

## OPTOGENETIC INHIBITION OF PURKINJE CELL ACTIVITY REVEALS CEREBELLAR CONTROL OF BLOOD PRESSURE DURING POSTURAL ALTERATIONS IN ANESTHETIZED RATS

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**Abstract**—The cerebellar uvula (lobule IX), a part of the vestibulocerebellum, is extensively connected to the areas of the brainstem that participate in cardiovascular regulation and vestibular signal processing. This suggests that the uvula regulates blood pressure (BP) during postural alterations. Previous studies showed that lesions of the uvula affected the baroreceptor reflex and cardiovascular responses during postural alterations. To investigate the mechanisms underlying this BP regulation, it is necessary to have a method to selectively modulate the activity of Purkinje cells (PCs), the sole output neurons from the cerebellar cortex, without affecting other neuronal types such as local interneurons or nonlocal neurons that send their axons to the cerebellar cortex. We recently developed a novel technique using optogenetics to manipulate PC activity and showed that activation and inhibition of PCs in the uvula either decreased or increased the resting BP, respectively. This technique was employed in the current study to examine the roles of the uvula in BP regulation during postural alterations in anesthetized rats. Enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR), a light-driven chloride ion pump, was selectively expressed in uvular PCs using a lentiviral vector containing the PC-specific L7 promoter. The eNpHR-expressing PCs were then illuminated by orange laser (593 nm) either during 30° head-up or 30° head-down tilts. The eNpHR-mediated photoinhibition of the uvula attenuated the extent of BP recovery after a BP increase induced by postural changes during head-down tilts. By contrast, photoinhibition had no statistically significant effect on BP recovery during head-up tilts. The effects of photoinhibition on BP during tilts were significantly different from those observed during the resting condition, indicating that cerebellar control of BP during tilts is dynamic rather than static. Taken together, these results suggest that PCs in the uvula dynamically regulates BP maintenance during postural alterations. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: BP, blood pressure; eNpHR, enhanced *Natronomonas pharaonis* halorhodopsin; EYFP, enhanced green fluorescent protein; gDNA, genomic DNA; HEK, human embryonic kidney; LSD, least significant difference; MAP, mean arterial pressure; NTS, solitary tract nucleus; PC, Purkinje cell; sL7 promoter, shortened L7 promoter; sL7-eNpHR, Lenti-sL7-eNpHR-EYFP-WPRE; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

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10.1016/j.neuroscience.2012.03.014

**Key words:** cerebellum, halorhodopsin, lentiviral vector, L7 promoter, blood pressure, postural alteration.

The cerebellar uvula (lobule IX) participates in cardiovascular control (Ito, 1984; Bradley et al., 1991; Nisimaru, 2004). Anatomically, Purkinje cells (PCs) in this region project to the lateral parabrachial nucleus, which connects to the solitary tract nucleus (NTS) (Paton and Spyer, 1990). Since NTS neurons receive primary afferent fibers from the arterial baroreceptors, it has been suggested that cardiovascular controls by the uvula are elicited through these connections. Indeed, lesioning of the uvula significantly alters the baroreceptor reflex to increased blood pressure (BP) in decerebrate rabbits (La Noce et al., 1991). PCs in the uvula also project to the inferior and medial vestibular nuclei, which mediate vestibular effects on the sympathetic nervous system during postural alterations (Yates et al., 2000). Lesioning of the uvula affects cardiovascular responses to postural alterations in cats (Holmes et al., 2002), suggesting that the uvula regulates vestibular effects on cardiovascular responses during postural alterations.

However, chronic cerebellectomy affects not only PCs, but also other cell types (e.g. stellate/basket, Golgi cells) including nonlocal cells that send their axons to the cerebellar cortex. It is thus still unclear whether the effects of lesioning of the uvula on cardiovascular control are actually due to the absence of PCs.

Optogenetics is a rapidly growing technique that enables us to probe functions of specific cell types in diverse model organisms (Zhang et al., 2007; Adamantidis et al., 2007; Abbott et al., 2009; Arrenberg et al., 2009; Diester et al., 2011; Feno et al., 2011). Recently, we have developed a novel technique using optogenetics for the selective and rapidly reversible manipulation of cerebellar PC activity *in vivo* (Tsubota et al., 2011). In that study, we used enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR) (Gradinaru et al., 2008) for optogenetic inhibition of PCs, where eNpHR was targeted to PCs using a lentiviral vector containing the shortened version of the PC-specific L7 promoter (sL7 promoter) (Oberdick et al., 1990; Sawada et al., 2010). As a result, eNpHR expression was observed in most PCs (95.3%), as determined by immunohistochemical analysis, and *in vivo* single-unit recordings of PCs revealed that almost all of the PCs (93.8%) that express eNpHR were inhibited during orange light illumination. This technique is suitable for probing the func-

tions of specific cerebellar areas, since it can be used to manipulate the output of the cerebellar cortex alone.

In this study, we applied this technique for investigating the precise roles of PCs in the uvula in posture-change-related cardiovascular regulation. We demonstrated that the eNpHR-mediated photoinhibition of PCs in the medial uvula dynamically altered BP maintenance during postural alterations in urethane-anesthetized rats, providing direct evidence that PCs within the uvula participate in the cardiovascular regulation during postural alterations.

