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Research report

Discrimination learning with light stimuli in restrained American lobster

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ABSTRACT

Operant discrimination learning has been extensively utilized in the study on the perceptual ability of animals and their higher order brain functions. We tested in this study whether American lobster *Homarus americanus*, which was previously found to possess ability of operant learning with claw gripping, could be trained to discriminate light stimuli of different intensities. For the current purpose, we newly developed a PC-controlled operant chamber that allowed the animal under a body-fixed condition to perform operant reward learning with claw gripping. Lobsters were first reinforced when they gripped the sensor bar upon presentation of a light cue. Then they were trained to grip the bar only when the light stimulus of a specific intensity was presented to obtain food reward while the stimuli of three different intensities including the reinforced one were presented in a random order. Finally, they were re-trained to grip the bar only when the light stimulus of another intensity that was not rewarded in the preceding training to obtain food while other intensities including the one that was rewarded previously were not rewarded any more. In these training procedures, the operant behavior occurred more frequently in response to the rewarded cue than to the non-rewarded one. The action latency for the reinforced stimuli showed a significant decrease in the course of training. These data demonstrate that lobsters can be trained with the light cues of different intensity as discriminative stimuli under a restrained condition that would allow application of electrophysiological techniques to the behaving subjects.

1. Introduction

Animals learn to discriminate signals for reward from those for nonreward [1]. Operant discrimination learning has been a powerful tool for studying not only the sensory or perceptual ability of animals but also their ability of higher order learning and cognitive behavior mainly in mammals and birds [1,2]. Especially, in their investigation into neural mechanisms underlying cognitive brain functions including decision-making and category/concept formation, many neuroscientists have used discrimination tasks with a restrained monkey that was trained to manipulate a lever when an appropriate light cue was presented [3–5]. Recent studies utilizing the operant discrimination learning have demonstrated that some invertebrates also possess the ability of higher order, non-elemental learning [6,7]. Their brains, called “microbrains” [8] or “minibrains” [9], are characterized by not only their size but also the cytoarchitecture and organization of neurons that are small in their population and large in their individual cell size. Intensive behavioral and molecular biological studies have been done on the learning ability in many invertebrates including insects [6,7,10–32] and crustaceans [15,33–38]. Electrophysiological techniques have been applied to the study of learning mechanisms in some invertebrates [39–47]. However, neurophysiological mechanisms responsible for their brain functions remain to be clarified at the level of identifiable nerve cells in the future chiefly because of experimental difficulties in recording their activities from freely behaving animals.

The American lobster *Homarus americanus* has a nervous system that is easily accessible with a variety of neurophysiological techniques [48–52] and yet can perform a precise limb movement that is recommended as an operant response in most learning experiments [53,54]. The lobster has a pair of bilaterally asymmetrical claws as the first thoracic appendage: the crusher is a stout, molar-toothed, slow-acting claw while the cutter is a slender, incisor-toothed, fast-acting claw. Which type grows on which side depends on the life history of individual animals [55]. The former type is usually used for breaking clamshells by gripping (defined by [56]) to eat shellfish meat so that its action can be precisely controlled regarding the direction of movements and the grip force [54,57]. Thus lobsters can perform manipulative behavior that, as in the case of octopus tentacles and mammalian hands, requires nervous control by more complex mechanisms than those mediating locomotion and posture [5]. If we can train lobsters to perform operant discrimination learning, then it would pave the way for intensive neurophysiological analysis of higher order brain functions, including rule learning and working memory [6,7], at the cellular level taking advantage of the microbrain system consisting of identifiable neurons.

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The present study was undertaken to test whether lobsters can achieve operant discrimination learning using visual stimuli of different intensity. Although lobsters have been known to be monochromatic in their vision [58], many physiological studies have demonstrated that they can discriminate between light intensities in the neural activities of receptor (or sensory) neurons [59–62]. We demonstrated in a previous study that lobsters could be trained by operant reward learning involving acquisition and extinction procedures with the gripping behavior even under the force control [54]. It remains unknown, however, if lobsters can perform more advanced form of learning. Since the behavior of lobsters, having a relatively long life [63,64] in invertebrates, is more likely to be affected by past experiences than that of less long-lived animals, we could expect that they have potential ability of higher order learning including operant discrimination learning.

