# 足裏感覚と学習
## ーマウスを用いた修正型抑制条件付け法の予備的検討ー

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# Modified CMIA method to understand acquired learning via plantar cutaneous sensation in C57BL/6J mice.

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**Abstract**

Plantar cutaneous sensation is crucial not only for posture maintenance, but also for motor skill acquisition in animals, including humans. In animal models, one-trial passive avoidance (OPA) task (inhibitory avoidance task) has been often used for assessment of the behavioral learning associated with plantar cutaneous sensation. OPA task is fairly useful to analyze animals' learning and memory ability, however, this task shows large variance in retention performance analysis, and cannot separate acquisition process from retention process of learning. In the present study, we developed a new inhibitory avoidance task using the C57BL/6J mouse model. In the acquisition trial, mice were individually put into a light box of the apparatus from which they ran into the dark box. One-second long foot stimulus was repeated every 10 seconds until the animal ran out from the dark box and stayed for a total of 200 seconds in the light box. In the retention test, mice were again individually put into light box until the animal ran into the dark box, or it timed out (300 seconds). During the acquisition trial, mice required more foot-shock stimuli to meet the acquisition criteria in the low stimulus-intensity condition than that in the high stimulus intensity condition. Furthermore, a significant negative correlation was observed between the number of foot-shock stimuli and the latency of the transition from the light box to the dark box in the retention test. These results suggest that the number of received foot-shock stimuli may provide an index for the acquisition of learning as well as the number of entries into the dark box.

***Keywords*:** plantar cutaneous sensation, learning and memory, inhibitory avoidance, acquisition, mice

## I. Introduction

Although, the soles lie the farthest from the brain in humans, their sensory functions, such as maintenance of posture and balance, center for gravity, and bipedal locomotion are indispensable from human behavior. When the soles are paralyzed, or the dominant sole-nerves are cut off, then it becomes difficult to maintain the posture and the bipedal locomotion [1, 2]. The motor skill acquisition, controlled by sole sensation, may largely depend on learning ability of animals. Thus, elucidation of the learning mechanisms underlying the behaviors elicited by sole sensation may be helpful for prevention and/or treatment of gait disturbance as well as for improvement of motor skills.

The control mechanisms that sole stimulation execute on the behavioral responses have been mainly studied with animal models. Medium to high intensity electric shock (0.1 mA to 0.3 mA, or more) arouses fear in animals that finally induces an escape response. Consequently, they

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start to avoid such stimulus after several times of paired presentation with other external signals. Shuttle box avoidance (active avoidance) task and one-trial passive avoidance (OPA) task have been often used to assess the anxiety profile and learning abilities of animals [3-5]. On the other hand, a brief electric shock to the hind paw of mice may produce a reward signal without arousing an anxiety and/or a fear response in a restricted condition [6]. Although, OPA task has an appreciable advantage in terms of time and cost for conducting experiments, it shows a large variance in the retention performance analysis, and also cannot distinguish the acquisition process from the retention process of the learning mechanism. One of the modified tasks developed to address these concerns is called continuous multiple-trial inhibitory avoidance (CMIA) task [7-11]. In the acquisition phase of this task, when animals run into the dark box, electric foot shocks are administered until they run back to the start box where no shocks are delivered. The total number of trials (number of entries into the shock-delivered box) required to reach the acquisition criterion (e.g. animals were required to stay in the light box for 200 seconds) is used as an acquisition index.

The CMIA task provides an acquisition index for inhibitory avoidance tasks, however, the total duration of delivered foot shock cannot be controlled, and thus, can become relatively very long (maximum 5 seconds in [8], 4 seconds in [11], and 2 seconds in [7 and 10]). Although, the longer duration of foot shocks can reduce the variance in the retention performance analysis, it may lead to a floor effect in the acquisition performance analysis (animals needed two or three trials in order to reach acquisition criteria in the previous studies [7-9, 11]). Thus, a modified procedure, in which the shock duration can be controlled, should be developed to avoid the floor effect in the acquisition trial as well as to decrease pain in animals. In the present study, we have applied a modified CMIA task to the C57BL/6J mice. The modified CMIA task would be useful to understand the underlying mechanisms of motor learning *via* plantar cutaneous sensation in animals, including humans.