## EXPERIMENTAL PROCEDURES

### Lentivirus production

The lentiviral vector, Lenti-sL7-eNpHR-EYFP-WPRE (sL7-eNpHR), was prepared using a previously described construct and method (Ohashi et al., 2011; Tsubota et al., 2011). Human embryonic kidney (HEK) 293T cells (obtained from RIKEN BioResource Center, Cell No. RCB2202) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> atmosphere. A lentiviral vector was produced by co-transfection of HEK293T cells with a mixture of four plasmids (pCL20c sL7-eNpHR-EYFP-WPRE, pCAGkGP1.1R, pCAG4RTR2, and pCAG-VSV-G) (Hanawa et al., 2002) using the calcium phosphate precipitation method. Sixteen hours after the transfection, the cells were washed with phosphate-buffered saline twice and then cultured for additional 24 h. The medium containing vector particles was harvested 40 h after transfection. The medium was cleared by low-speed centrifugation, filtered using a 0.22 μm low-protein-binding membrane, and centrifuged at 25,000 rpm for 90 min using an SW-28 rotor (Beckman Coulter, CA, USA). After centrifugation, viral pellets were resuspended with 4 °C PBS.

### Titration of lentiviral vectors

Titers of virus stocks were determined by the DNA titration method (Sastry et al., 2002). Serial dilutions of each lentiviral vector solution were added to 4×10<sup>5</sup> HEK293T cells growing in a monolayer in six-well plates in a total volume of 2 ml of culture medium. After 24 hours, the medium was changed, and culture was continued for another 2 days. Then the genomic DNA (gDNA) from approximately 1×10<sup>5</sup>–10<sup>6</sup> vector transduced cells was isolated using FastPure DNA Kit (Takara Shuzo, Co, Ltd.). To estimate the number of virus DNA molecules in gDNA, quantitative real-time PCR analysis was performed by using TaqMan MGB Probe pWPRE (5'-FAM-CAT GGC TGC TCG CCT-MGB-3') (Applied Biosystems, CA, USA), and the primers WPRE-F (5'-CGG CTG TTG GGC ACT GA-3') and WPRE-R (5'-GAG GGC CGA AGG GAC GTA-3'). PCR reaction and data analysis were performed using StepOne™ Real-Time PCR system (Applied Biosystems). Amplification of WPRE sequence-containing plasmid DNA for generation of a standard curve was performed using concentrations of plasmids ranging from 10<sup>7</sup> molecules/μl to 10<sup>2</sup> molecules/μl. Copy numbers of gDNA were estimated using TaqMan Copy Number Reference Assays (Applied Biosystems) and were used for the calculation of the final DNA titers of vectors.

### Lentivirus injection

Eight- to twelve-week-old male Wistar rats (Nihon SLC, Shizuoka, Japan) were used. Each rat was anesthetized with ketamine/xylazine (90 and 10 mg/kg, respectively). A small hole was drilled 14.0 mm posterior to bregma and on the midline. A glass pipette (~50 μm tip diameter) was attached to a 32-gauge blunt end needle, and the needle was then attached to a 10 μl gas-tight

Hamilton syringe (Hamilton Company, NV, USA). The glass pipette was tilted 20° forward in the sagittal plane and lowered into the cerebellum to a depth of 4.1 mm from the dura mater (Paxinos and Watson, 2007). Virus solution (6 μl), at a titer of >10<sup>10</sup> genome copies/ml, was injected at a flow rate of 200 nl/min using a micropump (UltramicroPump III; World Precision Instruments, FL, USA) and microprocessor-based controller (Micro4; WPI). The needle was left in place for an additional 10 min before being retracted from the brain. Animals were maintained for more than 8 days before use in physiological experiments. All procedures were performed in accordance with a protocol approved by the University of Tokyo Animal Care Committee. All efforts were made to minimize the number of animals used and their suffering.

### Arterial blood pressure measurement and laser illumination

Arterial BP recording and laser illumination methods were described previously (Tsubota et al., 2011). Each rat was anesthetized with ethyl carbamate (1.2 g/kg). Body temperature was maintained at 37.5 °C using a homeothermic heating pad (BioResearch Center, Aichi, Japan). A catheter (inner diameter, 0.5 mm; outer diameter, 0.9 mm; polyethylene tube SP35, NATSUME SEISAKUSHO, Tokyo, Japan) was inserted into the left femoral artery and connected to a blood pressure transducer (DX-360, NIHON KOHDEN) to continuously measure BP (Abbott et al., 2009). BP signals were amplified using a blood pressure amplifier (AP-641G, NIHON KOHDEN), digitized at 1 kHz, and stored using the Recorder software (Neural Data Acquisition System, Plexon, TX, USA). Stored data were analyzed offline using MATLAB (MathWorks, MA, USA). The vision of each rat was occluded during experiments so that visual information would not affect the BP. Furthermore, care was taken to minimize sound throughout the experimental procedure.

The orange light source was a diode pumped solid state laser (peak wavelength at 593 nm, 200 mW; beam diameter, 3.0 mm; CNI Optoelectronics, Changchun, China). The outgoing beam was passed through an ND filter (Thorlabs, NJ, USA) and an electrically controlled mechanical shutter (hole diameter, 2.0 mm; Model LS3, UNIBLITZ, NY, USA). The beam was then passed through two lenses (focal length: 1st lens 50 mm, 2nd lens 10 mm) to thin the beam diameter. The beam next entered into an optic fiber (core diameter, 62.5 μm) through a fiber collimator. The output beam from the fiber was also passed through another collimator (focal length, 4.50 mm; beam waist diameter, 0.9 mm). The optic fiber was held in a micromanipulator to control the position of the beam spot on the surface of the cerebellum. Light intensity was set at ~50 mW/mm<sup>2</sup> (Cardin et al., 2010) as measured by a power meter (PM100D, Thorlabs). To effectively illuminate the medial part of lobule IXb where electrical stimulation evokes the strongest cardiovascular responses in anesthetized rabbits (Bradley et al., 1987), a tungsten microelectrode was positioned at lobule IXb (coordinates from the bregma: AP, 15.2 mm; ML, 0.0 mm; DV, 6.0 mm) and used to record PC activity. The position of light illumination was then adjusted so as to maximize the effect of light illumination on that cell.

