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 eyelink 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 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.

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 Ca²⁺ influx to Purkinje cells through voltage-gated Ca²⁺ channels that are activated by CF activity [9]. Moreover, Ca²⁺ 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 eyelink responses in rabbits [14,15]. Thus, IO inputs to the cerebellum through CFs play a key role in the acquisition/expression of conditioned eyelink 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.

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

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, °Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo and Laboratory for Neuronal Architecture, RIKEN, Brain Science Institute, Saitama, Japan

Correspondence to Dai Yanagihara, PhD, Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

Tel: +81 3 5454 6857; fax: +81 3 5454 4317; e-mail: dai.y@daten.c.u.tokyo.ac.jp

Present address: Hiroko Kotajima and Kazuhisa Sakai: National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira-Shi, Tokyo 187-8502, Japan

Present address: Tsutomu Hashikawa: Support Unit for Neuromorphological Analysis, RIKEN, Brain Science Institute, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan

Received 27 November 2013 accepted 29 January 2014

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives 3.0 License, where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially.

DOI: 10.1097/WNR.0000000000000135
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 stereotactic 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 (4 µm) 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 second day of habituation, IO mice showed significantly poorer 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 = 200 µm. (c) Number of IO cells in control and IO mice. Error bars show SEM. ***P<0.001.

---

**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.
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 × 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 × 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 × mice, second day, F(4, 32) = 12.62, P < 0.001; time × 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±0.3 s, IO 7.0±0.5 s, t(8) = 0.672, P = 0.521; 110°C: control 4.2±0.4 s, IO 3.3±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.
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.

**References**

1. Ito M. Cerebellar long-term depression: characterization, signal transduction, and functional roles. *Physiol Rev* 2001; 81:1143–1195.
2. Watson TC, Koutsikou S, Cerminara NL, Flavell CR, Crook JJ, Lumb BM, et al. The olivo-cerebellar system and its relationship to survival circuits. *Front Neural Circuits* 2013; 7:72.
3. Rondi-Reig L, Delhaye-Bouchaud N, Mariani J, Caston J. Role of the inferior olivary complex in motor skills and motor learning in the adult rat. *Neuroscience* 1997; 77:955–963.
4. Fernandez AM, de la Vega AG, Torres-Aleman I. Insulin-like growth factor I restores motor coordination in a rat model of cerebellar ataxia. *Proc Natl Acad Sci USA* 1998; 95:1253–1258.
5. Aoki H, Sugihara I. Morphology of single olivocerebellar axons in the denervation–reinnervation model produced by subtotal lesion of the rat inferior olive. *Brain Res* 2012; 1449:24–37.
6. Ito M, Miyashita Y. The effects of chronic destruction of the inferior olive upon visual modification of the horizontal vestibulo-ocular reflex of rabbits. *Proc Jpn Acad* 1975; 51:716–720.
7. Montarolo PG, Palestini M, Strata P. The inhibitory effect of the olivocerebellar input on the cerebellar Purkinje cells in the rat. *J Physiol* 1982; 332:187–202.
8. Coesmans M, Weber JT, De Zeeuw CI, Hansel C. Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. *Neuron* 2004; 44:691–700.
9. Sukurai M. Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression. *Proc Natl Acad Sci USA* 1990; 87:3383–3385.
10. Freeman JH, Steinmetz AB. Neural circuitry and plasticity mechanisms underlying delay eyelink conditioning. *Learn Mem* 2011; 18:666–677.
11. Aiba A, Kano M, Chen C, Stanton ME, Fox GD, Herrup K, et al. Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* 1994; 79:377–388.
12. Schonenwille M, Belmeguenai A, Koekkoek SK, Houtman SH, Boele HJ, van Beugen BJ, et al. Purkinje cell-specific knockout of the protein phosphatase PP2B impairs potentiation and cerebellar motor learning. *Neuron* 2010; 67:618–628.
13. Thompson RF, Steinmetz JE. The role of the cerebellum in classical conditioning of discrete behavioral responses. *Neuroscience* 2009; 162:732–755.
14. Welsh JP, Harvey JA. Acute inactivation of the inferior olive blocks associative learning. *Eur J Neurosci* 1998; 10:3321–3332.
15. Zbarska S, Bracha V. Assessing the role of inferior olivary sensory signaling in the expression of conditioned eyeblinks using a combined glutamate/ GABA<sub>B</sub> receptor antagonist protocol. *J Neurophysiol* 2012; 107:273–282.
16. Nisimaru N. Cardiovascular modules in the cerebellum. *Jpn J Physiol* 2004; 54:431–448.
17. Nisimaru N, Yamamoto M, Shimoyama I. Inhibitory effects of cerebellar cortical stimulation on sympathetic nerve activity in rabbits. *Jpn J Physiol* 1984; 34:539–551.
18. Supple WF Jr, Leaton RN. Cerebellar vermis: essential for classically conditioned bradycardia in the rat. *Brain Res* 1990; 509:17–23.
19. Supple WF, Kapp BS. The anterior cerebellar vermis: essential involvement in classically conditioned bradycardia in the rabbit. *J Neurosci* 1993; 13:3705–3711.
20. Saade NE, Jabbar SJ. Nociceptive behavior in animal models for peripheral neuropathy: spinal and supraspinal mechanisms. *Prog Neurobiol* 2008; 86:22–47.
21. Yoshida M, Kondo H. Fear conditioning-related changes in cerebellar Purkinje cell activities in goldfish. *Behav Brain Funct* 2012; 8:52.
22. Achari NK, Downman CB. Autonomic effector responses to stimulation of nucleus fastigius. *J Physiol* 1970; 210:637–650.
23. Sacchetti B, Scello B, Tempia F, Strata P. Long-term synaptic changes induced in the cerebellar cortex by fear conditioning. *Neuron* 2004; 42:973–982.
24. Barmack NH, Shojaku H. Vestibular and visual climbing fiber signals evoked in the uvula-nodulus of the rabbit cerebellum by natural stimulation. *J Neurophysiol* 1995; 74:2573–2589.
25. Badura A, Schonenwille M, Voges K, Galliano E, Renier N, Gao Z, et al. Climbing fiber input shapes reciprocity of Purkinje cell firing. *Neuron* 2013; 78:700–713.