observations, it is possible that Na^+-rich aCSF in the RVLM increases sympathetic nerve activity and MAP.

The pressor effect was greater in SHRSPs than in WKY rats, and this difference between the strains might have been related to the different neural responsiveness to Na^+-rich aCSF via ENaCs in the RVLM. This pressor effect was partially blocked by the MR blocker eplerenone in SHRSPs, indicating that the effect of Na^+ might be mediated by MR activation. ICV infusion of the MR blockers, spironolactone[46] or benzamil,[47] prevented Na^+-induced sympathoexcitatory and pressor responses in WKY rats. It has also been reported that ICV infusion of eplerenone attenuated ENaC expression in mice with pressure overload.[30] Taken together, these findings suggest that MRs mediate Na^+ via ENaCs or transporters on the cell surface of neurons in the RVLM.

Study limitations

MRs are largely occupied by the glucocorticoid corticosterone,[48] which is present in a higher concentration than aldosterone in the brain.[41] The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which is distributed the brainstem, including the nucleus tractus solitarius,[49] rapidly converts corticosterone to an inactive metabolite. Thus, the coexpression of 11β-HSD2 with MRs may identify brain regions that are particularly sensitive to aldosterone. Although the precise expression of 11β-HSD2 in the RVLM has not yet been determined, we found that aldosterone in the RVLM increased blood pressure, and this pressor response was prevented by the MR blocker eplerenone. Therefore, our findings suggest that aldosterone acts on the MRs in the RVLM. We still cannot exclude the possibility that corticosterone, instead of aldosterone, may act on the MRs in the RVLM. Together with the origin of aldosterone in the RVLM as well as the central nervous system, the study regarding ligand-specifying mechanisms has just begun. In addition, we did not determine whether ENaC activity is involved in the neural responsiveness to Na^+-rich aCSF in the RVLM, because we did not measure ENaC activation in the RVLM. However, it is possible that MRs and ENaCs in the RVLM may be involved in this mechanism.

In conclusion, these findings indicate that MRs in the RVLM contribute to the neural mechanisms of hypertension via sympathetic nerve activity, and that increased activity of MRs may be involved in the elevation of blood pressure in SHRSPs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Oxidative stress in the rostral ventrolateral medulla modulates excitatory and inhibitory inputs in spontaneously hypertensive rats

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Objectives **The rostral ventrolateral medulla (RVLM) of the brainstem and the paraventricular nucleus (PVN) of the hypothalamus play crucial roles in central cardiovascular regulation. In hypertensive rats, an imbalance of excitatory and inhibitory inputs to the RVLM enhances central sympathetic outflow. Increased reactive oxygen species (ROS) in the RVLM also contribute to sympathoexcitation, leading to hypertension. The aim of the present study was to elucidate whether ROS in the RVLM modulate synaptic transmission via excitatory and inhibitory amino acids and influence the excitatory inputs to the RVLM from the PVN in spontaneously hypertensive rats (SHRs).**

Methods and results **We transfected adenovirus vectors encoding the *manganese superoxide dismutase* (*AdMnSOD*) gene to scavenge ROS in the RVLM both in Wistar-Kyoto rats and SHRs. The decreases in blood pressure and renal sympathetic nerve activity (RSNA) evoked by injecting kynurenic acid, a glutamate receptor blocker, into the RVLM were attenuated, and the increases in blood pressure and RSNA evoked by injecting bicuculline, a γ-amino butyric acid (GABA) receptor blocker, into the RVLM were enhanced in *AdMnSOD*-transfected SHRs compared with adenovirus vectors encoding the *β-galactosidase* (*AdLacZ*) gene-transfected SHRs. Furthermore, the increases in blood pressure and RSNA evoked by injecting bicuculline into the PVN were attenuated in *AdMnSOD*-transfected SHRs compared with *AdLacZ*-transfected SHRs.**

Conclusion **These findings suggest that ROS in the RVLM enhance glutamatergic excitatory inputs and attenuate GABAergic inhibitory inputs to the RVLM, thereby increasing sympathoexcitatory input to the RVLM from the PVN in SHRs. *J Hypertens* 30:97–106 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: brain, hypertension, hypothalamus, oxidative stress, rostral ventrolateral medulla, sympathetic nervous system

Abbreviations: ***AdLacZ***, adenovirus vectors encoding the β-galactosidase gene; ***AdMnSOD***, adenovirus vectors encoding the manganese superoxide dismutase gene; AT1, angiotensin type 1; GABA, γ-amino butyric acid; MAP, mean arterial pressure; PVN, paraventricular nucleus; ROS, reactive oxygen species; RSNA, renal sympathetic nerve activity; SHRs, spontaneously hypertensive rats; SHRSP, stroke-prone SHR; TBARS, thiobarbituric acid-reactive substances; uNE, urinary norepinephrine; WKY rats, Wistar-Kyoto rats

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Introduction

The central nervous system plays a key role in the regulation of cardiovascular function [1,2], and accumulating evidence indicates that activation of the sympathetic nervous system is involved in the pathogenesis of hypertension [2]. Among several important autonomic nuclei involved in cardiovascular function [3], the rostral ventrolateral medulla (RVLM) contains the presympathetic neurons that maintain the baseline sympathetic tone [4]. The tonic drive that the RVLM exerts on sympathetic activity appears to be increased in several models of hypertension [5–7]. Excitation of the RVLM by excitatory amino acid neurotransmitters [8] and by reduced inhibitory amino acid neurotransmitters [9,10] is involved in the increased central sympathetic outflow in experimental hypertension [11]. The RVLM receives excitatory inputs from the paraventricular nucleus (PVN) of the hypothalamus, another important nucleus for central cardiovascular regulation [12–14].

