

# Effects of inferior olive lesion on fear-conditioned bradycardia

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The inferior olive (IO) sends excitatory inputs to the cerebellar cortex and cerebellar nuclei through the climbing fibers. In eyeblink conditioning, a model of motor learning, the inactivation of or a lesion in the IO impairs the acquisition or expression of conditioned eyeblink responses. Additionally, climbing fibers originating from the IO are believed to transmit the unconditioned stimulus to the cerebellum in eyeblink conditioning. Studies using fear-conditioned bradycardia showed that the cerebellum is associated with adaptive control of heart rate. However, the role of inputs from the IO to the cerebellum in fear-conditioned bradycardia has not yet been investigated. To examine this possible role, we tested fear-conditioned bradycardia in mice by selective disruption of the IO using 3-acetylpyridine. In a rotarod test, mice with an IO lesion were unable to remain on the rod. The number of neurons of IO nuclei in these mice was decreased to ~40% compared with control mice. Mice with an IO lesion did not show changes in the mean heart rate or in heart rate responses to a conditioned stimulus, or in their responses to a painful stimulus in a tail-flick test. However, they did show impairment of the acquisition/expression of conditioned bradycardia and attenuation of heart rate

responses to a pain stimulus used as an unconditioned stimulus. These results indicate that the IO inputs to the cerebellum play a key role in the acquisition/expression of conditioned bradycardia. *NeuroReport* 25:556–561 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

*NeuroReport* 2014, 25:556–561

**Keywords:** cerebellum, fear-conditioned bradycardia, inferior olive, mouse

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Received 27 November 2013 accepted 29 January 2014

## Introduction

The neurons of the inferior olive (IO) regulate neuronal activity and plasticity in the cerebellar cortex through their climbing fiber (CF) inputs [1,2]. CFs are one of the two excitatory inputs to the Purkinje cells in the cerebellum; the other input comes from parallel fibers (PFs). It has been demonstrated that the IO–CF system plays a key role in motor control and learning. For example, administration of 3-acetylpyridine (3-AP) induces lesions of the IO and impairs the postural and locomotor control [3–5] and vestibulo-ocular reflex adaptation [6]. When Purkinje cells are deprived of CF inputs, they show a marked increase in simple spikes activity [7]. At the PF–Purkinje cell synapses, CF plays a critical role in controlling the induction of both long-term depression (LTD) and long-term potentiation (LTP) [8]. Induction of LTD requires dendritic Ca<sup>2+</sup> influx to Purkinje cells through voltage-gated Ca<sup>2+</sup> channels that are activated by CF activity [9]. Moreover, Ca<sup>2+</sup> influx triggered by CF inputs to the Purkinje cell governs

LTD/LTP induction in PF–Purkinje cell synapses [8]. The observations from previous studies indicate that CF inputs originating from IO neurons act as a controller of the firing activities of Purkinje cells and of the induction of synaptic plasticity in these cells.

Eyeblink conditioning provides a model of associative motor learning that is dependent on the cerebellum [10]; both LTD-deficient mice and LTP-deficient mice showed impairments in the acquisition of a conditioned-eyeblink response [11,12]. Moreover, it has been proposed that information related to a conditioned stimulus (CS) is transmitted by the mossy fibers to the cerebellum, whereas that of an unconditioned stimulus (US) is transmitted to the cerebellum by the CFs [13]. Inactivation of the IO prevents the acquisition or expression of conditioned eyeblink responses in rabbits [14,15]. Thus, IO inputs to the cerebellum through CFs play a key role in the acquisition/expression of conditioned eyeblink responses.

The cerebellum is thought to be involved in the control of the autonomic nervous system [16]; thus, electrical stimulation of the cerebellar vermis can produce marked changes in the heart rate [17]. The cerebellar contribution to the control of heart rate has been investigated

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using fear-conditioned bradycardia. Lesions in the cerebellar vermis of rats or rabbits impair the acquisition/expression of conditioned bradycardia without disrupting mean heart rate responses or unconditioned responses [18,19]. To date, however, the neural circuitry within the cerebellum that is responsible for fear-conditioned bradycardia has not been determined. In the present study, we focused on the IO because it is thought to be important for producing synaptic plasticity at the PF–Purkinje cell synapses. To examine the role of the IO, we chemically induced a lesion in the IO of mice and examined the effect of this lesion using fear-conditioned bradycardia.