To investigate the effects of eNpHR-mediated photoinhibition on BP during postural alterations, orange light was illuminated for 10 or 20 s continuously in each trial (a trial was defined as 1-min period centered on the time of light onset). Thirty-degree head-up (0 → 30° head-up) and head-down (0 → -30° head-down) tilts were performed by manually rotating a tilt table (a generous gift from Dr. Nisimaru) (Nisimaru et al., 1998) on which the stereotaxic frame was fixed. Each tilt was initiated at 1 or 10 s after the onset of illumination. A postural change experimental session consisted of the following successive manipulations: 1) head-up tilt with orange light → 2) head-down tilt with light → 3) head-up tilt without light → 4) head-down tilt without light. Each manipulation was separated from the others by at least 2 min, and at least 20

sessions were performed for each rat. All rats were perfused with saline followed by 4% paraformaldehyde in phosphate buffer at the end of the experiment, and their EYFP fluorescence within lobule IXab was confirmed by stereoscopic fluorescence microscope analysis.

Mean arterial pressure (MAP) was calculated as diastolic pressure plus one third of the pulse pressure for each beat. Baseline-normalized MAP was calculated in each trial by normalizing to average MAP during 0–5 s before light onset.  $\Delta$ MAP in head-up and -down conditions was defined as differences in baseline-normalized MAP between light-ON and light-OFF conditions.  $\Delta$ MAP in resting condition was calculated by subtracting baseline value (=1) from baseline-normalized MAP.

To estimate the effects of photoinhibition on the recovery from tilt-induced changes in BP,  $\Delta$ MAP was averaged using a 3-s time window (10–13 s after tilt-onset for the head-up condition, 5–8 s after tilt-onset for the head-down condition). These time windows were set at steady-state phases immediately after transient tilt-induced BP changes. Averaged  $\Delta$ MAPs were then further normalized to the maximum BP change during tilts. Maximum BP changes were calculated using data from the light-OFF condition for each rat; the maximum BP change was regarded as the difference between the baseline BP and the earliest minimum (for the head-up data) or maximum (for the head-down data) value of the tilt-induced BP change.

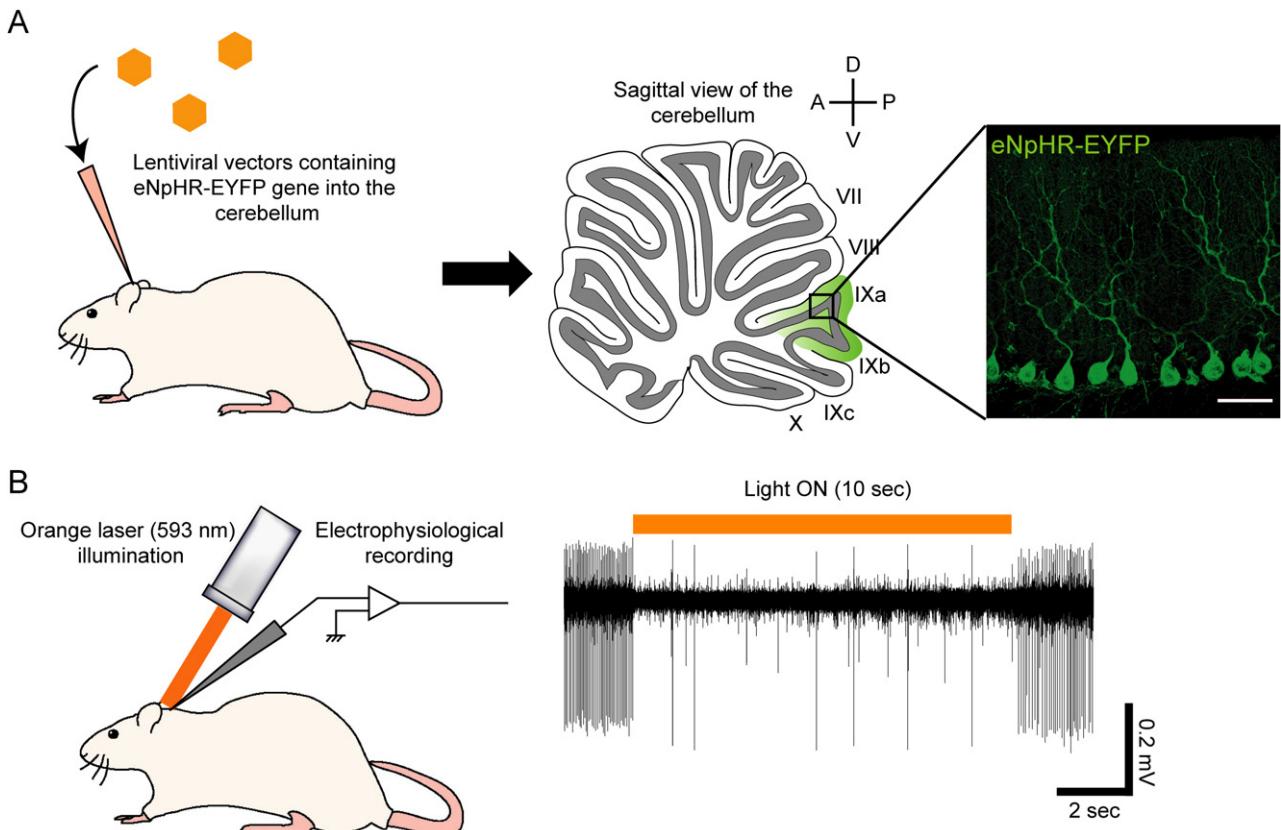
All statistical tests were performed using MATLAB and the R software. Error bars in all figures indicate the SEM, unless otherwise specified.