Recent studies indicate that increased oxidative stress in the RVLM contributes to the enhanced central sympathetic outflow in experimental animal models of hypertension [15,16], such as spontaneously hypertensive rats (SHRs) [17,18], stroke-prone SHRs (SHRSPs) [19], and two-kidney one-clip Goldblatt hypertensive rats [20,21]. Stimulation of angiotensin type 1 (AT1) receptors activates NAD(P)H oxidase, thereby producing superoxide anions [22–24]. In fact, AT1 receptor activation in the RVLM is involved in the enhanced central sympathetic outflow in SHRs [6] and SHRSPs [25]. Although AT1 receptor blockade in the RVLM reduces blood pressure (BP) in SHRs [6], it is clear that the RVLM also receives glutamatergic excitatory inputs

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because the blockade of glutamate receptors by injecting kynurenic acid into the RVLM also markedly reduces BP in SHRs, but not in normotensive Wistar-Kyoto (WKY) rats [8]. The antihypertensive effects of an AT1 receptor blocker and kynurenic acid injection into the RVLM are suggested to be independent of each other because the responses of these drugs are additive in SHRs [6]. No studies, however, have specifically addressed this issue. One of the major input pathways derives from PVN neurons [12–14,26]. Whether increased reactive oxygen species (ROS) production in the RVLM alters synaptic transmission in the RVLM, thereby increasing BP through central sympathetic outflow, however, is not clear. In addition, RVLM neurons also receive attenuated γ-amino butyric acid (GABA)-ergic inhibitory inputs in SHRs, probably due to the baroreflex-mediated caudal ventrolateral medulla neurons [1,9,10,27]. Therefore, the aim of the present study is to determine whether chronic reduction of oxidative stress in the RVLM modifies the glutamatergic excitatory inputs as well as the GABA-ergic inhibitory inputs. We performed experiments with adenovirus-mediated gene transfer of manganese superoxide dismutase (*AdMnSOD*) and injection of kynurenic acid or bicuculline into the RVLM. Bicuculline was also injected into the PVN to increase the excitatory inputs from the PVN to the RVLM neurons.

Methods

Animals and general procedures

Male WKY rats and SHRs (280–340 g; 14–18 week old) were obtained from SLC Japan (Hamamatsu, Japan). The study was reviewed and approved by the Committee of Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences and was conducted according to the guidelines for animal experiments of Kyushu University.

In-vivo gene transfer of manganese superoxide dismutase into the rostral ventrolateral medulla

Adenovirus vectors encoding the *MnSOD* (*AdMnSOD*) or *β-galactosidase* (*AdLacZ*) genes were transfected into the bilateral RVLM, as described previously [19,28,29]. The vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa [30,31]. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Immunohistochemical staining for manganese superoxide dismutase

To confirm the expression and localization of gene transfer into the RVLM, we performed immunohistochemical staining [29,32]. On day 7 after gene transfer, the rats were deeply anesthetized with an excessive dose of sodium pentobarbital, perfused with 4% paraformaldehyde in PBS, and then the brain was removed and stored in 10% paraformaldehyde for 48 h. After 48 h, coronal sections (50-μm thick) containing the RVLM were incubated with mouse anti-immunoglobulin G (IgG) (1 : 1000; Millipore Corporation, Billerica, Massachusetts, USA) at 4°C for 48 h followed by goat antimouse IgG (1 : 2000; Alexa Fluor 488, Molecular Probes, Invitrogen Corp., Carlsbad, California, USA).

Western blot analysis

To confirm the expression of MnSOD protein in the RVLM, we performed western blot analyses before and at day 7 after the gene transfer. The MnSOD protein was detected by a mouse IgG monoclonal antibody to MnSOD (1 : 2500; BD Biosciences, Franklin Lakes, New Jersey, USA), then with an antimouse IgG-horseradish peroxidase. We used signal from a rabbit IgG polyclonal antibody to β-tubulin as the loading control. The densitometric average was normalized to the values obtained from the analysis of β-tubulin protein. For details, see online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substance (TBARS) levels of the RVLM and PVN were measured as an indicator of oxidative stress, as previously described [19,32]. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Monitoring of blood pressure and heart rate

A UA-10 telemetry system (Data Sciences International, Saint Paul, Minnesota, USA) was used to measure mean arterial pressure (MAP) and heart rate (HR) in awake rats [19,28]. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Measurement of urinary norepinephrine excretion

Urinary norepinephrine (uNE) concentrations were measured using HPLC before and at day 7 after the gene transfer and uNE excretion was calculated as described previously [19]. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Experimental procedures

Previous studies demonstrated that BP and HR become stabilized at day 7 after the injection of adenovirus vectors [28,29]. Therefore, microinjection of additional agents into the RVLM or PVN was performed at day 7 after the viral injection. The rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally followed by 20 mg/kg per h intravenously), as described previously [19,28]. Following the insertion of a tracheal cannula for artificial ventilation, and a catheter (PE-50 tubing) into the femoral artery to record arterial BP and HR, renal sympathetic nerve activity (RSNA) was recorded. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Microinjections into the rostral ventrolateral medulla

To explore the mechanisms by which the ROS in the RVLM affect MAP and sympathetic nerve activity, we microinjected either kynurenic acid (2.7 nmol per 100 nl) or bicuculline (200 pmol per 100 nl) into the bilateral RVLM at day 7 after gene transfer with *AdMnSOD* or *AdLacZ*. The doses of these drugs were determined based on the results of a previous study [28]. Furthermore, to examine the effects of a ROS scavenger on responses to glutamatergic or GABAergic blockade, Mito-TEMPO (a mitochondrial ROS scavenger; ALEXIS Biochemicals Inc., San Diego, California, USA) (1 nmol per 100 nl) was microinjected into the RVLM bilaterally, thereafter either kynurenic acid (2.7 nmol per 100 nl) or bicuculline (200 pmol per 100 nl) was injected into the RVLM of SHRs [33]. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141). Each drug in the present experiment was dissolved in artificial cerebrospinal fluid.

Microinjections into the paraventricular nucleus

To explore the effect of ROS reduction in the RVLM on sympathetic outflow induced by PVN activation, we microinjected bicuculline (100 pmol per 100 nl) unilaterally into the PVN [26] at day 7 after gene transfer with *AdMnSOD* or *AdLacZ*. The tip of the micropipette was in a track located 1.4–2.2 mm posterior and 0.5 mm lateral to the bregma and at a depth of 7.5–8.0 mm below the dura.

Evaluation of the blood pressure-lowering effects of hydralazine on γ-amino butyric acid receptor blockade in the paraventricular nucleus of spontaneously hypertensive rats

AdMnSOD gene transfer into the RVLM in SHRs chronically decreased BP in awake rats. Therefore, to elucidate the BP-lowering effect on the GABAergic mechanisms within the PVN in SHRs, hydralazine (10 mg/kg per day) was administered by gastric gavage. After confirming the BP-lowering effect of hydralazine, we microinjected bicuculline (100 pmol per 100 nl) into the PVN in hydralazine-treated SHRs, as described above.

Histology

At the end of each experiment, we evaluated the injection sites by Evans blue dye (100 nl) staining. For details, see the online Supplemental Digital Content 1 (http://links.lww.com/HJH/A141).