## 2. Materials and methods

*2-1. Animals*: Eight-week-old male C57BL/6J mice (n=30) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were acclimated to the breeding conditions for one week prior to the behavioral experiments. The breeding and experiment rooms were air-conditioned (22 ± 1°C, 50-60% humidity), and the mice were kept on a 12-h light/dark cycle (lights on at 08:00 hours). Food and water were freely available in the breeding cages. Six mice were excluded from the experiment owing to skin injury caused by fighting instances or to low body weight (minimum body weight was 24.0 g), and twenty-four mice were used in the study. The behavioral experiments were conducted during the light cycle (between 13:00 and 17:00 hours). All animal experimental procedures in the present study were performed in strict accordance with the guidelines of the Institute of Physical and Chemical Research (RIKEN), and were approved by the institute's Animal Investigation Committee.

*2-2. Apparatus*: A small light-dark box (STC-001M: Muromachi Kikai, Tokyo, Japan) and a shock generator-scrambler (SGS-003DX: Muromachi Kikai, Tokyo, Japan) were used. The light box was made of white plastic [90 (W) × 115 (D) × 150 (H) mm] with a transparent plastic lid (250 Lux at the center of the box). The dark box was made of black plastic [140 (W) × 175 (D) × 150 (H) mm] with a black plastic lid. There was a tunnel with a sliding door in the center panel (3 × 5 cm) to allow the mice to transition between the light and the dark boxes. The grid floor was wired to the shock generator-scrambler.

*2-3. Procedure*: On the training day, an acquisition trial was conducted. Each mouse was introduced into the light box facing away from the sliding door. When the mouse stopped moving and turned to the sliding door, then the

door was opened manually. The door remained open throughout the training period. As the mouse stepped with all four paws into the dark box, electric foot shocks (1 second duration) were delivered at 10 second intervals, until the mouse escaped back into the light box. In the present study, the mice were divided into three independent groups on the basis of shock intensities (Low: 0.13 mA, Medium: 0.20 mA, High: 0.25 mA; n=8 in each group). During the acquisition trial, shock was delivered whenever the mouse returned into the dark box. The mouse was retained in the light-dark box system until it continuously remained in the light box for 200 seconds. Following this, the mouse was returned to its home cage. The total number of trials (entry into the dark box), and the number of shocks required to reach the acquisition criteria were recorded. After 48 hours of resting, a retention test was conducted on day 3. Each mouse was again introduced into the light box facing away from the sliding door, and their latency to step into the dark box was recorded (cut-off time was 300 seconds) without delivering foot shocks. After each trial (both acquisition trial and retention test), the light-dark box system was washed with tap water, cleaned with 70% ethanol, wiped, and dried with a paper towel.

*2-4. Statistical method*: Statistical analyses were conducted using SPSS™ version 25 statistical software (Advanced Analytics, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to compare the mean of each index (the light-dark latency in the acquisition trial, the number of entries to the dark box, the number of shocks received, the light-dark latency in the retention trial) among the three groups. The comparison between the acquisition and the retention trials was conducted using a repeated measures ANOVA (general linear model: GLM). Tukey HSD method was used for multiple comparisons. A Wilcoxon's sign-ranked test was used to compare the light-dark latencies of the acquisition trial with the retention test, because the cut-off time was set in the retention test.

## 3. Results

The method used in the present study was effective to induce inhibitory avoidance behavior in mice. Figure 1 shows the light-dark latency in the acquisition trial and in the retention test. GLM revealed significant effect of the trial ($F(1,42)=40.474$, $p<0.001$), but the effect of stimulus intensity ($F(2, 42)=1.840$, n.s.), and the interaction between trial and stimulus intensity were not statistically significant ($F(2, 42)=0.0525$,

