Involvement of the cerebellum in classical fear conditioning in goldfish

Masayuki Yoshida, Izumi Okamura, Kazumasa Uematsu

Graduate School of Biosphere Sciences, Hiroshima University, Higashihiroshima, Hiroshima 739-8528, Japan

Corresponding author:

Masayuki Yoshida

Graduate School of Biosphere Sciences, Hiroshima University,

1-4-4 Kagamiyama, Higashihiroshima, Hiroshima 739-8528, Japan

Telephone: +81-824-24-7982

Fax: +81-824-24-0790

E-mail: yosidam@hiroshima-u.ac.jp

Abstract

To investigate the cognitive role of the cerebellum of fish, we conducted experiments examining effects of cerebellar manipulations on fear-related classical heart rate conditioning in goldfish. We performed two types of manipulations, one was total ablation of the corpus cerebelli and the other was localized cooling of the corpus cerebelli for reversible inactivation of the cerebellar function. Both the cardiac arousal response to the first presentation of the conditioned stimulus and the cardiac reflex to the aversive unconditioned stimulus were not impaired by the ablation or cooling of the corpus cerebelli. On the other hand, inactivation of cerebellar function severely impaired the acquisition of a conditioned cardiac response in the fear-related conditioning. In addition, localized cooling of the corpus cerebelli reversibly suppressed the expression of established conditioned response. We suggest that the cerebellum of fish is not only being a motor coordination center but also is involved in emotional learning.

Keywords: cerebellum, goldfish, classical conditioning, emotional learning

1. Introduction

It is well known that the limbic system, especially the amygdala, is critically involved in fear-related classical conditioning in mammals [5,6,10,12,14]. On the other hand, ablation of the teleost telencephalon, which is phylogenetically related to the limbic system in land vertebrates, does not disrupt classical autonomic conditioning using an aversive procedure similar to that used in mammals [7,14,16,25].Recently, there is growing evidence that the mammalian cerebellum, in addition to being a motor coordination center, is involved in emotional behavior [2,3,7,23,27,28]. Vermal part of the cerebellum has been implicated in emotional or fear-related behaviors [2,28]. Bobee et al (2000) [3] have reported that the cerebellar vermis is involved in attentional capabilities and emotional behavior as well as in motor control. Although the mammalian cerebellum shows marked elaboration compared with that in fish, the basic design of internal circuitry is shared with fish [1,4,15]. The cerebellum of fish consists of three main parts, corpus and valvulla cerebelli and lobus vestibulolateralis. The piscine

cerebellar corpus is thought to be homologous with the vermal part of the cerebellum of higher vertebrates [9]. The cerebellum of fish has been shown to be involved in motor coordination, including the regulation of locomotor movement, DLR and VOR [18-22,24]. However, it is yet to be unveiled a whole spectrum of the functions of the fish cerebellum, which occupies considerable part of the brain.

In the present study, to investigate the cognitive role of the cerebellum of fish, we conducted experiments examining effects of cerebellar manipulations on fear-related classical heart rate conditioning. We performed two types of manipulations, total ablation of the corpus cerebelli (CC) and localized cooling of the CC for reversible inactivation of the cerebellar function. Effects of these manipulations on delay classical conditioning using aversive procedure were examined. In the present experiments, we used goldfish paralyzed by an injection of curare. Since the expression of conditioned responses of goldfish in fear-related classical conditioning is established quickly, we were able to obtain a series of data from a naive subject in a paralyzed condition.

2. Materials and methods

2.1. Animals

Commertially obtained goldfish (*Carassius auratus*), 45.9-90.0 mm in body length, were kept in our laboratory on a 14:10-hr light-dark cycle at controlled room temperature (18-24 °C) for 3-4 weeks before use.