Statistical analysis

All of the values are expressed as mean ± SEM. Intergroup differences in the TBARS values, the MAP and HR values obtained using the radiotelemetry system, and the 24-h uNE values were compared using two-way analysis of variance (ANOVA). In the ANOVA, comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. The MnSOD protein expression values (western blot analysis) and the changes in the MAP and RSNA values after microinjection of each component were compared using an unpaired *t*-test. Values of P less than 0.05 were considered significant.

Results

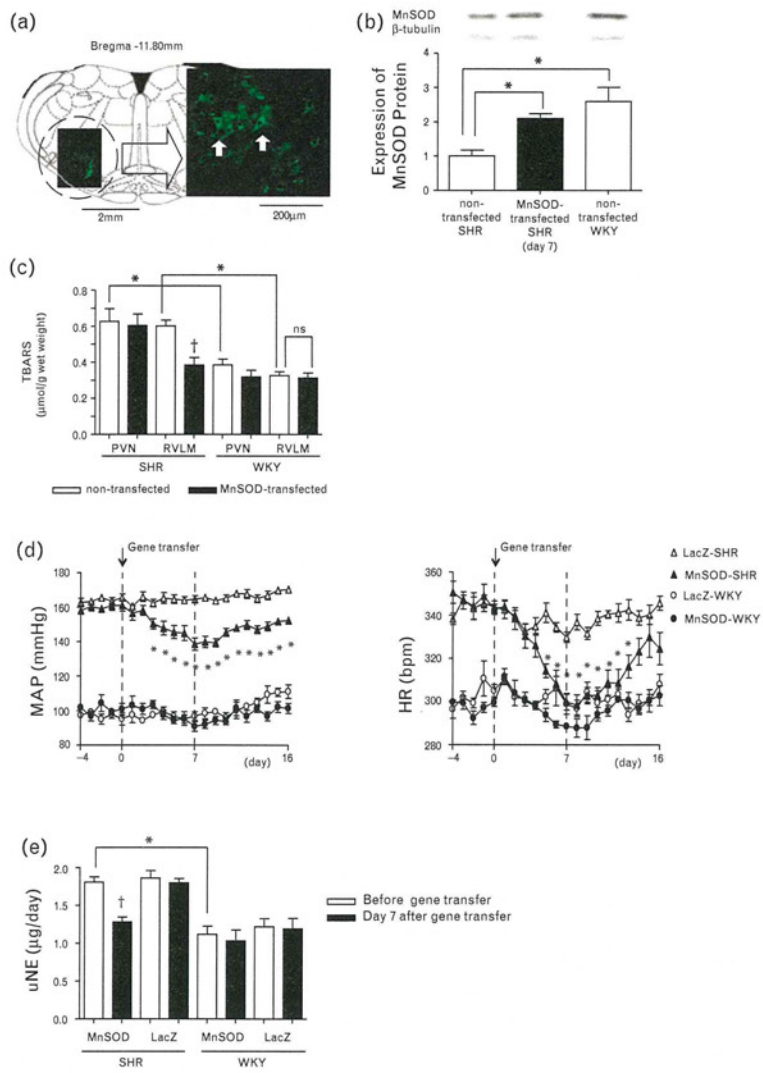
Overexpression of manganese superoxide dismutase in rostral ventrolateral medulla *in vivo*

Immunohistochemical staining for MnSOD at day 7 after the gene transfer revealed that MnSOD gene expression (Fig. 1a) localized in the RVLM, and not in the other sites. Western blot analysis revealed that MnSOD expression was significantly decreased in the RVLM tissue of nontransfected SHRs compared with that of WKY rats, but MnSOD expression in the RVLM tissue of *AdMnSOD*-transfected SHRs was significantly increased compared with that of nontransfected SHRs and at almost the same levels as that of WKY rats at day 7 after gene transfer (Fig. 1b). The TBARS levels were significantly higher in the PVN and RVLM of SHRs compared with WKY rats. MnSOD gene transfer into the RVLM suppressed TBARS levels only in the RVLM, and not in the PVN, of *AdMnSOD*-transfected SHRs and was at almost the same levels as that in WKY rats at day 7 after the gene transfer (Fig. 1c). At day 7 after the gene transfer, telemetry-monitored MAP and HR of *AdMnSOD*-transfected SHRs were significantly decreased compared with those in *AdLacZ*-transfected SHRs, but not those in WKY rats (Fig. 1d). The uNE excretion was significantly higher in SHRs compared with WKY rats. At day 7 after the gene transfer, uNE excretion was significantly decreased in *AdMnSOD*-transfected SHRs, but not in *AdLacZ*-transfected SHRs (Fig. 1e). The uNE excretion was not changed in either the *AdMnSOD*-transfected or *AdLacZ*-transfected WKY rats (Fig. 1e).

Effects of manganese superoxide dismutase overexpression on mean arterial pressure and heart rate

Baseline MAP and HR before microinjections of each drug into the RVLM or PVN are shown in Table 1. Overall, baseline MAP and HR were significantly lower in *AdMnSOD*-transfected SHRs than in *AdLacZ*-transfected SHRs in each experiment. Baseline MAP and HR did not differ between the *AdLacZ*-transfected SHRs and the nontreated SHRs. In WKY rats, baseline MAP and HR did not differ significantly between *AdMnSOD*-transfected WKY rats and *AdLacZ*-transfected WKY rats before microinjection of bicuculline into the PVN (Table 1). Because the MAP baseline values before microinjection differed between *AdMnSOD*-transfected and *AdLacZ*-transfected SHRs, the maximal changes in MAP are expressed as the percentage change from baseline.

