented during the pre-training, training (conditioning) and test trials. A single red LED (625 nm, E100; Gilway Technical Lamp Co.), a wavelength not visible to the cockroach, was positioned alongside the green LED for spatial continuity and was used in a control test to determine if sounds from the light switches were being detected.

Food odors (peanut butter; Skippy; Bestfoods Co., Eaglewood Cliffs, NJ, USA) were presented through an odor delivery system consisting of a syringe needle and a polyethylene tube (1 mm inner diameter) that were connected to odor sources. Pure air puffs (charcoal filtered; air pressure 10 Pa; stimulus duration 1 s) were blown through a cartridge containing the odor and controlled by a solenoid valve

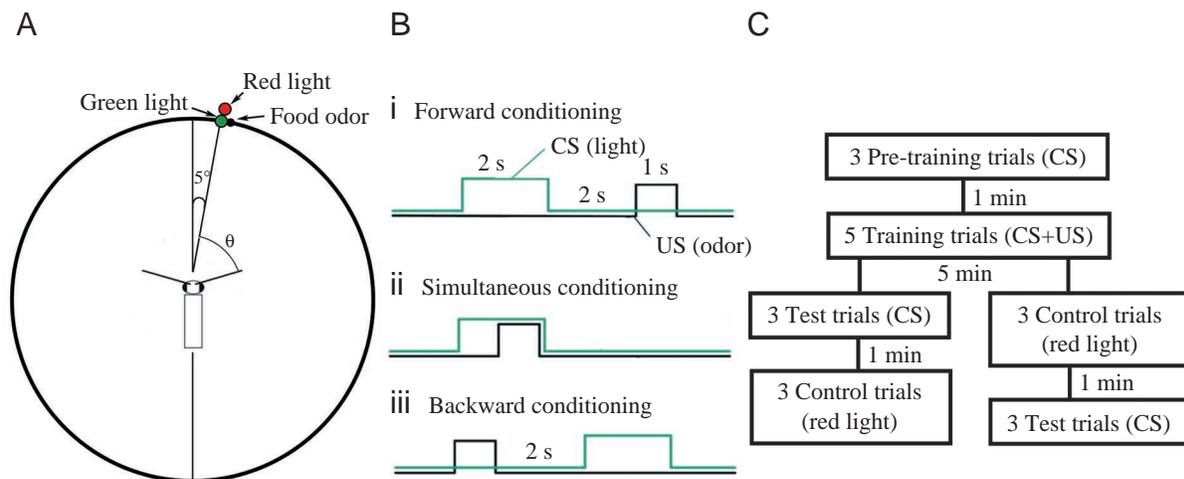


Fig. 1. The visual associative learning paradigm employed to train restrained cockroaches. (A) Experimental set-up. Restrained cockroaches were positioned at the center of the arena. The distance from the head to the position of visual and olfactory cues was 15 cm. Green and red LEDs (I.D. 3 mm) as visual cues were positioned in parallel with an odor cue approximately 5° from the midline of the head. (B) Learning performance was determined from classical conditioning procedures. These are forward conditioning, simultaneous conditioning and backward conditioning. The duration of the conditioned stimulus (CS; light cue) was 2 s and that of the unconditioned stimulus (US; odor cue) was 1 s. The time between the onset of the CS and the US indicates inter-stimulus intervals (ISI). The ISI in simultaneous conditioning was 1 s. The CS and US overlap and cease simultaneously. In forward conditioning, the CS is given in its entirety and, after a 2-s ISI, is followed by the US. In backward conditioning, an ISI of 2 s separates the US from the following 2 s-long CS. There is no overlap between the CS and US in forward and backward conditioning. (C) Basic training regimens consisted of three pre-training trials, five training trials and either three testing trials and three control testing trials, or three control testing trials and three testing trials. A 2-s exposure to a red diode (650 nm) was used to control for other incidental stimuli (see text).

(General Valve Co., Fairfield, NJ, USA). Permanent air flow was provided by an exhaust fan system placed above and behind the arena to remove odors from the inside of the arena between trials. Peanut butter odor was used as the unconditioned stimulus (US). Odor was delivered from immediately above the green LED used for conditioning trials.

