Method for Establishment and transfer of classical eyeblink conditioning using electrical microstimulation of the hippocampus as the conditioned stimulus

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Abstract

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Citation: Jian-feng Sui Method for Establishment and transfer of classical eyeblink conditioning using electrical microstimulation of the hippocampus as the conditioned stimulus. **protocols.io**
dx.doi.org/10.17504/protocols.io.hsib6ce
Published: 27 Apr 2017

Protocol

Subjects

Step 1.

A total of 48 adult male albino Dunkin-Hartley guinea pigs, weighing 500–600 g (4–5 months old) at the time of surgery, were included in the study. Before experiments and between conditioning sessions, these animals were individually housed in standard plastic cages on a 12:12 light/dark cycle with free access to food and water ad libitum. The room temperature was maintained at 23 ± 1 °C. The procedures were approved by the Animal Care Committee of the Third Military Medical University and were performed in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

Step 2.

Approximately 1 week before training, guinea pigs were removed from their home cage and anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). In brief, all animals were fitted with a headstage and a loop attached to the apex of the left upper eyelid. In the current study, this loop was utilized to attach the left upper eyelid to a movement-measuring device. The tension of the thread linking the eyelid loop and the transducer is so weak that this small resistance does not hinder the normal eyelid movement. Moreover, for each animal in every group, one small hole (diameter: 1.0 mm) was drilled on the right side of the skull centered on the right hippocampus at the following stereotaxic coordinates: anteroposterior (AP) +6.0 mm, mediolateral (ML) 5.0 mm relative to the frontal zero plane, and the midline sinus, respectively. Then, a bipolar stimulating electrode (No 792500, A-M Systems, Sequim, WA, USA; coated diameter: 332.00 µm, bare diameter: 254.00 µm) was implanted into the right hippocampus through the hole and the electrode’s tip was directed to the following stereotaxic coordinates: AP +6.0 mm, ML 5.0 mm, dorsoventral (DV) -4.5 mm to the skull surface. After the surgery, animals were allowed 1 week of

recovery.

Behavioral procedures

Step 3.

Animals were firstly adapted to the experimental environment for three sessions at 30 min per session, immediately followed by early training (or pseudo-training) sessions (stage I), transfer training sessions (stage II), and recall session (stage III). During these sessions, animals were restrained in a Plexiglas container (25 cm × 15 cm × 15 cm) located in a sound- and light-attenuated chamber, and their heads were secured with blunt ear bars that pressed on the head stages. The left eye of the animal was held open in a comfortable position, with the nylon loop sutured into the left upper eyelid, which was linked to the high-resolution potentiometer (JZ101, XH, Beijing, China). The voltage level represented the eyelid position, with baseline manually calibrated to a constant value. Moreover, the animal's left lower eyelid was taped open. These measures ensured continual exposure of the animal's left cornea.

The 48 male guinea pigs were divided into 8 groups, including 4 groups for study of delay paradigm and the other 4 for trace paradigm. For both delay and trace paradigm studies, 2 groups (1 for experiment and 1 for control) were included for study of learning transfer from central to peripheral and another 2 for study of learning transfer from peripheral to central. In studies of learning transfer from central to peripheral (including for both dEBC and tEBC), animals firstly received electrical stimulation of the right hippocampus as CS (CS1, central or Hip-CS) and paired (for experimental group) or pseudo-paired (for control group) with corneal air-puff US, which lasting for 6 daily sessions in delay paradigms or 12 daily sessions in trace paradigms (stage I). Then, CS was switched from hippocampus stimulation to tone stimulation (CS2, peripheral or tone-CS) and paired with US (6 daily sessions for both experimental and control groups, stage Ⅱ). Finally, CS was shifted from peripheral to central again and paired with US (1 daily session, stage III) to test the EBC memory recall to CS1. In studies of learning transfer from peripheral to central, corresponding procedures were included as described above except the difference in CS patterns, i.e., CS1 was tone stimulation, and CS2 was hippocampus stimulation.