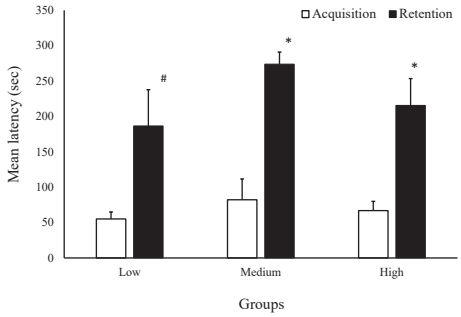


Figure 1 Results of the inhibitory avoidance learning. Data represent mean light-dark latency in the acquisition trial (unfilled bars) and in the retention test (filled bars). Error bars represent standard error of the mean (SEM). *: $p<0.05$, ♯:$0.10<p<0.05$

n.s.). Mice in the medium and the high groups showed significantly increased light-dark latency in the retention test compared with that in the acquisition trial (Medium: T=0, $p<0.05$; High: T=2, $p<0.05$). Mice in the low group also showed an increased light-dark latency in the retention test, but the *p*-value was not statistically significant ($T=6$, $0.10<p<0.05$). One-way ANOVA revealed no significant effect of stimulus intensity on the light-dark latency ($F(2, 21)=0.488$, n.s.), on the number entries the dark ($F(2, 21)=2.780$, $p=0.085$, n.s.), on the number of electric shocks ($F(2, 21)=2.263$, n.s.) in the acquisition trial, and on the light-dark latency in the retention test ($F(2, 21)=1.364$, n.s.). Multiple comparisons between groups with respect to each index did not reveal any group differences (the light-dark latency: low vs medium, $p=0.594$;

low vs high, $p$=0.904; medium vs high, $p$=0.845; the number entries the dark: low vs medium, $p$=0.119; low vs high, $p$=0.136; medium vs high, $p$=0.997; the number of electric shocks: low vs medium, $p$=0.259; low vs high, $p$=0.136; medium vs high, $p$=0.923, respectively). Figure 2 shows mean number of entries to the dark box and the administered foot shocks. Although, there were no statistical differences, both the number of entries to the dark box and the number of administered electric shocks were decreased in the medium and the high intensity groups. Figure 3 shows the correlation diagram between the number of shocks delivered in the acquisition trial and the light-dark latency in the retention test. There was a statistically significant negative correlation between the number of shocks delivered in the acquisition trial, and the mean light-dark latency in the retention test (Pearson's correlation coefficient = -0.404, $p$=0.05).

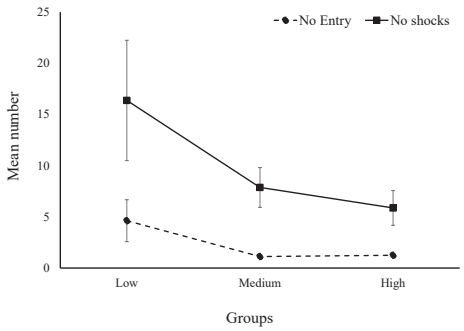


Figure 2 Results of the acquisition trial. Data represent mean number of entries to the dark box (filled circles) and the number of shocks delivered (filled squares). Error bars represent standard error of the mean (SEM).

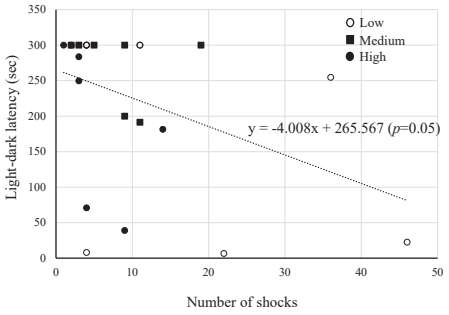


Figure 3 Statistically significant negative correlation between the number of shocks delivered in the acquisition trial and the mean light-dark latency in the retention trial (Pearson's correlation coefficient = -0.404, $p$=0.05).

## 4. Discussion

In the present study, we successfully demonstrated the acquisition process of inhibitory avoidance learning using a modified CMIA procedure similar to the one used in previous studies [7-11] (Figure 1). In addition, we revealed that mice can learn inhibitory avoidance learning through the modified CMIA procedure using both brief and low intensity electric-shocks (Figure 1 and 2).

The results also showed that the number of shocks delivered in the acquisition trial may be useful to predict the retention performance indexes, such as the light-dark latency (Figure 3).

Therefore, our study opens up a new possibility in inhibitory avoidance learning paradigm in mice.