Fig. 1



(a) Schematic drawing of a section that includes the rostral ventrolateral medulla (RVLM) (bregma −11.80 mm). The RVLM is encircled with a dashed line. Immunohistochemical staining for manganese superoxide dismutase (MnSOD) was detected by a confocal laser scanning microscope at day 7 after the gene transfer. MnSOD-immunopositive sites were observed locally in the bilateral RVLM of adenovirus vectors encoding the MnSOD (*AdMnSOD*)-transfected rats after staining with anti-MnSOD antibody (green, visualized with fluorescein isothiocyanate-conjugated fluoroprobe). Small vertical arrows indicate the RVLM neurons overexpressing MnSOD. (b) Western blot analysis demonstrating the expression of MnSOD protein in the RVLM tissue. MnSOD protein was significantly increased in the RVLM tissue of *AdMnSOD*-transfected spontaneously hypertensive rats (SHRs). The densitometric average was normalized to the values obtained from the analysis of β-tubulin. *Statistically significant difference at $P < 0.05$ between the values of nontransfected SHRs and day 7 after the gene transfer in SHRs ($n = 5$ for each) and between the values of nontransfected SHRs and nontransfected Wistar-Kyoto (WKY) rats ($n = 5$ for each). Data are shown as mean ± SEM. (c) Thiobarbituric acid-reactive substance (TBARS) levels in the RVLM and paraventricular nucleus (PVN) of WKY rats or SHRs. TBARS levels in both the RVLM and PVN were significantly higher in SHRs than in WKY rats (*$P < 0.05$, $n = 5$ for each). TBARS levels in the RVLM tissue in *AdMnSOD*-transfected SHRs were reduced at day 7 after the gene transfer. †Statistically significant difference at $P < 0.05$ between the values on day 0 and 7 after the gene transfer in the tissue of RVLM in SHRs. Data are shown as mean ± SEM. (d) Time course of mean arterial pressure (MAP) (mmHg) (left graph) and heart rate (HR) (beats/min) (right graph) in *AdMnSOD*-transfected or adenovirus vectors encoding the β-galactosidase (*AdLacZ*) gene-transfected rats before and after the gene transfer. *$P < 0.05$ between the values for *AdMnSOD*-transfected and *AdLacZ*-transfected SHRs ($n = 5$ for each). Open or filled black triangles represent the mean values for *AdLacZ*-transfected or *AdMnSOD*-transfected SHRs, respectively, whereas the open or filled black circles represent the mean values for *AdLacZ*-transfected or *AdMnSOD*-transfected WKY rats, respectively ($n = 5$ for each). (e) Urinary norepinephrine (uNE) excretion levels before and after the gene transfer in each strain ($n = 5$ for each). Open columns represent mean values before gene transfer, and filled black columns represent those after the gene transfer (at day 7). The uNE levels were significantly higher in SHRs than in WKY rats (*$P < 0.05$, $n = 5$ for each). †Statistically significant difference at $P < 0.05$ between the values before and at day 7 after the *MnSOD* gene transfer in SHRs.

Table 1 Baseline mean arterial pressure and heart rate just before microinjection of each drug into the rostral ventrolateral medulla or paraventricular nucleus

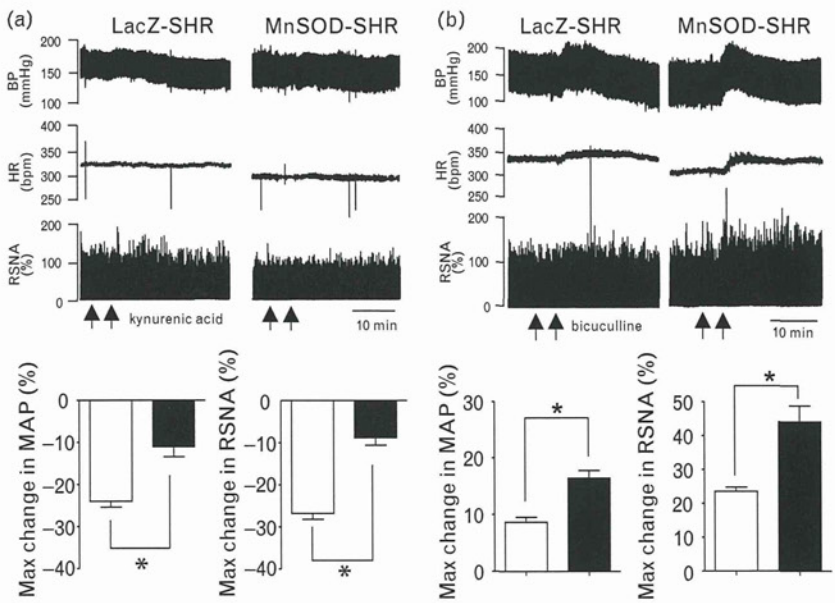
		Baseline	
Group	Compound	MAP (mmHg)	HR (beats/min)
LacZ-transfected SHRs	Kynurenic acid RVLM (5)	163 ± 3	337 ± 7
	Bicuculline RVLM (5)	164 ± 1	335 ± 9
	Bicuculline PVN (6)	170 ± 4	346 ± 5
MnSOD-transfected SHRs	Kynurenic acid RVLM (5)	141 ± 3*	302 ± 6*
	Bicuculline RVLM (5)	139 ± 4*	297 ± 7*
	Bicuculline PVN (6)	155 ± 3*	303 ± 6*
Control SHRs	Bicuculline PVN (5)	172 ± 2	345 ± 6
Hydralazine-treated SHRs	Bicuculline PVN (5)	151 ± 3*	358 ± 7
LacZ-transfected WKY rats	Kynurenic acid RVLM (4)	91 ± 2	288 ± 4
	Bicuculline RVLM (4)	98 ± 2	298 ± 4
	Bicuculline PVN (5)	88 ± 5	307 ± 9
MnSOD-transfected WKY rats	Kynurenic acid RVLM (4)	90 ± 3	290 ± 6
	Bicuculline RVLM (4)	98 ± 3	295 ± 5
	Bicuculline PVN (5)	85 ± 2	313 ± 4

Values are the mean ± SEM and represent mean arterial pressure (MAP) and heart rate (HR) before injection of each drug in each group. Values in parentheses indicate the number of animals. LacZ, ; MnSOD, manganese superoxide dismutase; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto. * $P < 0.05$ versus baseline values of adenovirus vectors encoding the β-galactosidase gene-transfected SHRs before injection of the same drug and area.