During behavior training with Hip-CS, the electrical constant current pulse train (350 ms duration) consisted of 70 pulses with single pulse width of 0.1 ms (cathodal, monophasic square, 200Hz) was repeatedly delivered via a stimulator (YC-2, Cheng Yi, Chengdu, China) and paired or pseudo-paired with the US. Current levels for electrical stimulation were adjusted to 40% of the minimum currents of eliciting measurable eyeblink responses, usually 100-300 μA for most animals. A binaural tone (2 kHz, 85-90 dB SPL, 5 ms rise/fall time) with duration of 350 ms was used as tone-CS, which was produced by a speaker placed 50 cm above the animal. A plastic pipe was placed 1.0 cm from the animal's left eyeball for delivering a 100 ms duration air-puff (3.0 psi, measured at the end of pipe) to stimulate animal's cornea and effect as US. Signals of eyelid-movement and CS/US were filtered with a bandpass of DC 100 Hz or 0.1 3 kHz, respectively, digitized by a data-acquisition system (RM6280, Cheng Yi, Chengdu, China) at a sample rate of 10 kHz, and recorded simultaneously using the system's built-in software (v 2.4). For delay paradigm in this study, the US co-terminated with the offset of the CS and a 250 ms delay interval between onsets of CS and of US emerged; for trace paradigm, a stimulus-free trace interval of 250 ms was interposed between the CS offset and the US onset. For the CS-US paired or pseudo-paired training, 60 trials were performed per day in a sound- and light- attenuated chamber, with inter-trial intervals varying randomly between 20 and 40 s. In the CS-US pseudo training paradigm, the US was presented at a random interval between 1 and 10 s after the CS onset.

Histology

Step 4.

At the end of stage III, guinea pigs were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with saline and 4% paraformaldehyde. The brains were removed and fixed in fresh paraformaldehyde solution for several days. Four days prior to sectioning, the brains were transferred to a 30% sucrose/4% paraformaldehyde solution. Frozen coronal sections at 30 μm in thickness were taken from the sites of the electrode implantation. The slices were stained with cresyl violet. The recognizable electrode tip tracks were examined carefully using a light microscope (SMZ1500, Nikon, Tokyo, Japan) with a digital camera (DXM1200F, Nikon, Tokyo, Japan) and were drawn onto plates using a stereotaxic atlas of the guinea pig brain. Data from animals were excluded if the location of electrode tip could not be determined with a high degree of confidence.

Behavioral data analysis

Step 5.

Each CS-US paired or pseudo-paired trial during recording was subdivided into three discontinuous analysis periods: (1) a “baseline” period, which occurred at 0–800 ms before the CS onset; (2) a “CR” period, which occurred at 200 ms before the US onset; and (3) a “UR” period, which occurred at 0–300 ms after the US onset. A significant eyelid movement was defined as an increase in the mechanogram amplitude that was greater than the mean baseline amplitude, plus four times the standard deviation of the baseline activity. In addition, a significant eyelid movement was also required to have a minimal duration of 15 ms. Any significant eyelid movement during the latter two periods as defined above was counted as a CR or a UR, respectively. The percentage of CR (CR %) was defined as the ratio of the number of trials containing the CR to the total number of valid trials. To avoid disturbance in CR calculation from higher baseline noise, trials with sudden increased baseline signals of greater than the mean baseline amplitude plus four times the standard deviation of the baseline activity and lasted more than 15 ms will be excluded from analysis. Only trials with qualified baseline are defined as valid ones.

Statistical analysis

Step 6.

All data were presented as means ± SEM. Statistical significance was determined by a least significant difference (LSD) post-hoc test following a two-way repeated measures analysis of variance (ANOVA), a separate one-way repeated measures ANOVA, or a separate one-way ANOVA using statistical software SPSS 18.0. A value of $P < 0.05$ was considered statistically significant for all tests.