## Methods

### Animals and lesion of the inferior olive

Male C57BL/6J mice (6–8 weeks of age) were used in the present experiments. The animals were housed individually and maintained under a 12 h light/12 h dark cycle with food and water available *ad libitum*. Lesion of the IO in eight mice was produced by intraperitoneal injection of 3-AP (Sigma-Aldrich, Tokyo, Japan; 50 mg/kg), followed by injection of nicotinamide (500 mg/kg, 3–3.5 h after the 3-AP injection). Control mice ( $n = 8$ ) were injected with saline. Three of the eight mice in each group were used for Nissl staining and cell counting to confirm the induction of an IO lesion. The other five mice were used for a rotarod test and for fear-conditioned bradycardia. All experimental protocols were approved by the Ethics Committee for Animal Experiments at the University of Tokyo and were performed according to the Guidelines for Research with Experimental Animals of the University of Tokyo and the Guide for the Care and Use of Laboratory Animals (NIH Guide), revised in 1996.

### Rotarod test

The rotarod tests were performed to confirm whether the lesion in the IO produced an effect. Mice were placed on a 5 cm (diameter) rod rotating at a rate of 8 rpm. Maximum retention time was set to 120 s. Each mouse performed the rotarod test over 10 trials. Before the test, mice were placed on the static rod for up to 120 s to habituate them to the rod.

### Conditioning procedures

After anesthetizing with 2% isoflurane, mice were restrained in a stereotaxic instrument and the scalp was incised. A chronic electrode was anchored with dental acrylic. Fine stainless steel wires were inserted subcutaneously and sewn into the sides of the mice. The animals were allowed to recover for at least 2 days before conditioning began. The conditioning apparatus consisted of a restraining device enclosed within a darkened sound-attenuating chamber. The chamber contained two speakers mounted on a two-tier rack. The heart rates of the mice being conditioned were amplified using the

implanted chronic electrode connected to an amplifier (MEG-2100; Nihon Kohden, Tokyo, Japan). The output signal from the amplifier was divided into two outputs: one was monitored on an oscilloscope (VC-6725; Hitachi, Tokyo, Japan) and the other was digitized using an analog to digital converter (MacLab 8s; AD Instruments, Dunedin, New Zealand) and stored on a computer at a 1 kHz sampling frequency. A conditioned and unconditioned (tone and electrical shock) stimulus paradigm was delivered using a programmable pulse generator (Master 8; A.M.P.I., Jerusalem, Israel). The tone stimulus was generated by a synthesizer (1941-Wave-Factory; NF Corporation, Yokohama, Japan), amplified by a two-channel power amplifier (SRP-P150; Sony, Tokyo, Japan) and delivered to the mice through two speakers. The sound intensity was measured with a sound-level meter. The US was delivered using an electrical stimulator (SEN-2201; Nihon Kohden) connected to the shock electrodes consisting of two electrodes secured around the tail of each mouse. Each mouse was habituated to restraint by placing it in a standard mouse restrainer and attaching heart rate electrodes and tail-shock electrodes; this was carried out twice daily for 60 min. Heart rate was sampled at 90-s intervals throughout each habituation. After habituation, each mouse received 2 consecutive days of unreinforced CS with a fixed 180-s interstimulus interval. The CS was a 5 s, 80 dB, 2.5 kHz tone. The conditioning phase was carried out for 3 consecutive days, followed by 50 paired presentations of a CS and a US (consisting of a 500 ms, 0.3 mA tail shock) presented on a fixed 180-s interstimulus interval. In all procedures, except for habituation, the first daily trial was initiated 10 min after the mouse was placed into the chamber. After the conditioning procedures, the mice underwent US-alone phase and a tail-flick test to confirm their response to pain stimuli. The US-alone phase consisted of 20 trials in a single day in which only the US was presented. Changes in heart rate were assessed by measuring the interbeat interval, defined as the time between successive R-waves (R–R interval) of the cardiac cycle. R-waves of the cardiac cycle were analyzed using LabChart Software (v.3.6.1/s; AD Instruments). The topography of the response to the stimulus (CS) was determined by comparing the mean pre-CS heart rate with each 1-s interval during the 5-s tone. The topography of the response to the US was determined by comparing the mean pre-US heart rate with each 1-s interval of the 6 s following the offset of the US. In the tail-flick test, mice received two types of thermal radiation (80 and 110°C) to the tail and the response latency was measured.