Stimuli and their sequences were controlled by a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA). Light and odor cues used for training trials were 15 cm from the cockroach head and at an angle of 5° with respect to the midline of the head (Fig. 1A).

Monitoring and video recording of antennal movements

Antennal movements were video recorded with either an 8 mm Camcorder (Sony) or a digital video camera (Panasonic). Video sequences of test cockroaches were digitized every 167 ms for 20 s using Motus software (Peak Performance Technologies, Inc., Englewood, CO, USA), which produced ~120 images per trial. From the digitized images, antennal angles were measured from the tip and base of the right antenna and the green light position (Fig. 1A).

Responses to sensory stimuli

Antennal responses to odor, light, mechanosensory and auditory cues were tested to evaluate the unconditioned arousal responses of cockroaches. This was done to control for arousal due to sensitization. Odor cues were given in the form of peanut butter; light cues were in the form of a green LED; mechanosensory cues were in the form of high-current air puffs; auditory cues were given at a frequency of 1.8 kHz (Shaw, 1994). In the absence of any other stimuli, a cue was presented for 5×1 s, with a 1 min interval.

Training

Cockroaches were first trained to project their right antenna towards a green light as the CS, coupled with a food odor as the US. Procedures were forward, simultaneous and backward conditioning (Fig. 1B). In all three procedures, the duration of visual and odor stimuli was 2 s and 1 s, respectively. In forward conditioning, the CS was presented for 2 s and, after a 2 s interval, the US was presented for 1 s (Fig. 1Bi). In simultaneous conditioning, the CS was presented for 2 s and, 1 s into the presentation, the US was presented for 1 s (Fig. 1Bii). In backward conditioning, the US was presented for 1 s followed by a 2 s interval and then 2 s CS (Fig. 1Biii). Experimental procedures consisted of three pre-training trials, in which only the CS was presented, followed by five training trials (which was determined to be the optimal number of training trials; data not shown) in which CS and US were presented. Five minutes after the last training trial, either (1) three test trials in which the CS was presented followed by three control trials in which red light was presented or (2) three control trials of red light followed by three test trials of the CS (Fig. 1C) were performed. The inter-trial intervals of all trials were 1 min.

Memory retention

Initial experiments showed that simultaneous conditioning was most effective (Fig. 4). Using simultaneous conditioning, short-term memory retention was measured at 5 min, 10 min, 20 min and 30 min after training. Long-term memory retention was measured at 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h after training. Throughout the tests, cockroaches remained restrained and were provided with water to prevent dehydration.

APR as a measure of learning

An APR by an exploring cockroach is defined as a directed movement towards the location of a specific stimulus, such as an odor, that is then followed by local sampling movements of the antenna in the vicinity of the location to which the antenna extended. Baseline movements of the antenna are small deflections of the antenna that do not involve a defined movement towards a stimulus. The conditioned response is the induced APR towards the position of the CS. Baseline movement and antennal position are determined by analyzing three 10-s time frames before beginning the training protocol. The APRs during pre-training, training, testing and control are compared to this baseline. Responses are scored as a '1' if an APR is elicited and is significantly different from the baseline and '0' if there was no APR 10 s after stimulation. Percentages of APRs were calculated by summation of all scores during a given trial, as assessed by video observation.

Statistics

The Friedman test was used to compare APRs within subjects. Once a significant difference was established, the Wilcoxon signed-rank test was applied to compare each value of the repeated measurements. The Mann-Whitney *U* test was used to test differences between two groups. Values shown depict the responses '0' or '1' in percentages. Statistics were carried out using Statistica 5.5 for Windows and results were regarded as 'not significant' if $P > 0.05$.

Results

Patterns of antennal movements to stimuli

Stimulus-elicited antennal movements of a single cockroach are shown in Fig. 2. The sequences of antennal angles (θ in Fig. 1A) were measured from digitized images and the data are smoothed using five-point adjacent averaging. The polar plots illustrate the antennal position during 10 s. Spontaneous antennal movements during presentation of the green light cue presented alone (duration 2 s) before training (A1–A3 in Fig. 2). The training phase (B1–B5 in Fig. 2) associates food odor with the visual cue. During this phase, strong APRs can occur to the cue position, although, as shown in B3 and B5, these are not invariable. During the conditioning phase, when APRs are elicited, APRs show high precision, directed as in B4. Antennal projections when the visual cue is again presented alone (C1–C3 in Fig. 2) are significantly increased with respect to antennal projections that occur during pre-

training (A1–A3 in Fig. 2). Control tests to red light stimuli did not elicit APRs (D1–D3 in Fig. 2).