Mean arterial pressure and renal sympathetic nerve activity responses to blockade of glutamate or γ-amino butyric acid receptors in spontaneously hypertensive rats

Microinjection of kynurenic acid bilaterally into the RVLM at day 7 after the gene transfer induced a gradual decrease in MAP and RSNA in *AdLacZ*-transfected SHRs (Fig. 2a). In *AdMnSOD*-transfected SHRs, microinjection of kynurenic acid into the RVLM induced a slight decrease in MAP and RSNA, and the changes in MAP and RSNA were much smaller than those in *AdLacZ*-transfected SHRs. In WKY rats, the changes in MAP and RSNA induced by the injection of kynurenic acid into the RVLM did not differ between the *AdLacZ* and *AdMnSOD*-transfected groups [ΔMAP/baseline MAP (%) -7.0 ± 1.6 versus $-7.6 \pm 1.5\%$, $P > 0.05$, $n = 4$ for each;

Fig. 2



Changes in mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) induced by the microinjection of either kynurenic acid (2.7 nmol) (a) ($n = 5$ for each) or bicuculline (200 pmol) (b) ($n = 5$ for each) bilaterally into the rostral ventrolateral medulla at day 7 after the gene transfer [adenovirus vectors encoding the *manganese superoxide dismutase* (MnSOD) (*AdMnSOD*) or *β-galactosidase* (*AdLacZ*) genes]. *Statistically significant difference at $P < 0.01$ between the two groups. Data are shown as mean ± SEM. Open columns represent mean values for the *AdLacZ*-transfected groups, and filled black columns represent mean values for the *AdMnSOD*-transfected groups. BP, blood pressure; HR, heart rate; SHR, spontaneously hypertensive rat.

change in RSNA (%baseline) -5.5 ± 0.9 versus $-6.3 \pm 1.0\%$, $P > 0.05$, $n = 4$ for each).

Microinjection of bicuculline bilaterally into the RVLM at day 7 after the gene transfer induced an increase in MAP and RSNA in both *AdMnSOD*-transfected and *AdLacZ*-transfected SHRs (Fig. 2b). In *AdMnSOD*-transfected SHRs, however, the pressor response and the change in RSNA evoked by microinjection of bicuculline were significantly greater than those in *AdLacZ*-transfected SHRs. In WKY rats, these responses did not differ between the *AdLacZ* and *AdMnSOD*-transfected groups [ΔMAP/baseline MAP (%) 47.2 ± 2.8 versus $46.4 \pm 3.0\%$, $P > 0.05$, $n = 4$ for each; change in RSNA (%baseline) 112.7 ± 2.8 versus $111.8 \pm 2.2\%$, $P > 0.05$, $n = 4$ for each).

Microinjection of Mito-TEMPO (1 nmol) into the RVLM bilaterally significantly decreased MAP, HR, and RSNA in SHRs [ΔMAP -25.5 ± 2.4 mmHg; ΔHR -40.8 ± 3.0 beats/min; change in RSNA (%baseline) $-24.4 \pm 2.4\%$; $P < 0.01$, $n = 4$]. The depressor and sympathoinhibitory responses to kynurenic acid into the RVLM were significantly attenuated after Mito-TEMPO injection [ΔMAP/baseline MAP (%)-5.9 ± 0.4 versus $-24.4 \pm 1.1\%$, $P < 0.001$, $n = 5$ for each; change in RSNA (%baseline) -11.1 ± 1.7 versus $-28.4 \pm 1.8\%$, $P < 0.001$; Mito-TEMPO injection group $n = 4$, control group $n = 5$). In contrast, the pressor and sympathoexcitatory responses to bicuculline were significantly augmented after Mito-TEMPO injection [ΔMAP/baseline MAP (%)39.2 ± 5.5 versus $9.7 \pm 1.2\%$, $P < 0.001$; Mito-tempo injection group $n = 6$, control group $n = 5$; change in RSNA (%baseline): 83.3 ± 8.0 versus $24.8 \pm 1.8\%$, $P < 0.001$, $n = 5$ for each].

Effects of chronic reduction of reactive oxygen species in rostral ventrolateral medulla on sympathoexcitatory responses induced by paraventricular nucleus in spontaneously hypertensive rats

Microinjection of bicuculline into the PVN increased MAP and RSNA in all groups, consistent with a previous report [26]. MAP and RSNA began to increase gradually within 1–5 min after microinjection and reached a peak value within 5–15 min.

In SHRs, the pressor responses and the changes in RSNA were significantly smaller in *AdMnSOD*-transfected SHRs compared with *AdLacZ*-transfected SHRs (Fig. 3a). The adenovirus itself did not affect bicuculline-elicited responses because the changes in MAP and RSNA did not differ between *AdLacZ*-transfected SHRs and nontransfected SHRs (Fig. 3c). Each parameter gradually returned to the baseline pre-injected levels within 40 min.

In WKY rats, microinjection of bicuculline into the PVN at day 7 after the gene transfer induced an increase in MAP and RSNA in both *AdMnSOD*-transfected and *AdLacZ*-transfected WKY rats, but the degree of the change in MAP and RSNA did not differ between groups (Fig. 3b). The microinjection sites were almost all located within or on the border of the PVN, extending from a level 1.4–2.2 mm caudal to bregma (Fig. 4).

Effects of blood pressure-lowering with hydralazine on mean arterial pressure and renal sympathetic nerve activity responses to blockade of γ-amino butyric acid receptors in paraventricular nucleus of spontaneously hypertensive rats