OPA task has an appreciable advantage in terms of time and cost for conducting experiments, therefore it has been widely used to analyze learning mechanisms and/or to assess efficacy of drugs. However, there is often a large variation in light-dark latency in the retention test as well as in the acquisition trial, and thus, a large number of animals are required to obtain stable data. On the other hand, CMIA task in which longer and multiple shocks are administered in the training session provides robust retention performance [7]; it is thought to be a valid variant of OPA. Nevertheless, the CMIA task has not been used

commonly, because it inevitably causes more pain to the animals than the OPA task. Therefore, new modified methods that can reduce the pain caused to animals are required, from the animal welfare perspective. In the present study, three foot-shock intensities were used (0.13 mA, 0.20 mA and 0.25 mA), and successfully conditioned inhibitory learning was obtained in 0.20 mA and 0.25 mA conditions. Mice subjected to 0.13 mA foot-shock did not show statistically significant learning, but the difference in the light-dark latency in the retention test was marginally significant ($0.10<p<0.05$) (Figure 1). The stimulus intensities used in the present study were as per the standard or a little weaker than the previous mouse inhibitory avoidance and fear conditioning studies [13-18]. These results suggest that the shock delivery method used in the present study is effective to evaluate the role of sole stimulation on behavioral responses with a decreased foot-shock intensity and duration.

Separation and quantification of the acquisition process are considerably important for the analysis of the mechanisms underlying learning and memory. Since, OPA task needs only one trial for conditioning, it cannot separate the acquisition process from the retention process. CMIA task also needs one trial for conditioning, but the foot shocks are repeatedly administered to animals until they stay in the light (start) box, in which foot shocks were not delivered, for a designated duration (acquisition criterion). Thus, the number of transitions or the dark (shock delivered) box entries provides an index for acquisition performance [8, 9, 11]. Although, the animals meet the acquisition criterion within two or three trials in the CMIA task, they present a floor effect in the retention test [8, 9, 11]. On the other hand, in the present study, the mice met the acquisition criteria within a few trials in 0.20 mA and 0.25 mA conditions, as well as presented significant learning in the retention test. Instead of a single, long foot-shock, repeated short foot-shocks were adopted in the present study. As the mice were subjected to 1-second-long foot shocks every 10 seconds after they entered the dark box, the number of shocks administrated was also used as an index of acquisition performance. The number of shocks did not show a floor effect in the present study. Unfortunately, statistical differences among the three groups with different stimulus conditions were not observed in this study, but our method may be able to detect these differences after some metric modification.

In the present study, a statistically significant negative correlation was obtained between the number of shocks administered in the acquisition trial and the resultant light-dark latency in the retention test (Figure 3). Mice subjected to the weakest shock (0.13 mA) condition required greater number of shocks and exhibited lower retention performance. However, regardless of the shock intensity, the mice which were administered fewer shocks to meet the acquisition criterion exhibited better retention performance. The results suggest that the number of shocks delivered to mice in the acquisition trial may predict their performance in the retention test. It seems to be paradoxical that fewer aversive experiences lead to better learning performance. At present, we cannot ascertain whether the individual difference in the sensory ability of foot pads or in the formation of neural circuits is responsible for the observed results. Thus, further research to improve the prediction accuracy of this method is required.

Although, CMIA is an effective method to compensate for the disadvantages of OPA method, it has not been widely used in behavioral neuroscience. The ethical issue may be a reason because CMIA method uses long electric foot-shock stimuli. However, the method used in the present study can induce conditioning by the same or weaker stimulus intensity, and shorter stimulus duration as compared with the previous studies. The effect of an increased number of stimuli might be offset by enough inter stimulus interval to reduce the pain experienced by animals. In addition, the number of animals used may be reduced owing to the stable retention performance.

Therefore, the modified CMIA method used in the present study will be useful to understand the underlying mechanisms behind the relationship between plantar cutaneous sensation and learning. This study also provides a new experimental model for the treatment of human gate disturbance.

## 5. Conclusion

In the present study, we propose a new variation of CMIA method. Our modified method has several advantages over the OPA and the traditional CMIA methods: 1) reduces the pain caused to animals, 2) obtains stable retention performance, 3) requires fewer animals to assess the learning and memory function. In addition, it provides a new index to assess the acquisition process in CMIA method without being affected by the floor effect. Hence, the method developed in the present study will be useful to understand the sensory-motor learning mechanisms, and will also provide a new experimental approach for the treatment of human gate disturbance.

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