Arousal versus conditioning: antennal responses to sensory stimuli

Are antennal projection responses during the test phase due to arousal or conditioning? To determine this, different sensory stimuli were presented alone and the

APRs analyzed (Fig. 3). The only significant APR to any stimulus was to odor (Mann–Whitney U test, $P < 0.00001$); there was no significant antennal response to visual, mechanical or auditory stimulation alone (Mann–Whitney U test, $P > 0.5$). Thus, the pairing of the olfactory stimulus, which induces an antennal projection, to the non-arousing visual stimulus indeed provides a classical conditioning paradigm.

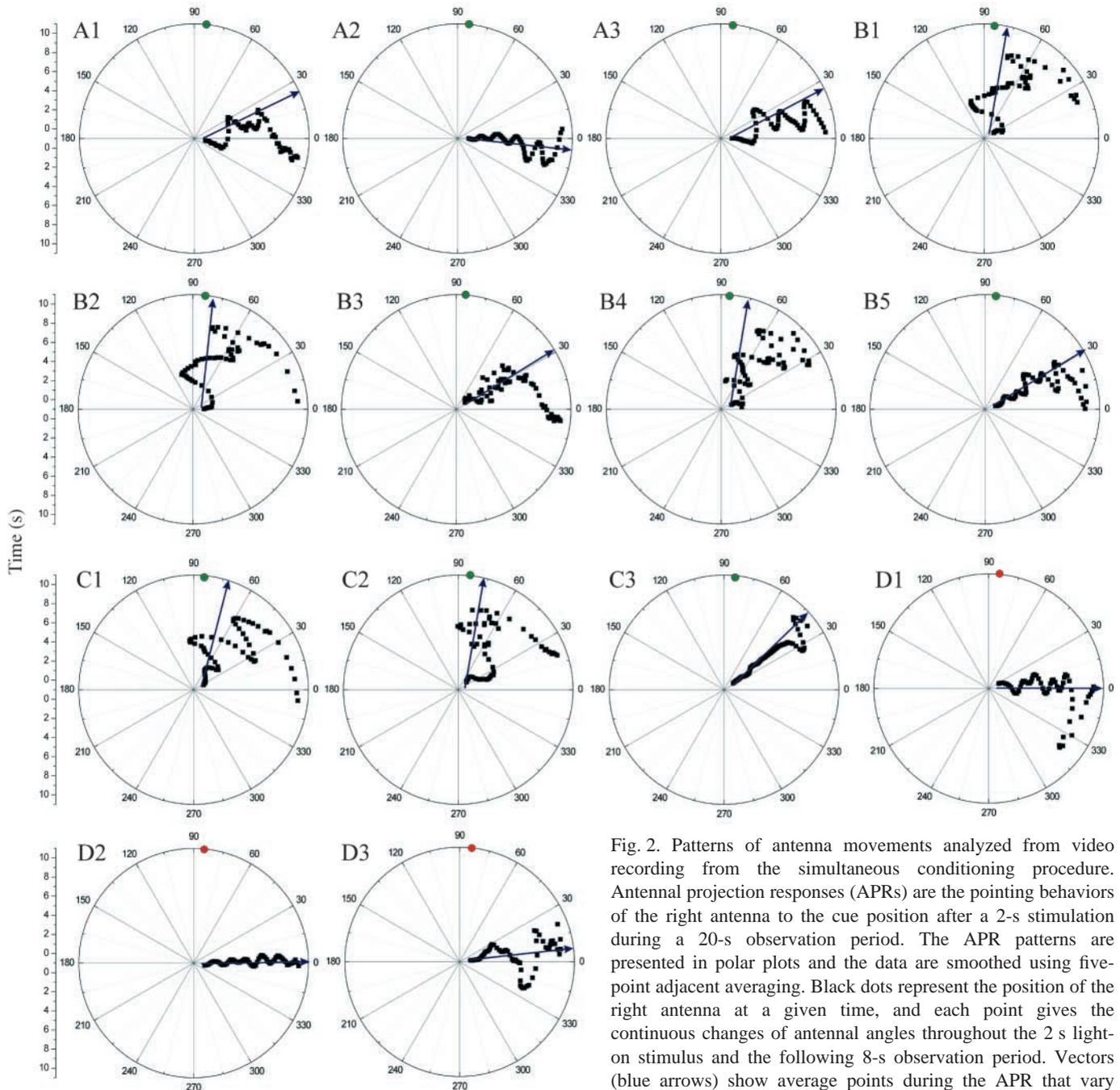


Fig. 2. Patterns of antenna movements analyzed from video recording from the simultaneous conditioning procedure. Antennal projection responses (APRs) are the pointing behaviors of the right antenna to the cue position after a 2-s stimulation during a 20-s observation period. The APR patterns are presented in polar plots and the data are smoothed using five-point adjacent averaging. Black dots represent the position of the right antenna at a given time, and each point gives the continuous changes of antennal angles throughout the 2 s light-stimulus and the following 8-s observation period. Vectors (blue arrows) show average points during the APR that vary significantly from baseline. During pre-training (A1–A3), there are spontaneous antennal movements but no APRs to the LED (green circle) position. During training (B1–B5), antenna movements after LED onset show an increasingly precise APR to the cue position. During testing (C1–C3), APRs were induced by the visual stimuli and were very similar to the APRs during olfactory stimuli. This animal showed no APR during the third trial of the test (C3). Control tests (D1–D3) did not result in APRs to red LED stimulation.

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Learning performance during different learning conditioning sequences