2.2. Ablation experiment

Goldfish were anesthetized in MS-222 (0.015%), and the cerebellar corpus (CC) was removed by aspiration through a small hole made in the scull. The hole was then covered with dental cement. Sham control fish were surgically operated in the same manner to the CC-ablated fish except for the aspiration of the brain. The operated fish were allowed to recover for 3 days before having the conditioning session described below. Normal, sham-operated or CC-ablated goldfish were anesthetized with MS-222 (0.015%) and immobilized by an intraperitoneal injection of curare (5 μg/g body weight) dissolved in phosphate-buffered saline. The gills were continuously irrigated with

aerated water. An electrocardiogram (ECG) was recorded using a pair of silver-plate electrodes placed on the surface of the trunk. After a 1- to 1.5-h adaptation period in the dimmed environment, the conditioning procedure began.

The conditioning procedure consisted of four sessions of habituation (10 trials), acquisition (20 trials) and extinction (20 trials). A 10.1-sec red light-emitting-diode (LED) light was presented from the right side as a conditioned stimulus (CS) that was coterminated with a 0.1-sec unconditioned stimulus (US) of mild electrical shock on the trunk surface. The intertrial interval was 100 sec. We had confirmed in a pilot experiment that repetitive presentation of the electrical shock at a regular interval (100 sec) did not cause any anticipatory response in the heart rate of goldfish. In the habituation and extinction sessions, the CS was presented alone. Magnitude of the conditioned bradycardic response was estimated by calculating the bradycardia index as follows:

Bradycardia index (%)=100 x {(number of heartbeats during the 10-sec period prior to CS presentation) - (number of heartbeats during the 10- sec period of CS presentation)}/(number of heartbeats during the 10-sec period prior to CS presentation)

After finishing the conditioning session, fish were deeply anesthetized and decapitated. The extent of the lesion was histologically examined. Among the CC-ablated group, only the fish in which more than about 80% of the CC was removed were accepted for later analyses. Data from the fish with any brain part other than the CC damaged were discarded.

2.3. Cooling experiment

Goldfish were anesthetized and immobilized as described above. The dorsal part of the CC was exposed, and the fish was placed in a recording chamber. The gills were irrigated continuously. An aluminium tube with a thin stainless-steel plate ending was gently placed on the dorsal surface of the CC. In the tube, water was continuously circulated via two polyethylene tubes, one for the inlet and the other for the outlet. The circulating water was quickly changed to ice-cooled water when the CC should be cooled. The cooling apparatus was kept in place throughout the experiment. ECGs were recorded as described above. The fish were allowed to recover from the

anesthesia for 1.5 h in a dimmed environment before starting the conditioning procedure.

The conditioning procedure consisted of 5 trials of habituation and 30 trials of acquisition. In the cooled group (n=14), cooling of the CC started 15 min before the conditioning procedure began and stopped just after the ending of the 15th trial of the acquisition session. Thus, in the latter half of the acquisition session, the CC was not cooled. In the control group (n=14), the cooling apparatus was set as in the cooled group but the CC was not actually cooled.

To examine the effect of CC cooling on acquired conditioned response, 5 well-conditioned fish in the control group were subjected to an additional experiment. Fifteen minutes after finishing the conditioning procedure, 3 trials of paired presentation of the CS and US were given to determine the retention of the conditioned bradycardia. Then the CC was cooled for 15 min and another 3 trials of the paired presentation were given under the CC-cooled condition. The fish were then recovered from the cooling for 15 min, and 3 trials of the paired presentation were given to determine the recovery of the response.

After finishing the experiment, fish were deeply anesthetized and decapitated.

2.4. Statistics

All statistical analyses were performed using the software package JMP (SAS Institute). Differences in learning curves between groups were analyzed using repeated-measure ANOVA, and the effect of the CC cooling on the acquired conditioned response was analyzed using a paired t-test. One-way ANOVA was used for analyzing the differences in the normal heart rate and responses to the first CS and US between groups. Differences were considered to be significant when $p<0.05$.

