List of Supplemental Digital Content

Supplemental Digital Content 1. Text documents that show methods for anatomical observation of blood vessels in the dorsomedial medulla oblongata, measuring cardiovascular parameters, and Quantitative RT-PCR. doc

Supplemental Digital Content 1

Methods

Anatomical observation of blood vessels in the dorsomedial medulla oblongata

To identify whether specific branches of the dorsal medullary veins (DMV) of the caudal medulla oblongata is one of the drainage systems from the dorsomedial region including nucleus tractus solitarii (NTS), we visualized blood vessels on the dorsal surface of the caudal medulla oblongata using a zoom stereo microscope (LOM, Konan Medical Inc, Japan) and magnified images were photographed using a scanning USB camera microscope (UM-02-01, MicroLinks Technology Co, Taiwan). Blood vessel structure within the NTS and the gracile nucleus were also identified immunohistochemically as follows. Animals ($n = 6$) were transcardially-perfused with 4% paraformaldehyde. The brain stem was removed, post-fixed with 4% paraformaldehyde for at least 24 h, and transferred to PBS containing 30% sucrose. Serial sections (40 μm) through the NTS were obtained using a freezing microtome (REM-710, Yamato kohki industrial Co, Japan). The sections were rinsed in PBS, placed in 10% serum with 0.3% Triton X-100 for 15 min at room temperature, rinsed again and then incubated with an endothelial cell marker (RecA1, Abcam plc, UK; dilution 1:200 in PBS with 1% serum and 0.3% Triton X-100). After overnight incubation at 4°C, the sections were rinsed in PBS and incubated with biotinylated anti-mouse IgG (Vector Laboratories, UK; dilution 1:500,) for 1 h. The sections were rinsed and then incubated in streptavidin conjugated Alexa-Fluor 594 (Molecular Probes, USA; dilution 1:500) for 1 h. Finally, sections were washed in PBS before mounting in Vectashield (Vector Laboratories). Sections were photographed using a scanning laser confocal microscope (LSM 5 Pascal, Carl Zeiss, Germany). See Figure S1 for the result.

Baroreflex testing

Baroreflex function was evaluated by measuring the bradycardia in response to phenylephrine injection (10–20 μg/kg, i.v.; Sigma-Aldrich, Steinheim, Germany) using a syringe pump (CFV-3200, Nihon Kohden); this raised mean blood pressure (MAP) between 40 to 60 mmHg. By measuring the peak changes in MAP (ΔMAP) and the corresponding peak reflex changes in HR (ΔHR), the ΔHR $-\Delta$MBP ratios (ΔHR/ΔMAP) were averaged and used as an index of baroreceptor cardiac reflex gain.

Baroreceptor reflex denervation

To eliminate a potential compensatory mechanism in regulating arterial pressure by the baroreflex, sino-aortic denervation (SAD) according to the Krieger method (1) was performed in 20 animals as we described previously (2). Complete baroreceptor denervation was confirmed by measuring the bradycardia in response to phenylephrine injection (10–20 μg/kg, i.v.; Sigma-Aldrich, Steinheim, Germany) using a syringe pump (CFV-3200, Nihon Kohden) before and after CCPV occlusions; this raised mean blood pressure (MAP) between 40 to 60 mmHg. None of the sino-aortic denervated rats showed a baroreceptor reflex mediated bradycardia confirming complete baroreceptor denervation. In addition, a sham operation in which a neck incision was made to expose (but not to section) carotid sinus and aortic depressor nerves was performed in 16 control rats (i.e. nerve intact rats).