Antennal projection responses elicited by forward ($N=13$), simultaneous ($N=21$) and backward ($N=11$) conditioning showed significant differences during pre-training, training and testing (Friedman test, $P<0.003$; Fig. 4). APRs to green LEDs were below 10–20% during the pre-training trials, during which cockroaches responded spontaneously, if at all, to the light cues. During the five training trials, animals showed clear evidence of learning to associate light cues with food odors and were not different from testing trials (Wilcoxon signed-rank test, $P>0.19$). APRs were significantly higher in simultaneous conditioning than those in forward and backward conditioning (Mann–Whitney U test, $P<0.001$) and showed no significant difference between forward and backward conditioning (Mann–Whitney U test, $P>0.2$). Five minutes after training, APRs of cockroaches to

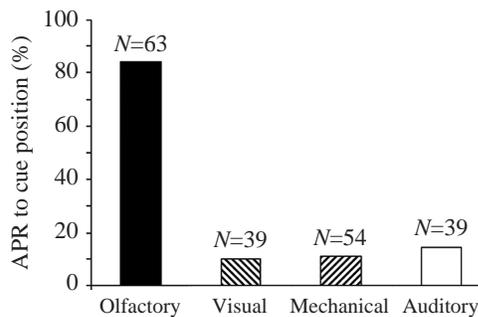


Fig. 3. Percentage of antennal projection responses (APRs) to olfactory, visual, mechanical and auditory stimulation. The APRs were analyzed to determine the unconditioned responses to different stimuli with possible arousing effects. APRs to olfactory stimuli differed significantly from those to visual, mechanical and auditory stimulation.

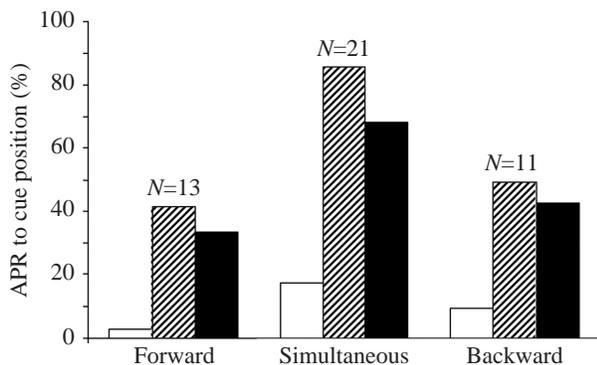


Fig. 4. Antennal projection responses (APRs) and learning performance of restrained cockroaches during forward, simultaneous and backward conditioning procedures. APRs during training (hatched bars) and testing (black bars) trials were increased significantly in all three conditioning procedures compared with pre-training (open bars) trials. APRs during training and testing in each conditioning procedure were not statistically different and showed no difference between forward and backward conditioning.

green LEDs were significantly increased compared with pre-training levels (Friedman test, $P<0.001$).