3. Results

3.1. Effects of CC ablation on classical heart rate conditioning

There were no significant differences in the heart rate prior to the start of the conditioning session between normal, sham-operated and CC-ablated goldfish. Thus,

surgical operation had no effect on the heart rate under resting conditions. Figure 1 shows the cardiac response to the first presentation of a US in normal and CC-ablated goldfish. The goldfish responded to a mild electrical shock in the trunk with cardiac deceleration (bradycardia) followed by slight acceleration (tachycardia), which were innate unconditioned responses. CC-ablated goldfish responded to the US presentation in the same way as did normal fish, indicating that the neural pathway underlying expression of the cardiac response induced by the nociceptive stimulus was intact after CC ablation. In the first trial of the habituation session, goldfish responded to the CS, i.e., LED light, with cardiac deceleration (Fig. 2). This arousal response to the novel stimulus was observed in all groups including normal, sham-operated and CC-ablated fish, and no significant differences were found between groups (Fig. 2). This result indicates that the perception of the CS was normal in CC-ablated fish. The arousal response to the CS was greatly reduced in the second trial and thereafter, and almost no response was observed in the latter half of the habituation session in all three groups (Fig. 2).

In the acquisition session, normal and sham-operated fish apparently acquired a conditioned bradycardic response within several trials. We found that about 15 paired stimuli were enough for the majority of normal and sham-operated fish to acquire the maximum conditioned response (Fig. 2). No significant difference was observed in the learning curve between normal and sham-operated goldfish. On the other hand, ablation of the CC greatly and significantly impaired the acquisition of the conditioned bradycardia ($p<0.05$) (Fig. 2). Because, as described above, both the perception of the CS and US and cardiac regulation directly related to those stimuli were not impaired by CC ablation, the effect of the ablation was on the plastic change in this type of learning. There seemed to be a tendency that the conditioning in the CC-ablated fish was in the course of development at the end of the acquisition session. Thus, it was not clear from the present experiment whether CC intactness is critical for the classical heart rate conditioning. However, the CC obviously plays a major part in the central circuit underlying the present type of conditioning. The conditioned response was reduced during the extinction session, in which the CS was never reinforced, in all three groups

(Fig. 2). However, the conditioned response was not completely extinguished even after 20 extinction trials (Fig. 3). In the CC-ablated goldfish, the extinction curve was somewhat shallow compared with that in normal and sham-operated goldfish (Fig. 2).

3.2. Effects of CC cooling on classical heart rate conditioning

The above results show that the integrity of the CC is important for learning performance in a classical heart rate conditioning situation. We further investigated the involvement of the cerebellum in heart rate conditioning by examining the effect of reversible inactivation of the CC function by localized cooling. Goldfish fitted with a cooling apparatus but not actually cooled were treated as the control group so that only the effect of cooling could be evaluated. It was confirmed that both the base heart rate and the unconditioned response to the US were normal during the CC cooling. Thus, the cooling of the CC had no effect on the unconditioned regulation of heart rate. In the cooling experiment, an acquisition session consisted of 30 trials of paired presentations of CS and US. In the CC-cooled group (n=14), cooling was started 15 min before the habituation session and stopped just after the 15th trial of the acquisition session. The arousal response to the first presentation of the CS was normally observed in the CC-cooled group (Fig. 3). However, we found that acquisition performance during cooling in the CC-cooled group was significantly lower than that in control fish (n=14) ($p<0.05$) (Fig. 3). It is obvious that the localized cooling of the CC greatly impaired acquisition of the conditioned response. After the cooling was stopped, a conditioned bradycardic response developed as if fish were naive (Fig. 3).

To examine the effect of CC cooling on acquired conditioned bradycardia, we subjected 5 fish that showed relatively high performance in the acquisition session in the control group to an additional experiment. Fifteen minutes after the acquisition session ended, conditioned bradycardia was well retained and the magnitude of the conditioned response was not significantly different from that in the last 5 trials of the acquisition session (Fig. 4). However, 15-min cooling of the CC greatly reduced the conditioned response (Fig. 4), indicating that the CC cooling affected certain stage(s) involved in the

expression of conditioned bradycardia. The conditioned response was completely recovered from the effect of CC cooling after a 15-min uncooled recovery period (Fig. 4).