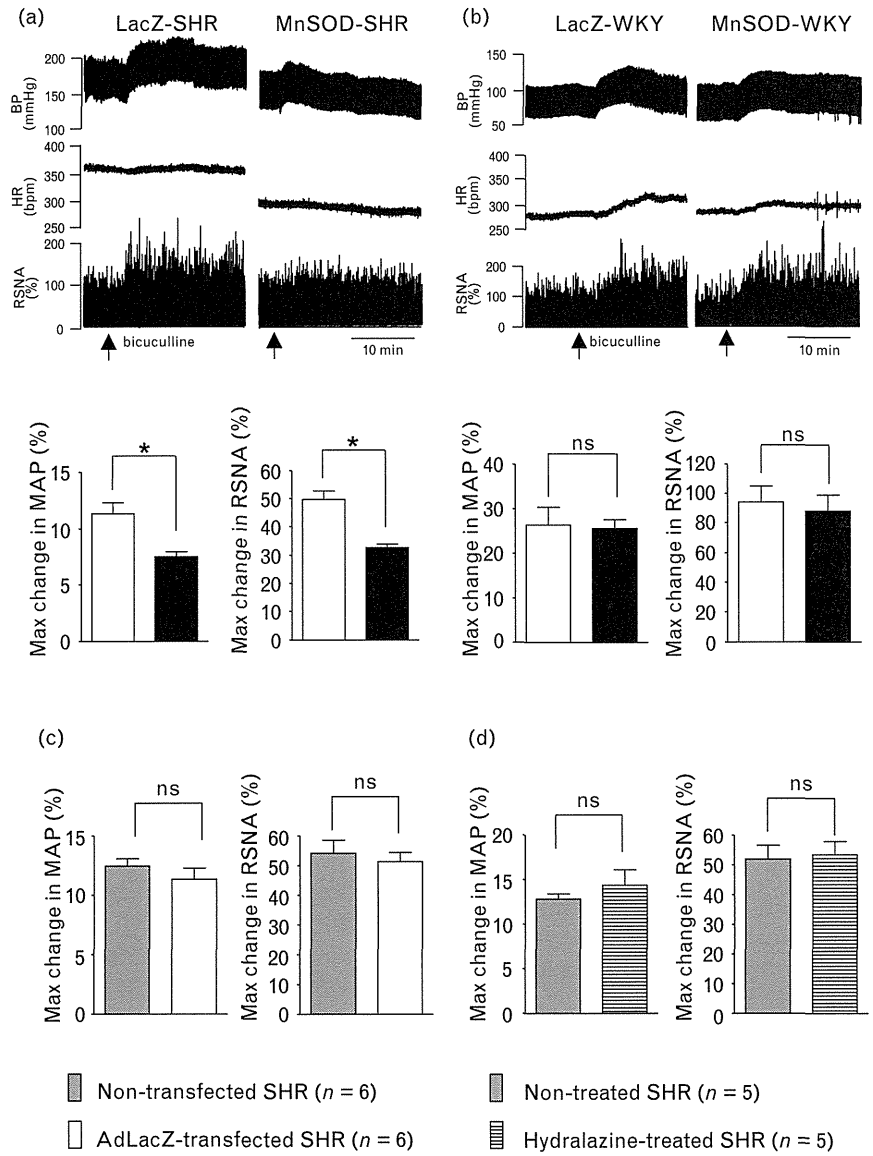
Telemetry-monitored BP began to decrease at day 1 or 2 after the administration of hydralazine and plateaued at day 5 in SHRs (MAP 165 ± 2–142 ± 3 mmHg, $P < 0.001$, $n = 5$ for each; change in MAP -23 ± 4 mmHg). At day 5, the microinjection study was performed. The changes in MAP and RSNA induced by the microinjection of bicuculline into the PVN did not differ between hydralazine-treated SHRs and nontreated SHRs (baseline cardiovascular values: Table 1, Fig. 3d).

Discussion

In the present study, ROS in the RVLM enhanced glutamatergic excitatory inputs and attenuated the GABAergic inhibitory inputs to the RVLM neurons in SHRs. Furthermore, ROS in the RVLM enhanced the pressor and sympathoexcitatory response induced by activation of the PVN neurons. In contrast, overexpression of MnSOD in the RVLM of WKY rats did not affect the pressor or sympathoexcitatory response induced by activation of the PVN neurons. Taken together, these findings suggest that increased ROS in the RVLM of SHRs contribute to hypertension by altering synaptic transmission in the RVLM through sympathoexcitation evoked by enhancing glutamatergic inputs to the RVLM from the PVN and attenuating the GABA-mediated sympathoinhibition in the RVLM.

Chronic inhibition of oxidative stress in the RVLM attenuated the glutamatergic excitatory inputs and enhanced GABAergic inhibitory inputs to the RVLM of SHRs in the present study. In WKY rats, overexpression of MnSOD in the RVLM did not alter the glutamatergic and GABAergic inhibitory inputs to the RVLM. ROS production was reduced by the overexpression of MnSOD in the RVLM, as previously described [19]. The time course of MnSOD expression in the RVLM and the changes in MAP and HR were consistent with the results of a previous study [19]. ROS production was decreased in the RVLM of SHRs based on the measurement of TBARS levels. Furthermore, we found that a mitochondrial ROS scavenger (Mito-TEMPO) also modulated the glutamatergic and GABAergic inputs to the RVLM in SHRs in the acute experiments, further supporting our data in MnSOD overexpression in SHRs. The excitatory inputs to the RVLM are enhanced and inhibitory inputs are reduced in SHRs, thereby increasing the central sympathetic outflow as one of the neural mechanisms

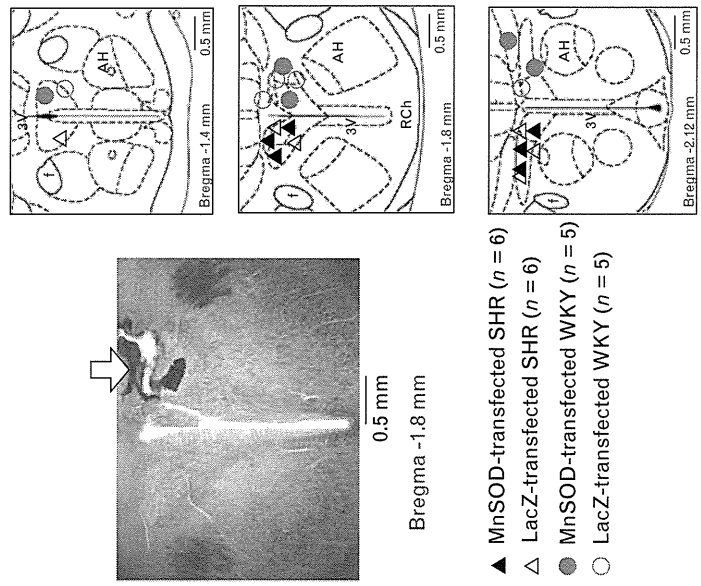
Fig. 3



Changes in mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) induced by the microinjection of bicuculline (100 pmol) [(a) $n = 6$ for each; (b) $n = 5$ for each; (c) $n = 6$ for each; and (d), $n = 5$] for each unilaterally into the paraventricular nucleus (PVN) at day 7 after the gene transfer [adenovirus vectors encoding the *manganese superoxide dismutase* (*AdMnSOD*) or *β-galactosidase* (*AdLacZ*) genes] in spontaneously hypertensive rats (SHRs) (a) groups, Wistar-Kyoto rats (b) groups, *AdLacZ*-transfected or non-transfected SHRs (c) groups and hydralazine-treated or non-treated SHRs (d) groups. Representative recordings showing blood pressure and RSNA responses to microinjection of bicuculline into the PVN. *Statistically significant difference at $P < 0.01$ between the two groups. Data are shown as mean ± SEM. Open columns represent mean values for the *AdLacZ*-transfected groups, and filled black columns represent mean values for the *AdMnSOD*-transfected groups.