In the current experiment, tethered lobsters were first trained in an aquarium to grip a vertical bar to obtain food reward when a light stimulus was applied in a dark condition. Then they were trained to grip the bar to obtain food in response to the light stimulus of a specific intensity while light stimuli of three different intensities were presented serially at random. We conducted rigorous control experiments including reversal discrimination test to make sure that lobsters really responded to the light stimulus specifically designated in each test by food reward. Every animal thus went through the same single line of experiments consisting of seven procedures. The results obtained in ten animals demonstrated their ability of operant discrimination learning with light stimuli in the body-fixed condition, suggesting the applicability of this type of training for the neurophysiological study of their learning ability and higher order brain functions at the level of identifiable neurons.

2. Materials and methods

2.1. Animals

Adult lobsters, *H. americanus*, of both sexes were purchased at a commercial retail market (Daisan-Nishizawa, Sapporo, Japan). They were imported from Canada and the United States, and kept in cooled aquariums for sale in the shop. In our laboratory, they were kept individually in separate aquariums filled with artificial or natural seawater at 10–15 °C under the condition of continuous filtration. Animals were fed every 4 or 5 days with small pellets of dried fish sausage. The food was chosen because of its low-cost availability, easy preservability and handy processability. The same type of food was consistently used in the keeping aquarium as food and in the experimental aquarium as reward because lobsters show a preference for odor of familiar food [65]. Acclimation was carried out at least two weeks prior to the training under a day/night rhythm of 12L/12D: the light period started at 6 o'clock in the evening while the dark period at 6 o'clock in the morning. All experiments were done in the subjective dark period for the animal because they are generally nocturnal [66].

When a naïve lobster was placed in the experimental aquarium for the first time, it was in a highly alert and vigilant state with restless movements and unresponsive rigidity. Such an animal did not eat diet nor show spontaneous gripping behavior in many cases. Therefore, we made the animal habituated to the experimental environment prior to the experimental procedures starting with the pre-shaping process. The animal was left undisturbed in the experimental aquarium under the body-fixed condition for at least 3 h/day for two or more days before experiment. The animal became calm in the restrained state and responsive after the habituation. Once the pre-shaping and experimental procedures were started, the habituation process was never resumed throughout all the experiment with the animal. Thus, the habituation was not targeted to the visual stimulus but to the general environment before experiment. We tested 28 animals in all. Four of them died before experiment and 14 were judged to be unfit for the current experiment chiefly because they did not positively feed on the pellets of dried fish sausage nor spontaneously act on the sensor bar. The judgment was made during the habituation period. As a result, only those ten lobsters that could grip the sensor bar and get food directly from the feeder pipe in this habituation period were used as experimental animals. During the experimental period, animals were fed only in the operant training procedure as the reinforcement. Animals ranged between 10.9 and 14.2 cm in carapace length and 477 and 565 g in weight.

2.2. Apparatus

In a glass aquarium (90 cm × 45 cm × 45 cm) the animal was physically fixed to an acrylic tether bar and a bolted-down plate glued to its carapace using a quick-drying adhesive (Aron Alpha, Toagosei, Tokyo). The animal could not move around but could freely move its appendages (Fig. 1A and B) and could be released by loosening bolts after experiment. The experimental animal was held in a position that it could grip the sensor bar with the crusher claw. The seawater was continuously filtered at 15 ± 1 °C, and maintained at the depth of about 3 cm above the lobster's eyes. The whole apparatus was placed in a Faraday cage covered with light-tight curtains that completely shielded the animal from the outside electromagnetic waves and any visual disturbance.