### Nissl staining and cell counting

The mice were deeply anesthetized with pentobarbital (100 mg/kg) and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The brains were removed from the

mice and embedded in paraffin wax after dehydration using graded ethanol and processed for the preparation of paraffin sections (4µm) using a rotating microtome (HM 355S; Thermo, Yokohama, Japan). Sections were mounted on glass microscope slides. For Nissl staining, the sections were immersed in distilled water and submerged in 0.1% cresyl violet solution for ~10–30 min until the desired depth of staining was achieved. The sections were rinsed in distilled water, dehydrated in a graded series of ethanol (70, 80, 90, and 100%), cleared in xylene, and covered with xylene. Sets of nine coronal sections from each animal were examined for cell counting. Each section was viewed using a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan), and the number of cells that presented a well-delimited, spherical form with a distinct nucleus and nucleolus was counted.

**Statistical analysis**

All data were analyzed with the statistical package for social sciences (SPSS, version 14.0; SPSS Japan Inc., Tokyo, Japan) using Student’s *t*-test, two-way analysis of variance for repeated measures and the Bonferroni post-hoc test. Results are presented as means±SEM and statistically significant differences are defined as *P* value less than 0.05.

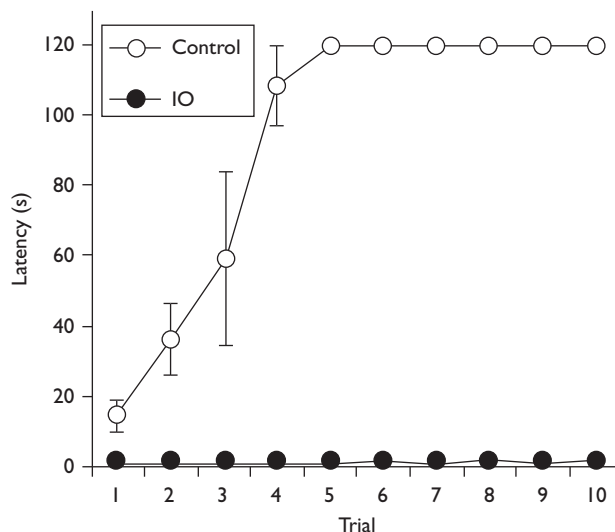
**Results**

The number of IO neurons in control and IO-lesioned mice (IO mice) were compared by analysis of Nissl-stained coronal brain sections (Fig. 1). IO mice had significantly fewer IO neurons than controls [control 2082±59, IO 1281±81, *t*(5) = 8.197, *P* < 0.001; Fig. 1c].

In the rotarod test to examine the effect of 3-AP injection on motor control, IO mice showed significantly poorer performance than the control mice [trial × mice, *F*(9, 72) = 21.76, *P* < 0.001; Fig. 2].

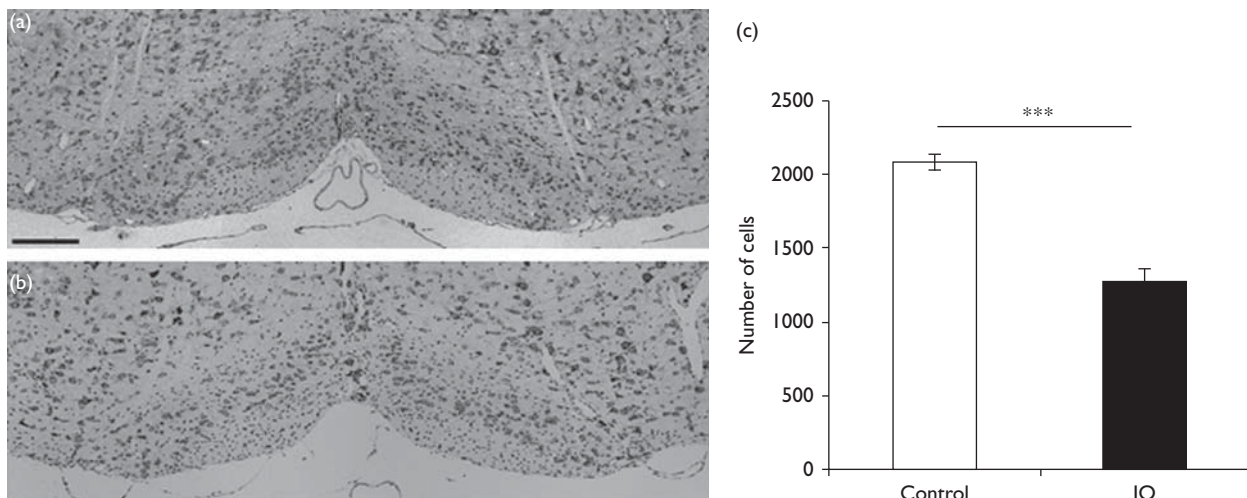
Control and IO mice had similar and consistent patterns of baseline heart rate on the second day of habituation (Fig. 3a) with no significant difference in mean heart rates between the two groups [control 671±18 beats/min, IO 713±12 beats/min, *t*(8) = -1.918, *P* = 0.091]. On the