of hypertension [6,8–10]. Particularly, the injection of kynurenic acid into the RVLM reduces MAP in SHRs [8] but not in WKY rats, suggesting enhanced activity of the RVLM neurons in SHRs. With regard to GABAergic inhibitory input to the RVLM, injecting bicuculline into the RVLM elicits a smaller pressor response in SHRs than in WKY rats [9,10]. Similar results were observed in the present study. The depressor and sympathoinhibitory responses to muscimol injection into the PVN are attenuated after the blockade of both excitatory and inhibitory amino acid receptors in the RVLM of SHRs [34], consistent with the ideas outlined above, although we cannot exclude the possibility that the bicuculline excited neurons through its effect on small-conductance Ca^{2+}-activated K^+ channels in addition to blocking GABA receptors [35]. Although ROS are suggested to affect neuronal activity, thereby directly altering the function of Ca^{2+} and/or K^+ channels [36,37], our findings suggest that the inhibition of ROS in the RVLM modulates synaptic transmission, whether or not channel

Fig. 4



Distribution of the centers of the injection sites in the paraventricular nucleus (PVN) in each experiment. Arrows indicate the injection site in the PVN. The distance of each section caudal to bregma is indicated according to the atlas of Paxinos and Watson. AH, anterior hypothalamic nucleus; f, fornix; 3 V, third ventricle; LacZ, β-galactosidase; MnSOD, manganese superoxide dismutase; RCh, retrochiasmatic area; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.

function is affected by synaptic transmission, and thus inhibits the increased RVLM neuronal activity and reduces the resting MAP in SHRs.

Another important finding of the present study was that chronic reduction of oxidative stress in the RVLM attenuated the pressor and sympathoexcitatory responses induced by activation of the PVN neurons. To activate PVN neurons, bicuculline was injected into the PVN [26,38] in *AdMnSOD*-transfected SHRs and *AdLacZ*-transfected SHRs. It is possible that the intensity of stimulation is altered after transfection of *AdMnSOD* due to the reduction in BP. This possibility is unlikely, however, because treatment with hydralazine did not alter BP or the RSNA responses evoked by injecting bicuculline into the PVN, despite the fact that hydralazine reduced BP to a level similar to that observed after the transfection of MnSOD into the RVLM. It is also worth noting that TBARS levels in the PVN did not differ between MnSOD-transfected and nontransfected rats, suggesting that PVN neurons are influenced by oxidative stress even after transfection of MnSOD into the RVLM of SHRs, although the findings of the present study do not allow us to completely exclude the possibility that PVN neurons were stimulated by a drop in BP. Because of the complex interaction between glutamate and GABA systems in the integration of sympathetic outflow by the PVN [39,40], we did not examine this issue further in the present study. Our findings are consistent with those of previous studies, indicating the importance of the pathway from the PVN to the RVLM neurons [26,34] and further suggest that a reduction of oxidative stress in the RVLM attenuates the excitatory input from the PVN to RVLM neurons.

Injection of kynurenic acid and the AT1 receptor blocker valsartan into the RVLM of SHRs elicits an additive depressor response compared with the injection of either drug alone [6]. This suggests that the antihypertensive effects of each drug are independent of each other. Because stimulation of AT1 receptors in the RVLM produces ROS via activation of NAD(P)H oxidase [22–24], blockade of AT1 receptors reduces BP via the reduction of ROS in SHRs or SHRSPs [17,19]. Treatment with the AT1 receptor blocker olmesartan attenuates the augmented pressor response to glutamate in the RVLM of SHRs [41], and the pressor response to angiotensin II injected into the RVLM is abolished, suggesting that oral treatment with olmesartan blocks AT1 receptors in the RVLM. The present results further suggest that there is a possible interaction between ROS production and amino acid-induced synaptic transmission.

In contrast to the results obtained in MnSOD-transfected SHRs, overexpression of MnSOD in the RVLM in WKY rats did not affect the pressor and sympathoexcitatory responses evoked by activation of PVN neurons. This finding is consistent with our [19] and others [17] previous studies. Injection of tempol, a superoxide dismutase mimetic, into the RVLM attenuates the sympathetic excitation induced by exposure to air-jet stress in awake rabbits [42]. Scavenging ROS in the RVLM attenuates

the pressor response to peripheral chemoreflex activation in normotensive rats [43]. These studies suggest that ROS in the RVLM are involved in mediating acute hypertensive responses. Furthermore, systemically administered tempol acts on both PVN and RVLM neurons, thereby reducing BP and RSNA, probably due to reduced ROS in those regions, in anesthetized normotensive Sprague-Dawley rats [44]. It should be noted, however, that tempol was administered acutely and the effects of tempol on BP, RSNA, and the PVN, and RVLM neuronal activity were transient. Furthermore, tempol can directly activate potassium currents [45] in addition to its SOD-like actions, which might affect blood vessels and neuronal excitability. Thus, it is unknown whether chronic reduction of systemic oxidative stress affects neuronal activity in the PVN and RVLM, thereby reducing the basal sympathetic activity. We did not explore the mechanisms by which ROS in the brain affect BP and sympathetic responses in normotensive rats in the present study. In addition, it is not clear whether increased oxidative stress in the PVN increases oxidative stress in the RVLM. Further studies are needed to address these questions.

The precise mechanisms by which ROS in the RVLM alter glutamatergic sympathoexcitation and enhance GABA-mediated sympathoinhibition are not known. We speculate that these responses might be mediated by an interaction between superoxide and nitric oxide (NO). Superoxide anions react rapidly with NO, forming peroxynitrite and decreasing the bioavailability of NO [46]. We previously demonstrated that NO in the RVLM causes hypotension and sympathoinhibition via GABA release [28]. The chronic reduction of ROS in the RVLM might increase the bioavailability of NO and enhance GABA release. Further studies are needed to clarify these issues.

In conclusion, our results suggest that ROS in the RVLM further enhance the excitatory glutamatergic inputs and attenuate GABAergic inhibitory inputs to RVLM neurons, thereby increasing BP through enhancing central sympathetic outflow in SHRs. In addition, ROS in the RVLM are suggested to augment the excitatory inputs from the PVN neurons to the RVLM neurons.

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Conflicts of interest

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There are no conflicts of interest and disclosure.

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Decreased brain sigma-1 receptor contributes to the relationship between heart failure and depression

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Aims Depression often coexists with cardiovascular disease, such as hypertension and heart failure, in which sympathetic hyperactivation is critically involved. Reduction in the brain sigma-1 receptor (S1R) functions in depression pathogenesis via neuronal activity modulation. We hypothesized that reduced brain S1R exacerbates heart failure, especially with pressure overload via sympathetic hyperactivation and worsening depression.