Quantitative RT-PCR to verify hypoxic condition in the NTS

The brain was rapidly removed from the cranium and the NTS between 1.0 mm rostral and 0.5 mm caudal to the calamus scriptorius from each animal was dissected out of the brainstem. The rostral ventrolateral medulla was also collected. The brain tissues were immediately frozen in dry ice and stored at −80°C. Total RNAs from each brain samples were isolated using TRIZOL reagent according to the protocol supplied by the manufacturer. The RNA from the NTS was treated with RNase-free DNase I (Invitrogen Life technologies). Quantitative RT-PCR was performed using designed primer sets for ß-actin, HO-1, HO-2 and HIF-1α, the SYBR® PrimeScript® RT-PCR Kit, and a Thermal Cycler Dice® Real Time System (all from Takara Bio Inc., Shiga, Japan), according to the manufacturer's recommendation. Expression of target genes was assessed in relation to the Ct value of the ß-actin using the

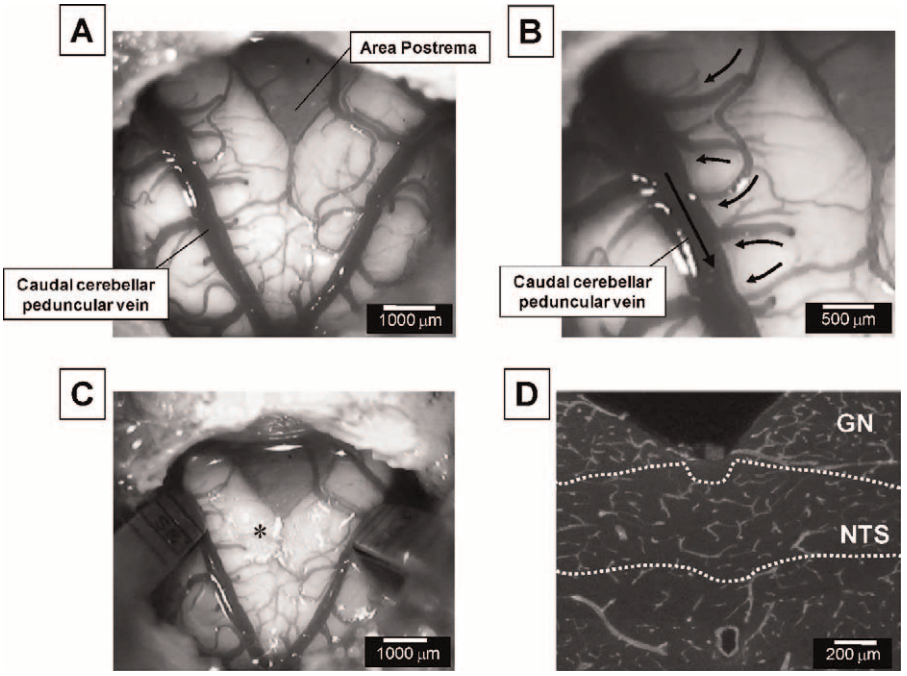
comparative ($2^{-\Delta\Delta Ct}$) method (3) and fold differences of gene expression levels between 2 groups of rats were calculated (4).

References

1. Krieger EM. Neurogenic hypertension in the rat, Circ Res. 1964; 15: 511–521.
2. Waki H, Kasparov S, Katahira K, Shimizu T, Murphy D, Paton JF. Dynamic exercise attenuates spontaneous baroreceptor reflex sensitivity in conscious rats. Exp Physiol. 2003; 88: 517-526.
3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25: 402-408.
4. Waki H, Liu B, Miyake M, Katahira K, Murphy D, Kasparov S, et al. Junctional adhesion molecule-1 is upregulated in spontaneously hypertensive rats: evidence for a prohypertensive role within the brain stem. Hypertension. 2007; 49: 1321-1327.

Supplemental Digital Content 2. Figure that demonstrates anatomical observation of blood vessels located on/within the dorsal part of the caudal medulla oblongata. doc

Supplemental Digital Content 2



Blood vessels located on/within the dorsal part of the caudal medulla oblongata.