Learned APRs after training

To determine memory retention, APRs were tested to green LEDs presented at 5 min, 10 min, 20 min and 30 min after training trials ($N=18$). APRs to the visual cue were retained for at least 30 min after training (Fig. 5A). APRs before and after training were significantly different (Friedman test, $P<0.0001$). A high percentage of APRs (80–90%) to the visual cue were retained at 5 min, 10 min, 20 min and 30 min following training and showed no difference in these intervals (Wilcoxon signed-rank test, $P>0.3$). A red LED was presented with the same duration as that of the green LED either before testing at 5 min or after testing at 5 min (Figs 1C, 5A). Cockroaches are insensitive to red light (Seelinger and Tobin, 1981), so that

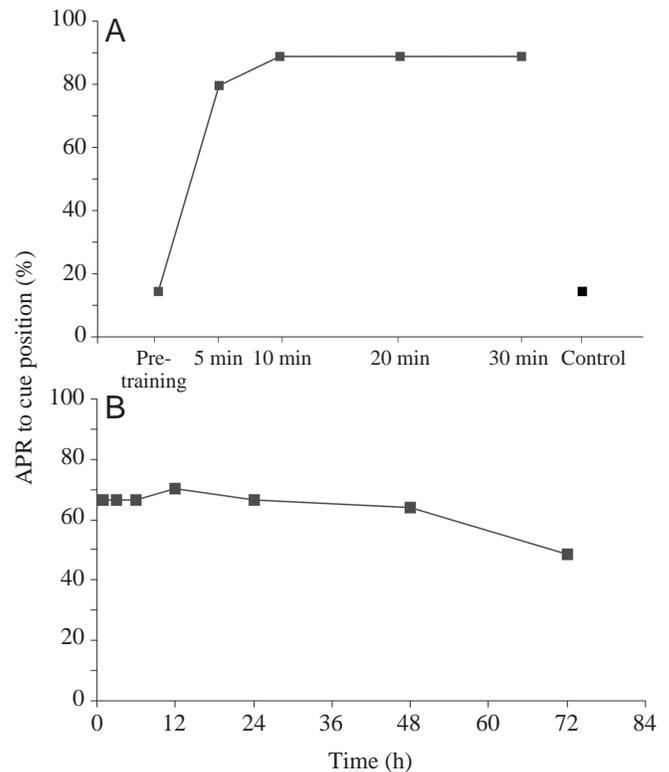


Fig. 5. (A) Learning performances [assessed by percentage of antennal projection responses (APRs)] were tested for up to 30 min after five training trials with simultaneous conditioning ($N=18$). A high percentage of APRs to the visual cue was retained 5 min, 10 min, 20 min and 30 min after training and showed no statistical difference in these intervals. The responses at these times were significantly different from pre-training and control test levels, which were not significantly different. (B) Tests for long-term memory of APRs ($N=9$). After training, APRs to the green light cue were tested for up to 72 h. APRs were significantly different from those at pre-training even at 72 h, showing that cockroaches retained this learned behavior for long periods. The response from 30 min to 1 h decreases by almost 30% but remains stable for up to 72 h. The response at 72 h has decreased to almost 40%, which is half of that observed at 30 min.

control tests with the LED should reveal whether or not the insect has learned to associate the odor with sensory modalities, other than the visual cue, that may have been present and not evident to the experimenters or whether the response was due to an increased antennal movement due to sensory arousal. Pre-training and control tests were not significantly different (Wilcoxon signed-rank test, $P > 0.09$). Only spontaneous antennal movements were observed in response to the red LED (Fig. 5A and Fig. 2 D1–D3), indicating that cockroaches learned to associate visual cues with the odor but not other concurrent sensory stimuli that may have been present and there was no increase in antennal movement due to arousal or sensitization.

Decay of the learned response over time

Antennal projection responses to the CS were tested three times at 5 min, 10 min, 20 min and 30 min after training and thereafter at 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h. Antennal projection responses decrease after 30 min from 90% to 60%, but even after 72 h were still significantly higher in four of nine animals than at pre-training (Friedman test, $P > 0.7$, $N = 9$). The persistence of this learned response to a visual cue may suggest the establishment of long-term memory (Fig. 5B).