4. Discussion

Classical conditioning in which emotional associative learning and systemic conditioned responses are involved is established relatively quickly compared with that involving discrete somatic motor learning [11,13]. In the present study, we found that the establishment of a conditioned bradycardic response was remarkably rapid in our preparations, supporting our belief that the present situation was not discrete somatic motor learning but so-called fear-related emotional learning. In goldfish, Overmier and Savage (1974) [17] have shown that, by using a delay conditioning procedure similar to that in the present experiment, conditioned bradycardic response reach an asymptotic state within 50 trials of paired stimulations. In the present experiments, we used goldfish paralyzed by an injection of curare, a neuromuscular blocking agent. Subjecting the immobilized animal to the experiment on brain mechanisms of learning is advantageous for further experiments involving the recording of neural activity and acute pharmacological treatment.

There could be a possibility that the surgical ablation or temporal cooling of the CC affected on the central circuits mediating the cardiac responses induced by vidual or mechanimal stimuli and hence caused an impairement of the conditioning. However, both the cardiac arousal response to the first presentation of the CS and the cardiac reflex to the aversive US were not impaired by the CC ablation and cooling. This result indicates that the impairements of the acquisition of the conditioned bradycardia in CC-ablated or CC-cooled goldfish were not due to blockade of the sensorimotor pathway mediating the innate cardiac responses.

Acquisition of the conditioned bradycardia was greatly impaired by the ablation of the CC. In contrast to the normal or sham-operated fish, acquisition curve of the CC-ablated fish did not reach an asymptotic level within 20 trials of the acquisition session. There seemed to be a tendency that the conditioning in the CC-ablated fish was in the cource of development at the end of the acquisition session. Thus, it was not clear from

the present experiment whether the intactness of the CC is critical for the classical heart rate conditioning. However, the CC obviously plays a major part in the central circuit underlying the present type of conditioning. The cerebellum of the goldfish consists of three major parts including the corpus and valvula cerebelli and vestibulolateral lobe. The CC and the medial valvula cerebelli share their major projection areas [8]. This might be a reason why the ablation of the corpus cerebelli did not totally abolish the acquisition of conditioned response. Otherwise, brain regions other than the cerebellum is also involved.

Cooling of the CC also impaired the acquisition of the conditioned bradycardia. The effect of cooling was almost compared to that of the CC ablation. In the procedure used in the present experiment, control fish were also fitted with the cooling aparatus in which circulating water was not cooled. Therefore, only the effect of the cooling on the acquisition can be evaluated. By cooling the localized part of the brain, we were able to depress the function of the CC reversibly. After the cooling was stoped, development of the conditioned response was observed as in naive fish. This result suggests that the assosiation of CS and US and/or memory storage is the role of the CC in classical heart rate conditioning. If the CC controls only the expression of the conditioned response, the magnitude of the conditioned bradycardic response would quickly reach the control level after the cooling was stoped. However, a possibility that the neural circuitry in the CC suffered from the after effect of the cooling for some period cannot be ruled out. Monitoring of the activities of the cerebellar neurons during the period of cooling and recovery from the cooling would be required in further experiment.

Here we demonstrated that the cerebellum in fish is involved in classical heart rate conditioning. The results presented here also support the idea from a functional aspect in demonstrating that the CC is homologous with the vermis of higher vertebrates [9]. It is interesting to note that the cerebella in teleost fish and mammals share an "emotional" function in an aversive situation. In mammals, it has been shown that integrity of both amygdala and the cerebellum is essential for classical fear-related conditioning including heart rate conditioning [6,10,11,14,23,25-27]. On the other hand, telencephalic ablation does not disrupt the acquisition of classically conditioned bradycardia in a teleost

goldfish [16,17]. The neural center for aversive learning in the limbic system might have developed during the course of the evolution of tetrapods.

Acknowledgements

This research was partly supported by Japan Society for the Promotion of Science.