(A) The dorsal medullary veins (DMV). The main branches of the rostral posteromedian medullary vein are also called the caudal cerebellar peduncular veins (CCPV) (1). (B) A magnified photograph showing a left CCPV. It has small branches penetrating the dorsal surface of the medulla oblongata. The directions of blood flow (indicated by arrows) were confirmed by visualization of red blood cell movement using a zoom stereo microscope and it has been identified that the CCPV drains the dorsal surface of the caudal medulla oblongata. (C) Both sides of CCPV were occluded by micro clamps and small branches of the CCPV were cut by fine forceps/dissecting scissors (see text for more details) to induce complete hemostasis. * indicates approximate position of the probe for tissue blood flow recording (D) A transverse section of the NTS at the level of the calamus scriptorius. Capillaries stained by an endothelial cell marker (RecA1, Abcam plc, UK) were shown. In consistent with a previous report (2), the NTS and the gracile nucleus (GN) were identified to be heavily vascularized compared to the other parts of brainstem, suggesting that the NTS exhibits a high level of oxygen demand. These observations suggest that the CCPV is likely to be one of the major drainage vessels from the NTS. In fact, following vessel occlusion BF in the medulla oblongata in which the NTS is located showed over 60% decrease (See text). This may result from blood congestion due to increased resistance of drainage

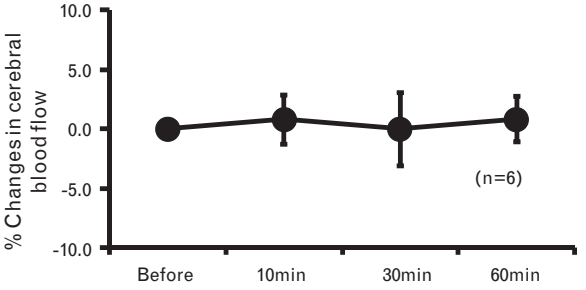
venules resulting in stagnant hypoxia. Moreover, immunohistochemical and mRNA evidence demonstrated that bilateral occlusions of the vessels induced highly localized hypoxia in the caudal part of the NTS (See text). All told, the CCPV of the caudal medulla is an important drainage system which is required for homeostatic function of the NTS.

References

1. Schünke M, Schulte E, Schumacher U. *Kopf und Neuroanatomie Prometheus LernAtlas der Anatomie*. Stuttgart, Germany: Thieme; 2006.
2. Gross PM. Morphology and physiology of capillary systems in subregions of the subfornical organ and area postrema. Can J Physiol Pharmacol. 1991; 69: 1010–1025.

Supplemental Digital Content 3. Figure that demonstrates effects of CCPV occlusion on regional blood flow in the cerebral cortex. doc

Supplemental Digital Content 3

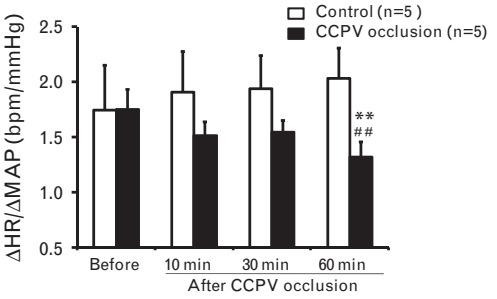


Effects of CCPV occlusion on regional cerebral blood flow.

Regional blood flow in the cerebral cortex was measured in baroreceptor afferent nerve intact animals but it did not change after CCPV occlusion, indicating that CCPV occlusion produced a relatively localized effect.

Supplemental Digital Content 4. Figure that demonstrates effects of CCPV occlusion on baroreceptor reflex function. doc

Supplemental Digital Content 4



Effects of CCPV occlusion on baroreceptor reflex function.

The baroreceptor bradycardic reflex gain was measured in nerve intact rats ($n = 5$). This value was not changed 10 min and 30 min after CCPV. The reduction became significant only 60 min after CCPV occlusion compared to the baseline level and the value in vessel intact animals. These results suggest that the pressor response induced by CCPV occlusions was not due to altered reflex gain, but may be due to a shift of the set point of arterial pressure.

**$p < 0.01$, compared to control rats.

##$p < 0.01$, compared to the baseline level.