**Fig. 2**



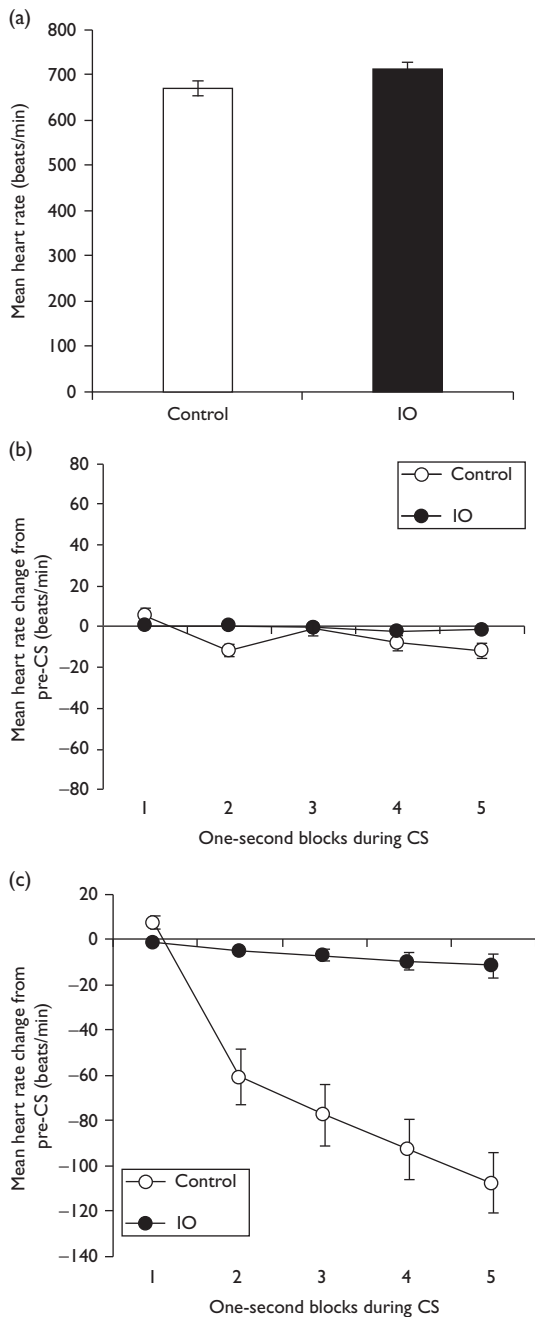
Rotarod test of control (open circles) and inferior olive (IO) mice (closed circles). The performance of each mouse was limited to a maximum retention time of 120 s/trial. IO mice showed poor performance compared with the control mice. Error bars show SEM.

**Fig. 1**



The effect of 3-acetylpyridine administration. Coronal sections of -3.08 mm from the interaural were stained with cresyl violet. (a) Control mice (n=3); (b) inferior olive (IO) mice (n=3). Scale bar=200µm. (c) Number of IO cells in control and IO mice. Error bars show SEM. \*\*\**P*<0.001.

Fig. 3



Effects of the inferior olive (IO) lesion on the mean heart rate, heart rate responses to the conditioned stimulus (CS), and acquisition/expression of conditioned bradycardia. (a) Bars show mean baseline heart rates on the second day of habituation in control (open bar) and IO mice (filled bar). (b) Mean changes in heart rate (beats/min) during 1-s intervals of the 5-s CS averaged from 50 trials on the second day of CS-alone testing in control (open circles) and IO mice (closed circles). The horizontal line indicates the mean heart rate before the 5-s CS averaged from 50 trials. (c) IO mice had impaired acquisition/expression of conditioned bradycardia. Mean heart rate changes from the pre-CS baseline during the 5-s CS averaged from 50 trials on the first day of the CS-unconditioned stimulus phase. Error bars show SEM.