## RESULTS

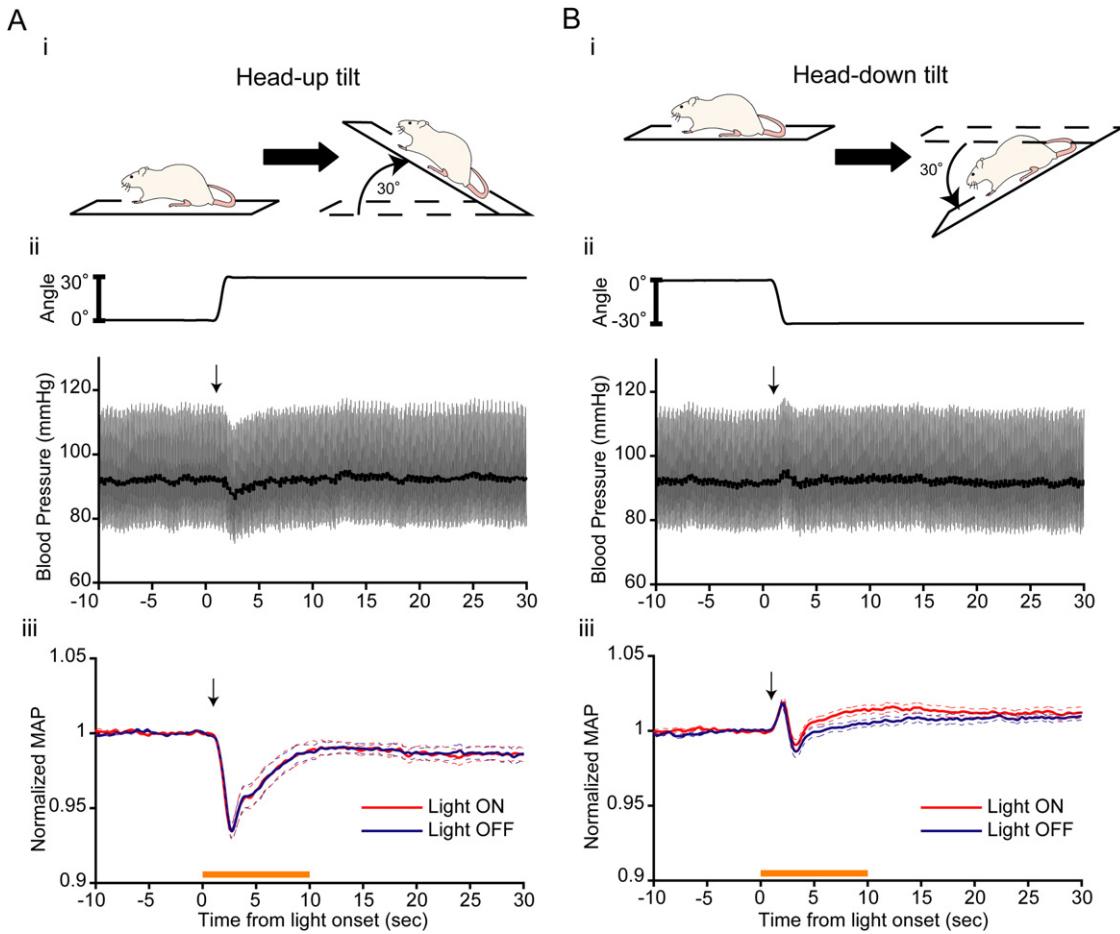
### Photoinhibition of uvular PCs attenuates BP recovery during head-down tilts

In this study, the optogenetic technique that we have developed recently for inhibiting PC activity (Tsubota et al., 2011) was used to examine the roles of the uvula in BP regulation. In the previous study, we reported a lentiviral vector coding eNpHR-EYFP under the control of the sL7 promoter (sL7-eNpHR), and showed that the viral vector was broadly infected to the cerebellar lobule IXab, where most of the PCs (95.3%) were selectively labeled by eNpHR-EYFP (Fig. 1A). We also showed that the simple spike activity of a PC in virus-infected lobule IXab was strongly inhibited by orange light illumination (Fig. 1B). Among eNpHR-expressing PCs, 93.8% decreased their simple spike frequency in response to light illumination, indicating that population PC activity in the sL7-eNpHR-injected uvula can be strongly inhibited by orange light (Tsubota et al., 2011).