Discussion

Learned antennal responses to visual cues

Directed antennal movements, such as antennal projections and subsequent scanning towards specific cues, have been previously demonstrated in honey bees, where antennal extension can be elicited by olfactory, tactile or visual cues such as moving stripes (Erber et al., 1993). Such antennal movements can be classically conditioned by simple association between a US and a sucrose reward or even with non-rewarding conditions. We also show that in cockroaches, as in honey bees, such movements are not due to an unascrivable mechanism of 'arousal' but are elicited either by an odor stimulus or after association between the odor stimulus and the visual cue. The present results show that, in cockroaches, directional movements by the antennae can be recruited through classical conditioning with no other reward than an attractive odor, which could be explained as intrinsically rewarding in itself.

Motor learning without a reward is not without precedent. After scanning an object within the range of their antennae, honey bees will continue to make antennal movements towards the position of the object even after it is removed and even without receiving a reward (Erber and Pribbenow, 1997).

Effects of stimulation intervals on learning performance

The inter-stimulus interval (ISI) and sequence of the unconditioned and conditioning stimuli strongly influence retention. In honey bees, olfactory learning, as assayed from the proboscis extension reflex, demonstrates that an optimal learning performance is achieved when the ISI between the presentation of the CS (odor) and the US (sucrose) is between

0 s and 5 s. If there is a longer ISI, inhibitory learning results upon the presentation of the two stimuli. This is shown by backward conditioning, where the ISI of the US and CS exceed 1 s and result in inhibitory learning (Menzel et al., 1993). Backward conditioning in honey bees showed that an ISI of 15 s between the US and CS induced maximum inhibitory learning (Hellstern et al., 1998), suggesting that contiguity between the CS and US is critical in reward-based learning performance. A reinforcer must be temporally connected to a stimulus. The acquisition of a gill-withdrawal reflex after using electric shock as a negative reinforcer in *Aplysia* showed that an ISI of 0.5 s between the backward pairing of the CS and US induces no learning (Hawkins et al., 1986).

In the present experiments, ISIs of 2 s between the CS and US in forward and backward conditioning result in a weak learning performance compared with simultaneous conditioning (Fig. 4). That these responses are learned responses rather than sensitization is demonstrated by the observation that animals do not show significant responses during control testing, indicating that APRs induced by the CS after conditioning are due to associative learning rather than non-associative effects. Interestingly, short ISIs between the odor and visual cue in backward conditioning elicited moderate learning performance, suggesting that temporally close stimuli can be learned and that concurrent stimuli are not a prerequisite. In nature, foraging animals detect salient cues before the reward, implying that the ISI is a critical factor in reinforcement-based conditioning. Visual learning with food odors used here suggests that cockroaches can learn to associate visual cues with food odors if the ISI is less than 2 s.

Effect of inter-trial interval on learning

Intervals between training trials (ITIs) have an important influence on learning and memory retention. Gerber et al. (1998) examined the effect of different ITIs (30 s, 1 min, 3 min or 20 min) on intermediate (1 day) or long-term (4 days) memory. These authors demonstrated that proboscis extension reflexes evoked during training using ITIs of 20 min and 1 min showed stable intermediate and long-term retention, while 3 min and 30 s showed stable intermediate but not long-term retention. The impairment of long-term memory during the 30 s intervals may reflect massed training results and habituation. Impairment using 3 min ITIs may be due to the disruption of consolidation of each training trial. These results suggest that there is an ITI dependence of the molecular mechanism involved. At the level of gene expression, spaced training of *Aplysia* results in the expression of new protein synthesis, which is essential for long-term memory formation, whereas massed training did not (Alberini, 1999).

In the present study, the interval between training trials was 1 min, creating a 'spaced training' protocol. Cockroaches showed a significant learning performance after five training trials (Figs 4, 5A). Although learning behaviors to varying ITIs were not tested, our results suggest that an ITI of 1 min with repeated presentation of multimodal information in the

absence of rewards is sufficient to support long-term retention (Fig. 5B) similar to that shown in the honeybee (Gerber et al., 1998).

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