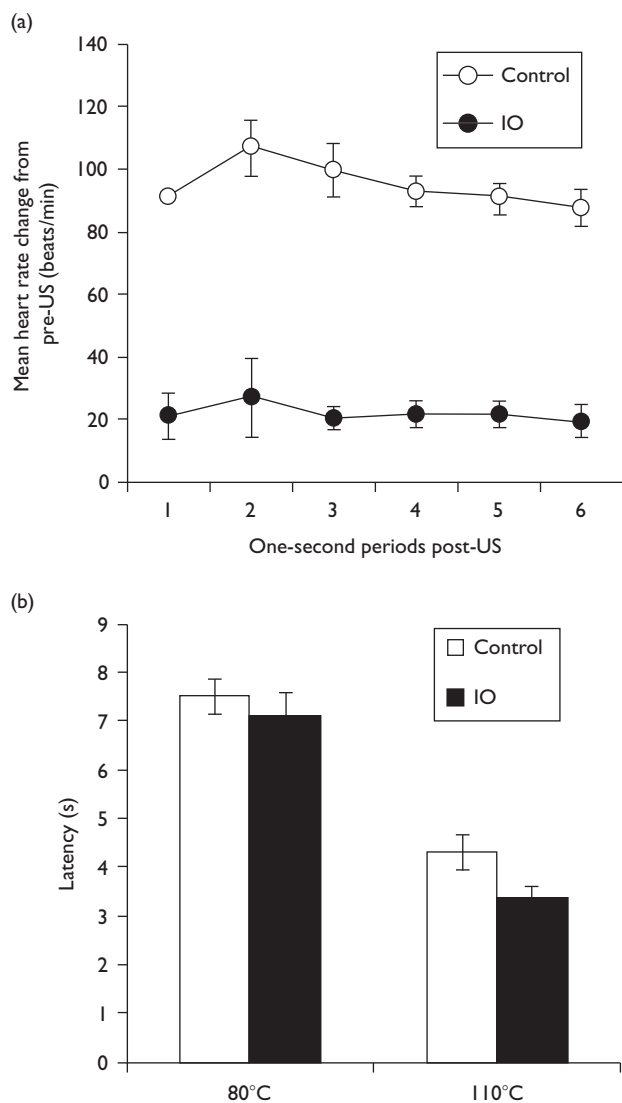
second day of the CS-alone phase, the topography of heart rate responses was examined at 1-s intervals during the 5-s CS in 50 trials. The aggregate patterns of the heart rate responses of the two groups of mice are shown in Fig. 3b; both groups showed similar heart rate responses [time  $\times$  mice,  $F(4, 28) = 1.947$ ,  $P = 0.130$ ]. Conditioned bradycardia was investigated for 3 consecutive days of the CS-US phase in control and IO mice; Fig. 3c shows the aggregate patterns from 50 trials. In control mice, the predominant response was a progressive decrease in heart rates during the 5-s CS. There were significant differences in the conditioned bradycardia responses between the control and IO mice [time  $\times$  mice,  $F(4, 28) = 36.994$ ,  $P < 0.001$ ]. This pattern continued on the second and third day of the CS-US phase, as reflected by a significant main effect for groups [time  $\times$  mice, second day,  $F(4, 32) = 12.62$ ,  $P < 0.001$ ; time  $\times$  mice, third day,  $F(4, 32) = 7.625$ ,  $P < 0.001$ , data not shown]. After completion of the conditioning procedure, the mice were subjected to a US-alone phase and to a tail-flick test to confirm their responsiveness to pain stimuli. IO mice showed a significantly higher attenuation of their tachycardiac response compared with control mice [mice,  $F(1, 7) = 98.524$ ,  $P < 0.001$ ; Fig. 4a]. There were no significant differences between the control and IO mice in the tail-flick test [80°C: control  $7.4 \pm 0.3$  s, IO  $7.0 \pm 0.5$  s,  $t(8) = 0.672$ ,  $P = 0.521$ ; 110°C: control  $4.2 \pm 0.4$  s, IO  $3.3 \pm 0.5$  s,  $t(7) = 1.590$ ,  $P = 0.156$ ; Fig. 4b].

### Discussion

As expected from a previous study in rats [3], IO mice had fewer IO cells than the control mice and they also performed more poorly in the rotarod test. IO mice showed impaired acquisition/expression of conditioned bradycardia without disruption of mean heart rate or heart rate responses to a CS. Moreover, IO mice had an attenuated tachycardiac response to an electrical shock used as the US in the US-alone phase. However, the IO mice had the same response as control mice to a pain stimulus in tail-flick test.