Using these techniques, we examined how eNpHR-mediated photoinhibition of the uvula affected BP regulation in anesthetized rats during postural alterations. The effects of photoinhibition were investigated during 30°



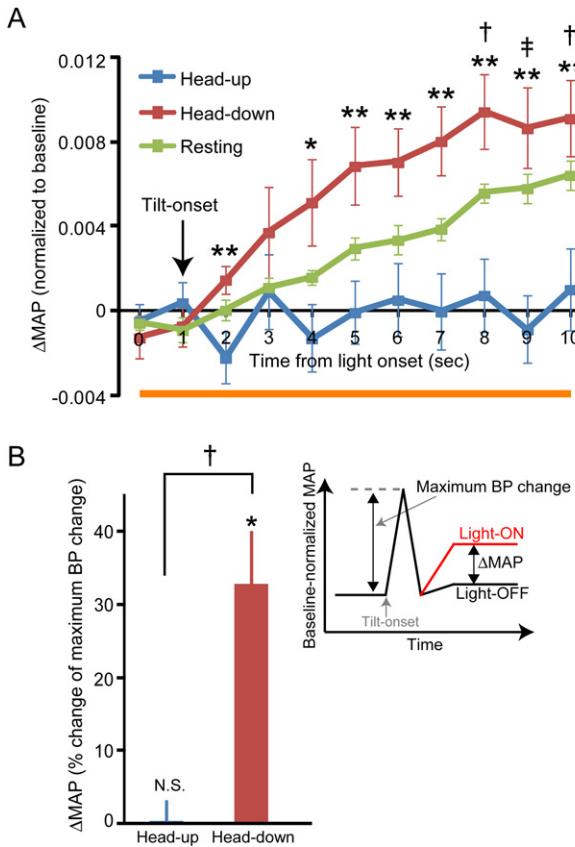
**Fig. 1.** Schematic drawings of lentiviral vector-based method for optogenetic inhibition of PCs. (A) The lentiviral vector containing the eNpHR-EYFP gene under the control of the shortened version of the PC-specific L7 promoter was injected into lobule IXab of the rat cerebellum, and PCs within lobule IXab selectively expressed eNpHR-EYFP, as shown in the confocal image of a cerebellar sagittal section (25  $\mu$ m slice). Scale bar, 50  $\mu$ m. (B) Simultaneous orange light illumination and electrophysiological recordings shows simple spike activity in an eNpHR-expressing PC was strongly inhibited in response to orange light illumination. (B) was modified from Tsubota et al. (2011) PLoS One 6: e22400, doi: 10.1371/journal.pone.0022400.



**Fig. 2.** The effects of 10-s photoinhibition of the uvula on BP during postural alterations in sL7-eNpHR rats. (A<sub>i</sub>, B<sub>i</sub>) Schematic diagrams of posture change experiments. (A<sub>ii</sub>, B<sub>ii</sub>) Sample traces of tilt angle (upper traces), raw arterial blood pressure (lower traces, gray), and MAP (lower traces, black) of a head-up tilt trial (A<sub>ii</sub>) and a head-down tilt trial (B<sub>ii</sub>). Arrows indicate the initiations of tilts. (A<sub>iii</sub>, B<sub>iii</sub>) Population average time courses of baseline-normalized MAP in head-up (A<sub>iii</sub>) and head-down tilt trials (B<sub>iii</sub>) with or without orange light illumination. Before averaging, MAP data of each trial was low-pass filtered at 0.5 Hz. Blue lines are of light-OFF conditions, and red lines are of light-ON conditions. Thin traces are mean±SEM. The periods of light illumination are indicated by horizontal orange bars.  $n=10$  rats.

head-up tilts as well as 30° head-down tilts (Fig. 2A<sub>i</sub>, B<sub>i</sub>). BP decreased or increased transiently after a head-up tilt or head-down tilt, respectively, but then recovered to the baseline level (Fig. 2A<sub>ii</sub>, B<sub>ii</sub>). These results are consistent with previous observations in anesthetized cats and rabbits (Doba and Reis, 1972; Nisimaru, 2004). We next compared the baseline-normalized MAP between two conditions, light-ON and light-OFF, in both head-up and head-down tilt trials. In the light-ON condition, illumination with orange light was initiated 1 s before the tilt-onsets and persisted for 10 s. Because the latency of eNpHR-mediated photoinhibition is millisecond-timescale (Zhang et al., 2007), orange light illumination strongly inhibits PC activity already at 1 s after onsets of illumination (Tsubota et al., 2011). In the light-OFF condition, no light was employed. Four different trials (light-ON/head-up, light-OFF/head-up, light-ON/head-down, and light-OFF/head-down) were performed within the same experimental session, and at least 20 sessions were performed for each rat (Fig. 2A<sub>iii</sub>, B<sub>iii</sub>). During head-down tilts, photoinhibition of the uvula resulted in a transient increase in BP in the light-ON condi-

tion compared with the light-OFF condition (Fig. 2B<sub>iii</sub>). By contrast, no effect of photoinhibition was observed during head-up tilts (Fig. 2A<sub>iii</sub>). To analyze the statistical significance of the eNpHR-mediated photoinhibition effects on BP, we compared differences in the baseline-normalized MAP ( $\Delta$ MAP) between light-ON and light-OFF conditions during the 10-s period corresponding to light illumination (Fig. 3). Because photoinhibition of the uvula also evokes pressor responses during the resting condition (Tsubota et al., 2011), we simultaneously compared  $\Delta$ MAP measured during the head-up, head-down, and resting conditions. Two-way repeated measures ANOVA [factor 1, tilt condition (head-up, head-down, or resting); factor 2, time] for  $\Delta$ MAP showed a significant interaction between tilt condition and time ( $F_{20,180}=4.11$ ,  $P<1\times 10^{-6}$ , Fig. 3A). Post hoc comparisons at each time point showed significant differences for  $\Delta$ MAP between head-up and head-down conditions ( $P<0.05$  at 4 s,  $P<0.005$  at 2 and 5–10 s, one-way ANOVA followed by Fisher's least significant difference (LSD) test, Fig. 3A). Furthermore, the effect of photoinhibition during head-up tilts was also significantly