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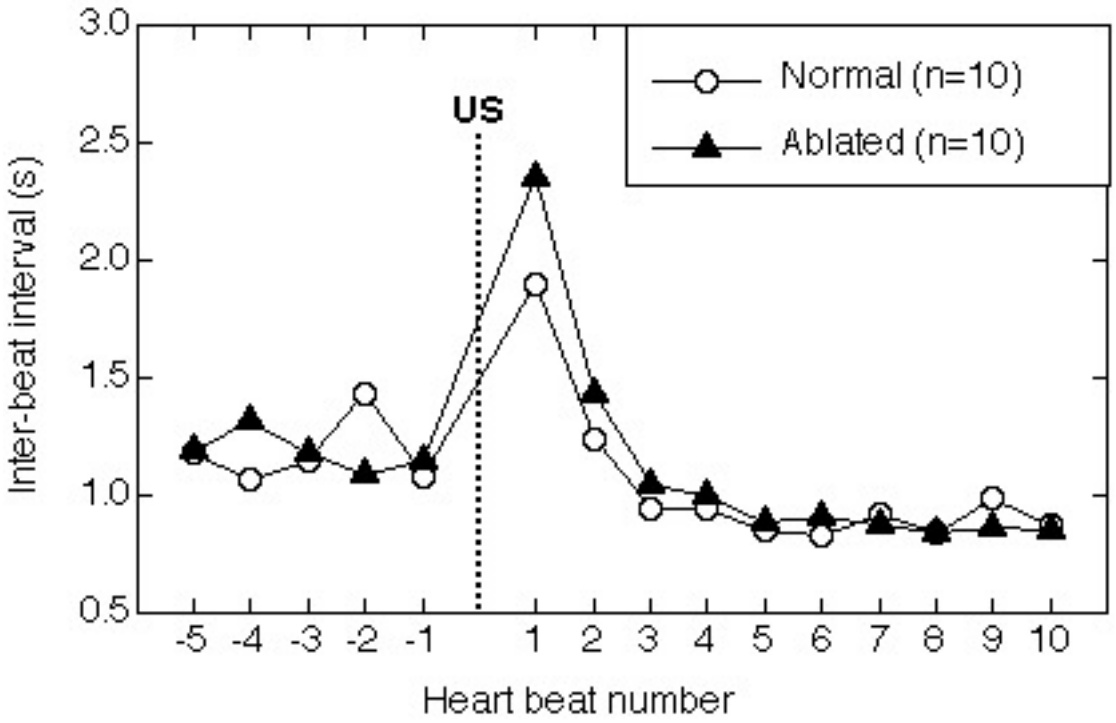

Figure 1. Cardiac response to the US. CC-ablated goldfish unconditionally responds to the US with cardiac deceleration followed by slight acceleration as in normal fish.