CF inputs to the Purkinje cells modulate LTD/LTP at the PF-Purkinje cell synapses [8]. Both LTD-deficient and LTP-deficient mice exhibit impaired acquisition of the conditioned eyeblink response [11,12]. In fear-conditioned bradycardia, a lesion of the cerebellar vermis did not show acquisition/expression of conditioned bradycardia without the disruption of mean heart rate, heart rate responses to the CS, and heart rate responses to the US [18,19]. Information regarding the CS is transmitted by the mossy fibers to the cerebellum, whereas information regarding the US is transmitted through the CF to the cerebellum during eyeblink conditioning [13]. In this study, we showed that the IO lesion impaired the acquisition/expression of conditioned bradycardia during the CS-US phase and attenuated tachycardiac responses

Fig. 4



Heart rate responses to an electrical tail shock given as an unconditioned stimulus (US) and responses to a pain stimulus in a tail-flick test. (a) Topography of the heart rate responses following application of the electrical tail shock in the US-alone phase. Mean changes in heart rate (beats/min) during each 1-s interval of the 6 s following the offset of the US averaged from 20 trials. Inferior olive (IO) mice show an attenuated tachycardiac response to the tail shock. (b) Tail-flick test. Both control and IO mice received two different thermal stimuli (80 and 110°C). Error bars show SEM.

during the US-alone phase. Results from previous studies and the present study are consistent with the idea that impairment of acquisition/expression in conditioned bradycardia is induced because of disrupted transmission of the US to the cerebellum as a consequence of the IO lesion. In the tail-flick test, there were no differences between the control and IO mice. This result indicates that both IO and control mice had normal spinally mediated nociceptive reflexes. Pain stimuli are conveyed from the spinal cord through the reticular formation and thalamus to the cerebral cortex, hypothalamus, and limbic

lobe [20]. We found that the IO is not associated with nociceptive reflexes in the tail-flick test but is important in the tachycardiac response to the US. A previous study on the acquisition of fear-conditioned bradycardia in goldfish found that cerebellar Purkinje cells have a simple spike response to the CS and complex spike response to the US [21]. Thus, inputs from the IO to the cerebellar cortex and/or cerebellar nuclei are thought to be involved in tachycardiac responses to the US. An important question is how the bradycardia response is induced during the CS in the CS-US phase and tachycardiac response to the US in the US-alone phase? The cerebellar vermis is connected to the fastigial nucleus, which is one of the cerebellar deep nuclei, and is involved in autonomic nervous system control [16]. It has been reported that electrical stimulation of the fastigial nucleus produces increased heart rate [22]. Thus, induction of the tachycardiac response to the US may require increased activity of the fastigial nucleus. Conversely, it is thought that activity of the fastigial nucleus is decreased in the conditioned bradycardia response during CS. One possible mechanism for the decreased activity of the fastigial nucleus is that LTP at the PF-Purkinje cell synapses is induced during fear-conditioned bradycardia. A previous study reported that LTP at the PF-Purkinje cell synapses is induced in the cerebellar vermis after the acquisition of conditioned responses in fear-conditioned freezing response [23]. This observation suggests that conditioned bradycardia during the CS might be induced because increased inhibitory output of Purkinje cells inhibits the activity of the fastigial nucleus. It has also been reported that complex spikes are induced in response to the US in Purkinje cells during acquisition of conditioned bradycardia; simple spikes induced by the CS are temporarily paused by the complex spikes in fear-conditioned bradycardia [21]. Previous studies have shown that the simple and complex spikes show reciprocal activity [24] and the CF inputs control this reciprocal behavior of complex and simple spikes in Purkinje cells [25]. This implies that the inhibition of simple spikes by complex spikes causes a decrease in the inhibitory output from Purkinje cells to the fastigial nucleus resulting in the induction of a tachycardiac response.

## Conclusion

Our observations here show that IO mice had impaired acquisition/expression of conditioned bradycardia and attenuated tachycardiac response to an electrical shock as the US in the US-alone phase. Therefore, we propose that the IO transmits the US through the CFs to the cerebellum in fear-conditioned bradycardia and the IO-CF system plays a key role in the acquisition/expression of fear-conditioned bradycardia.

## Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (C) and Priority Areas 'Emergence of Adaptive

## Motor Function through Interaction between Body, Brain and Environment' to D.Y.

### Conflicts of interest

There are no conflicts of interest.

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