**Fig. 3.** 10-s photoinhibition attenuates BP recovery during head-down tilts. (A) MAP differences between light-ON and -OFF conditions ( $\Delta\text{MAP}$ ) in the head-up (blue), head-down (red), and resting conditions (green), respectively. An arrow indicates the tilt-onset in the head-up and head-down conditions. A significant interaction ( $F_{20,180}=4.11$ ,  $P<1\times10^{-6}$ ) was revealed by two-way ANOVA with repeated measures on both factors [tilt condition (head-up, head-down, resting)  $\times$  time]. \*  $P<0.05$ , \*\*  $P<0.005$ , difference in  $\Delta\text{MAP}$  between the head-up and head-down conditions; †  $P<0.05$ , ‡  $P<0.005$ , difference in  $\Delta\text{MAP}$  between the head-up and resting conditions; one-way ANOVA followed by Fisher's LSD test. Width of time window used for calculating  $\Delta\text{MAP}$  at each time point is 1 s. A horizontal orange bar indicates the period of light illumination.  $n=10$  rats. (B) The effects of photoinhibition on the recovery from tilt-induced BP change.  $\Delta\text{MAP}$  was first averaged using 3-s time window (10–13 s after tilt-onset for head-up condition, 5–8 s after tilt-onset for head-down condition), and then further normalized to the maximum BP change during tilts. †  $P<0.005$ , paired  $t$  test. \*  $P<0.005$ ,  $t$  test (the null hypothesis stated that the mean was equal to 0). N.S., not significant. Inset, schematic diagram of estimation of the maximum BP change and  $\Delta\text{MAP}$  in the head-down condition.

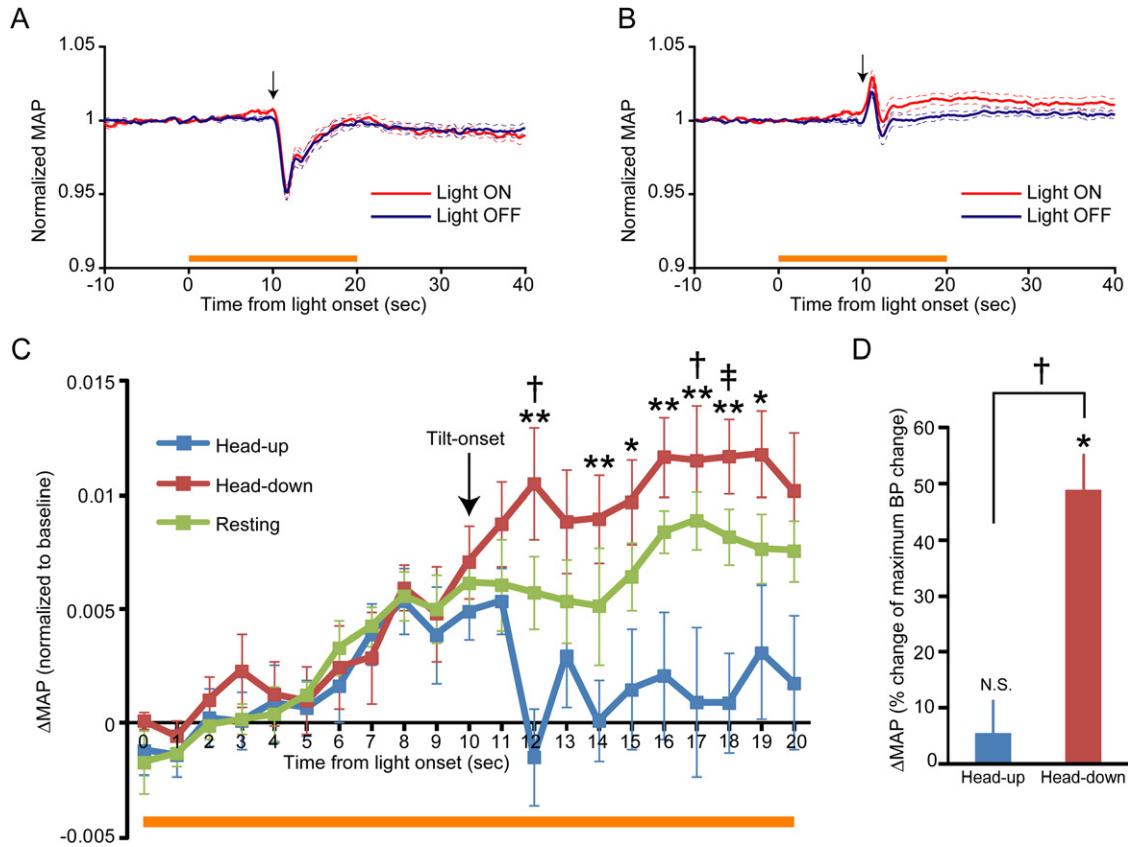
different from that during the resting condition ( $P<0.05$  at 8 and 10 s,  $P<0.005$  at 9 s, Fisher's LSD test, Fig. 3A). These results demonstrate the time-dependence of the effect of photoinhibition on BP between different tilt conditions. To investigate the functional role of this dynamic regulation of BP by the uvula, we next evaluated whether photoinhibition differentially affected the ability of rats to recover from tilt-induced BP changes in head-up vs. head-down conditions (see Experimental procedures for calculations).  $\Delta\text{MAP}$  values were normalized to reflect the ratio of  $\Delta\text{MAP}$  to the maximum BP change during tilts. This

analysis revealed a significant difference in BP recovery between head-up and head-down conditions ( $P<0.005$ , paired  $t$  test, Fig. 3B). Furthermore, the BP was unable to recover to the baseline level during head-down tilts ( $P<0.005$ ,  $t$  test) although a full recovery was observed during head-up tilts ( $P>0.8$ ,  $t$  test, Fig. 3B). These results suggest that PCs in the uvula dynamically regulate BP maintenance during head-down tilts.