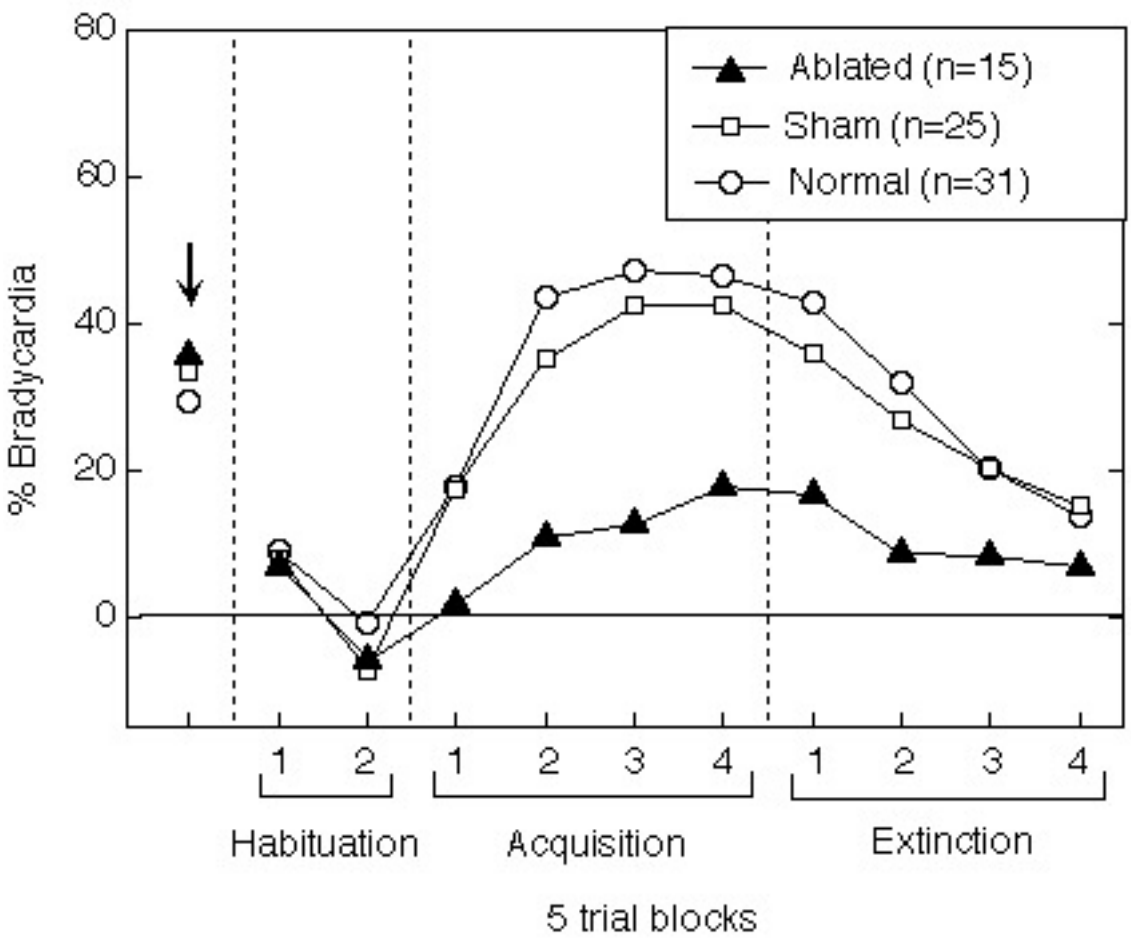

Figure 2. Effect of CC ablation on learning performance. The acquisition of conditioned bradycardia is impaired by the CC ablation, while arousal cardiac response to the first presentation of CS (arrow) is not affected. Each point other than the arousal response represents the averaged bradycardia index of 5 consecutive trials.