Lobsters were subjected to 12L/12D photoperiod as in the same manner during the acclimation period. The illuminance of white fluorescent lamp was maintained at 20–30 lx during the L period (night) and 0 lx during the D period (day) to avoid light adaptation of the animal. We carried out experiments during the day period under low-intensity red light to which lobsters are scarcely sensible (the sensitivity of the lobster visual pigment is the greatest near 525 nm, a wavelength corresponding to blue-green light [57,67,68]). A light cue for discrimination learning was presented by a white LED covered with a plastic hemisphere that was located immediately above the animals' head (Fig. 1A and B). The illuminance of the light stimuli was 20, 40 or 60 lx, depending on the experimental session, which can be discriminated by a closely related Astacidea crayfish species in the neural activity of its oculomotor fibers [69].

The animals obtained food, one pellet at one time, which was dropped into a water stream spouting out from a small pipe at its mouthpart. This feeder system provided the animal with food reward that was associated with the gripping behavior (see below). The detailed information of gripping sensor bar is described in [54]. The sensor was functionally coupled with the feeder motor (Oriental Motor CPL28), controlled by a personal computer (CPU1 in Fig. 1A): We measured the grip force of the lobster's claw with the sensor and digitized it every 15 ms by a 16-bit A/D converter (National Instruments USB-6009) connected to CPU1 which controlled the feeder and LED by a home-made program and to CPU2 which stored the original sensor data at the sampling rate of 1 kHz using a PowerLab 8RSP (ADInstruments, Tokyo, Japan). The data stored in CPU2 was analyzed with Chart software version 5.3 (ADInstruments, Tokyo, Japan) and R programming software (Fig. 1A).

The training program was written in Objective-C using Cocoa framework. A grip force threshold, called reinforcement threshold in this study, was set in CPU1 for providing food rewards from the feeder according to the experimental procedure when the grip force exceeded the threshold (Fig. 1C). The reinforcement delay, i.e., the latency from the time of threshold attainment to the time of pellet release, was within a second in every experimental session. One training session was finished when the sensor bar was manually removed from the set position at a scheduled time. We also observed the lobster behavior during experiment under a low-intensity red light.

2.3. Experimental design

All ten animals went through the same seven procedures including (1) pre-shaping, (2) shaping, (3) post-shaping, (4) light(+)/dark(−) discrimination, (5) dim(+)/middle(+)/bright(+) training, (6) dim(−)/middle(−)/bright(+) or dim(+)/middle(−)/bright(−) discrimination, and (7) reversal discrimination. We trained two animals per day rotationally in this study. The values of experimental parameters such as intensity and duration of light stimulation were determined by repeated pilot studies conducted before the reported experiment and were rigorously controlled throughout this study.

2.3.1. Pre-shaping procedure

In order to acclimate the experimental animals to the light stimulus, naïve lobsters were exposed to 5-min dark-situation and 5-min light-presentation (40 lx) alternately in a 30-min session under the body-fixed condition. Lobsters obtained no reward for their gripping actions. The procedure was performed for 2 day, three 30-min sessions per day.

2.3.2. Shaping procedure

Since naïve animals did not know that gripping the sensor bar would bring them food reward, we let them know it by this procedure. On the day following the pre-shaping procedure, we forced the animal to grip the sensor bar reflexively by holding the meropodite part of the claw close to it with a waterproof wire wound around them. In this situation, small amounts of food were dispensed when the lobster gripped the bar. The animals were exposed to 5-min dark-situation and 5-min light-presentation (40 lx) alternately in a 30-min session. These exercises were conducted repeatedly 5 times in each light and dark condition per one session and all 3 sessions were carried out. After this training, we observed that the animal showed gripping behavior more spontaneously than before (Fig. 2). The animal was regarded to have been "shaped" for the bar-gripping task. Those animals that did not present gripping behavior were excluded from the present study.

2.3.3. Post-shaping procedure

This procedure was carried out to test the possibility that the animal has developed a preference for either the light or dark condition during the shaping procedure. In this procedure, lobsters obtained no reward even when they gripped the bar spontaneously. The procedure was performed on the day following the shaping procedure in three 30-min sessions. Lobsters were exposed to 5-min dark-situation