#### Effects of 20-s photoinhibition on recovery from BP changes induced by postural alterations

Because the effects of photoinhibition on BP were most prominent toward the end of the 10-s period of orange light illumination (Fig. 3A), it is possible that the impact on BP immediately after tilt-onset was not fully evaluated with our initial photoinhibition protocol. We therefore performed an experiment in which the period of photoinhibition was prolonged so that its effect on BP was already prominent at tilt-onset. Uvular PCs were illuminated with orange light for a period of 10 s before tilt-onset for a total of 20 s in this experimental protocol. Comparison of baseline-normalized MAP between the light-ON and -OFF conditions confirmed that BP was actually increased already at the tilt-onsets in the light-ON condition (Fig. 4A, B). Consistent with the data of the 10-s illumination protocol, recovery from tilt-induced BP change was impaired by photoinhibition during head-down tilts (Fig. 4B), but not during head-up tilts (Fig. 4A). However, comparison of  $\Delta\text{MAP}$  between three tilt conditions (head-up, head-down, and resting) shows that the effects of 20-s photoinhibition immediately after the tilt-onset were more pronounced compared with those in the 10-s protocol (particularly demonstrated in a sudden dip of  $\Delta\text{MAP}$  after the tilt-onset observed in the head-up condition, Fig. 4C). Two-way repeated measures ANOVA [factor 1, tilt condition (head-up or head-down or resting); factor 2, time] for  $\Delta\text{MAP}$  showed a significant interaction between tilt condition and time ( $F_{40,200}=3.18$ ,  $P<1\times10^{-7}$ , Fig. 4C). This result was consistent with the results from the previous 10-s protocol. Post hoc comparisons at each time point showed significant differences in  $\Delta\text{MAP}$  between head-up and head-down conditions ( $P<0.05$  at 15 and 19 s;  $P<0.005$  at 12, 14, and 16–18 s; one-way ANOVA followed by Fisher's LSD test, Fig. 4C). Significant differences were also found between the head-up and resting conditions ( $P<0.05$  at 12 and 17 s;  $P<0.005$  at 18 s, Fig. 4C). These data suggest that the effects of photoinhibition were mostly consistent between the 10- and 20-s protocols. However, the dynamics of the photoinhibition effects in 20-s photoinhibition protocol, in which the variance between the head-up and the resting condition was already detected at 2 s after tilt-onset, were slightly different compared with those in the 10-s photoinhibition protocol.

Finally, we examined the effects of photoinhibition on BP recovery during tilts in the 20-s protocol. The extent of BP recovery was significantly different between the head-up and head-down conditions ( $P<0.005$ , paired  $t$  test; Fig. 4D). Moreover,  $\Delta\text{MAP}$  was significantly higher than zero in the head-down condition ( $P<1\times10^{-4}$ ,  $t$  test), but not in the head-up condition ( $P>0.27$ , Fig. 4D). These



**Fig. 4.** The effects of 20-s photoinhibition of the uvula on BP during postural alterations. (A, B) Population average time courses of baseline-normalized MAP in the head-up (A) and head-down (B) conditions with (red) or without (blue) orange light illumination. Before averaging, MAP data of each trial was low-pass filtered at 0.5 Hz. Thin traces are mean  $\pm$  SEM. Arrows indicate the initiations of tilts. The periods of 20-s orange light illumination are indicated by horizontal orange bars.  $n=6$  rats. (C) Same as Fig. 3A, but of the 20-s photoinhibition protocol. A significant interaction ( $F_{40,200}=3.18$ ,  $P<1\times 10^{-7}$ ) was revealed by two-way ANOVA with repeated measures on both factors [tilt condition (head-up, head-down, resting)  $\times$  time]. \*  $P<0.05$ , \*\*  $P<0.005$ , difference of  $\Delta\text{MAP}$  between head-up and head-down conditions; †  $P<0.05$ , ‡  $P<0.005$ , difference of  $\Delta\text{MAP}$  between head-up and resting conditions; one-way ANOVA followed by Fisher's LSD test. (D) Same as Fig. 3B, but of the 20-s photoinhibition protocol. †  $P<0.005$ , paired  $t$  test. \*  $P<0.005$ ,  $t$  test (the null hypothesis stated that the mean was equal to 0).

results further support the hypothesis that PCs in the uvula regulate BP maintenance during head-down tilts.

## DISCUSSION

In the present study, we targeted eNpHR selectively to PCs within the uvula using a lentiviral vector containing the PC-specific L7 promoter. PC activity within the uvula was strongly inhibited as a population by orange light. Using these techniques, we showed that photoinhibition of the uvula attenuated BP recovery during head-down tilts.