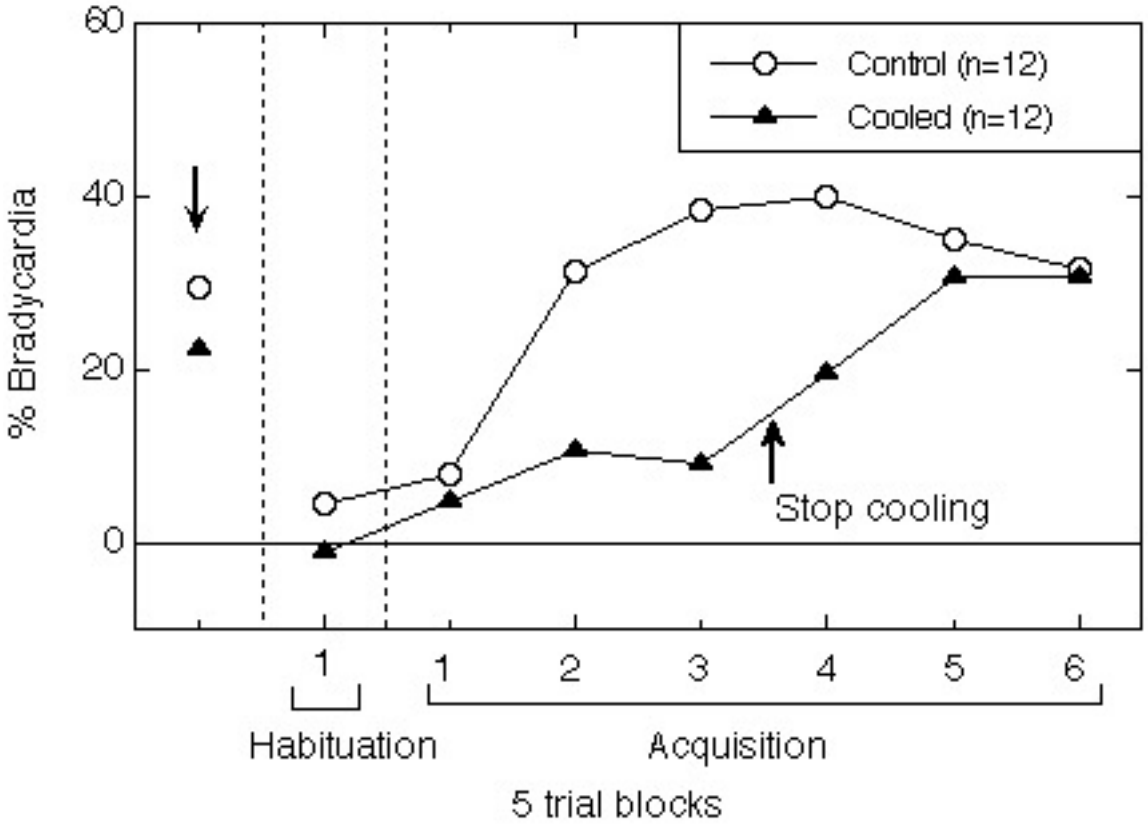

Figure 3. Effect of CC cooling on learning performance. Cooling of the CC impairs the acquisition of conditioned bradycardia ($P<0.05$), while arousal cardiac response to the first presentation of CS (downward arrow) is not affected. The conditioned response develops after the cooling is stopped (upward arrow). Each point other than the arousal response represents the averaged bradycardia index of 5 consecutive trials.

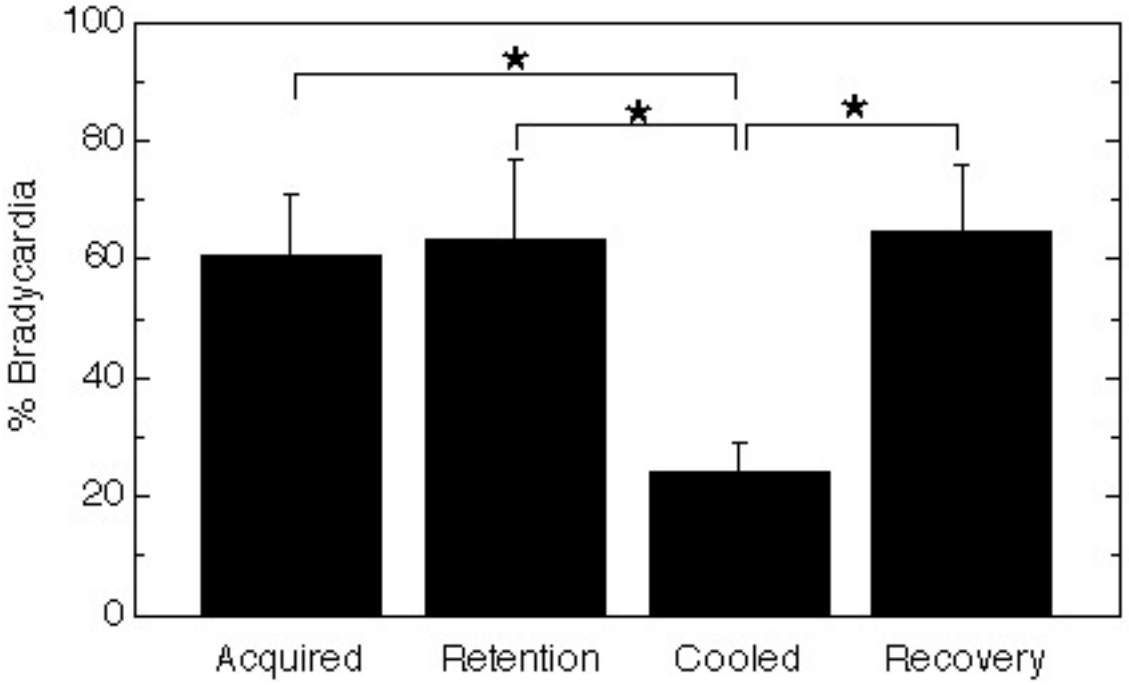

Figure 4. Effect of CC cooling on acquired conditioned bradycardia. Acquired conditioned bradycardia is retained over a 15-min resting period after the acquisition session (retention) and is suppressed by the CC cooling (cooled). Each bar represents the average of 3 trials. Error bars denote s.e.m. (*P<0.05).