Because the uvula receives vestibular information during postural alterations (Yakhnitsa and Barmack, 2006), it has been hypothesized that the uvula plays a modulatory role in the cardiovascular system during postural alterations. Indeed, in a previous study of cats, it was shown that the chronic lesioning of the uvula significantly alters cardiovascular regulation during postural alterations (Holmes et al., 2002). However, there are several drawbacks to cerebellectomy: 1) it lacks cell-type specificity, and fibers projecting to the cerebellar cortex are also injured, 2) it is not possible to reinstate the damaged region to obtain control measures, and 3) there are often neural compen-

sations that mask the original lesion-induced deficits (Lomber, 1999). In contrast, because eNpHR-mediated photoinhibition is rapidly reversible and can specifically control the activity of PCs, it can overcome these shortcomings of lesion experiments. The findings of the present study thus clearly show that PCs in the uvula regulate BP during postural alterations.

Since the effects of electrical stimulation of the uvula in rats are much smaller than those in cats and rabbits (Tsubota et al., 2011), it is reasonable to anticipate that the absolute value of the magnitude of the impact of photoinhibition on BP would be comparatively small in rats (Figs. 3A and 4C). However, when  $\Delta\text{MAP}$  was normalized to the maximal BP change during tilts (Figs. 3B and 4D), the extent of BP recovery during head-down tilts was reduced by 33% following photoinhibition for the 10-s photoinhibition condition (Fig. 3B) and by 49% for the 20-s photoinhibition condition (Fig. 4D). These results suggest that photoinhibition had a substantial impact on the BP maintenance during tilts.

Such BP regulation might be particularly important in quadrupeds such as rodents, because dorsiflexion and

ventroflexion movements of the head are very common behavior during locomotion, particularly during rearing and sniffing (Crusio et al., 1989). Rapid adjustments in circulation should take place during these movements in order to maintain stable cardiac output and brain perfusion. In the present study, the significant effects of uvula photoinhibition were observed as early as 2 s after posture changes (Figs. 3A and 4C). This suggests that the uvula plays an important role in rapid regulation of BP during locomotion behavior.

In the cardiovascular regulatory region located in the cerebellar nodulus-uvula transition zone, it has been shown that uvular stimulation affects BP by depressing  $\alpha$ -adrenergic outflow (Henry et al., 1989). In the cardiovascular region focused in the present study, it has been suggested that sympathetic nervous system (and therefore the adrenergic system) also plays an important role in regulation of BP (Bradley et al., 1991). Therefore, pressor responses that are induced by photoinhibition during the resting condition might potentially be explained by simple disinhibition of a tonic cerebellar effect. However, effects of photoinhibition during tilts cannot be explained by mere disinhibition of a tonic cerebellar effect, because the photoinhibition effects on BP were dynamically different between the three conditions employed in the current study (head-up, head-down, and resting, Figs. 3A and 4C). Furthermore, BP was not elevated by photoinhibition during the head-up tilt condition (Figs. 3A and 4C).

The dynamics of the BP response during head-up/down tilts is known to be divided into three phases: 1) the uncompensated phase corresponding to the initial elevation in BP, 2) the early compensated phase when BP is recovering, and 3) the steady-state adjustment phase when BP is stabilized in spite of the maintained tilt (Doba and Reis, 1972, 1974). In both 10- and 20-s photoinhibition experiments, the effects of photoinhibition were decidedly prominent during the early compensated and the steady-state adjustment phases (Figs. 3A and 4C). However, the effects of photoinhibition during the early compensated phase were even more pronounced in the 20-s compared with the 10-s experiments. This was particularly well-illustrated by the sudden drop in  $\Delta$ MAP after tilt-onset in the head-up condition (Fig. 4C). Because the baroreceptor reflex plays a fundamental role in BP recovery during both the early compensated phase and the steady-state adjustment phase (Koyama et al., 1981), one possible explanation for our observations in the 20-s experiments is that the early initiation of photoinhibition may in turn have caused an adaptation in the neurons related to the central baroreflex system before the tilt-onset, which accordingly limited the ability of the BP to recover during the early compensated phase.

In conclusion, together with the fact that PCs in the uvula are indirectly connected to the NTS and other brainstem nuclei that participate in the baroreceptor reflex (Bradley et al., 1991), it's tempting to suggest that PCs in the uvula dynamically act on the baroreceptor reflex and promote the recovery of BP to the baseline level in the steady-state adjustment phase.

**Acknowledgments**—The authors are indebted to Drs. Masao Ito (RIKEN Brain Science Institute, Saitama, Japan), Naoko Nisimaru (Oita University, Oita, Japan), and Yasuo Ohashi (Department of Public Health, The University of Tokyo School of Medicine, Tokyo, Japan) for helpful comments to the manuscript; Drs. Hideki Kashiwadani (Department of Physiology, The University of Tokyo School of Medicine), Masanori Matsuzaki (National Institute for Basic Biology, Aichi, Japan) for technical advice; Ms. Ayana Sato, Dr. Masae Yaguchi, and Ms. Ayumi Fukuda (Department of Physiology, The University of Tokyo School of Medicine) for technical assistance. The tilt table was kindly provided by Dr. Nisimaru. The St. Jude lentiviral vector system was kindly provided by St. Jude Children's Research Hospital (Dr. Arthur W. Nienhuis) and George Washington University. This work was supported by a Grant-in-Aid for specially Promoted Research from Ministry for Education, Culture, Sports, Science and Technology (MEXT) to Y.M. (19002010), Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms) from MEXT to Y.M., a grant from Takeda Science Foundation to Y.M., Grant-in-Aid for Young Scientists from MEXT to Y.O. (23700489), and Japan Society for the Promotion of Science (JSPS) Research Fellowships for Young Scientists to T.T. (235569), Y.O. (195225), and K.T. (211438).

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(Accepted 7 March 2012)  
(Available online 14 March 2012)