Methods and results Male Institute of Cancer Research mice were treated with aortic banding and, 4 weeks thereafter, fed a high-salt diet for an additional 4 weeks to accelerate cardiac dysfunction (AB-H). Compared with sham-operated controls (Sham), AB-H showed augmented sympathetic activity, decreased per cent fractional shortening, increased left ventricular dimensions, and significantly lower brain S1R expression. Intracerebroventricular (ICV) infusion of S1R agonist PRE084 increased brain S1R expression, lowered sympathetic activity, and improved cardiac function in AB-H. ICV infusion of S1R antagonist BD1063 increased sympathetic activity and decreased cardiac function in Sham. Tail suspension test was used to evaluate the index of depression-like behaviour, with immobility time and strain amplitude recorded as markers of struggle activity using a force transducer. Immobility time increased and strain amplitude decreased in AB-H compared with Sham, and these changes were attenuated by ICV infusion of PRE084.

Conclusion These results indicate that decreased brain S1R contributes to the relationship between heart failure and depression in a mouse model of pressure overload.

Keywords Sympathetic nervous system • Heart failure • Depression • Brain sigma-1 receptor • Pressure overload

1. Introduction

Numerous studies have demonstrated that heart failure and depression often coexist, and that depression is linked to the severity of heart failure symptoms.[1,2] Heart failure and depression are both individually associated with poor health outcomes, and depression also adversely affects heart failure outcomes.[3,4] Importantly, depression is associated with the risk of heart failure among patients with hypertension.[5] Furthermore, hypertensive heart disease is well recognized as the major cause of heart failure.[6] One of the physiological mechanisms involved in both depression and heart failure is high sympathetic tone caused by cardiovascular autonomic dysregulation.[7,8] The central nervous system contributes to the worsening of both heart failure[9] and hypertension.[10] The detailed mechanisms involved in this process remain unclear, but these findings suggest the presence of a common pathway for heart failure and depression in the brain.

Recently, a reduction in brain sigma-1 receptor (S1R) expression has been shown to play a key role in the pathogenesis of depression.[11] S1R ligands have been reported to have antidepressant activity in behavioural models of depression.[12] The S1R has been shown to modulate neuronal intracellular calcium levels[13] and *N*-methyl-D-aspartate-mediated response.[14,15] The results of these studies strongly suggest that the S1R contributes to the regulation of neuronal activity. Neurosteroids such as dehydroepiandrosterone (DHEA) and its sulfate conjugate (DHEAS) are recognized as endogenous S1R agonists,[16]

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and DHEAS was reported to decrease in heart failure.[17] Therefore, we hypothesized that expression of brain S1R is reduced in heart failure via decreased DHEAS, and that this reduced expression contributes to the exacerbation of heart failure via enhanced sympathetic activity and worsening of depression. Therefore, the aim of the present study was to assess the role of the brain S1R in the relationship between heart failure and depression.

Recently, we found that a pressure overload model with salt loading is a model of hypertensive heart disease leading to heart failure.[18] The relationship between hypertension and depression has been well recognized, and depression is associated with the risk of heart failure among patients with hypertension.[5] In addition, the pressure overload model was reported to decrease S1R expression in the left ventricle.[16,19] Therefore, the pressure overload model was used in the present study. Aortic banding (AB) was performed in mice then fed a high-salt (HS) diet to accelerate cardiac dysfunction.[18] We investigated (i) sympathetic activity by 24-h urinary norepinephrine (U-NE) excretion[18,20] and cardiac function by echocardiography[18,20]; (ii) brain S1R expression; (iii) the index of depression-like behaviour; (iv) the effects of intracerebroventricular (ICV) infusion of PRE084, a selective S1R agonist,[24,25] and BD1063, a selective S1R antagonist,[21,22] on sympathetic activity, cardiac function, brain S1R expression, and the index of depression-like behaviour and (v) the concentration of serum and brain DHEAS.

2. Methods

2.1 Animals

The study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male Institute of Cancer Research (ICR) mice (10 weeks old; SLC, Fukuoka, Japan) were used. Sodium pentobarbital was used as anaesthetic, and the adequacy of anaesthesia was confirmed by the absence of a withdrawal response to hindpaw nociceptive stimulation.

2.2 Mouse heart failure model

Mice under sodium pentobarbital [25–40 mg/kg intraperitoneal (ip)] anaesthesia were treated with AB at the suprarenal abdominal aorta[18,20] with 5–0 silk sutures guided by a blunted 27-gauge needle, which was withdrawn as quickly as possible. Sham-operated mice (Sham) served as controls. Four weeks later, both AB mice and Sham mice were fed a HS (8% NaCl) diet for 4 weeks (Sham-H or AB-H).[18,20] The survival rates after heart failure model preparation were as follows: 4 weeks after AB, 97%; 8 weeks after AB with HS diet (AB-H), 83%.

2.3 Evaluation of cardiac function

Cardiac function was evaluated by echocardiography[18,20] under light sodium pentobarbital anaesthesia with spontaneous respiration. An echocardiography system (SSD5000; Aloka, Tokyo, Japan) with a dynamically focused 7.5-MHz linear array transducer was used, and M-mode tracings from the short-axis view at the level of the papillary muscle were recorded. Left ventricle (LV) end-diastolic diameter (LVDD), LV end-systolic diameter (LVSD), and LV wall thickness (LVWT), calculated as the mean thickness of the interventricular septum and the posterior LV wall, were measured, and per cent fractional shortening (%FS) was calculated as follows:

$$\%FS = (LVDD - LVSD)/LVDD \times 100.$$

2.4 Evaluation of blood pressure, heart rate, and sympathetic activity

In acute experiments, sympathetic activity was evaluated by power spectral analysis.[24,25] Blood pressure (BP) and the heart rate were measured by right carotid artery cannulation with a stretched polyethylene tube (PE50). Data were recorded using the Powerlab system and Chart 5 software (AD Instruments), and the power spectrum of the beat-by-beat systolic blood pressure (SBP) time series and the beat-by-beat pulse interval time series was calculated using the maximum entropy method with MemCalc software (Suwa Trust Co, Ltd).[24] Two zones of interest for autonomic control of BP and the heart rate were observed. The first zone, covering the 0.15–0.6 Hz range of the SBP spectrum, was used as a low-frequency zone reflecting sympathetic control in mice. The second zone, covering the 2.5–5.0 Hz range of the pulse interval spectrum, was used as a high-frequency zone reflecting vagal control in mice.[25] In chronic experiments, sympathetic activity was evaluated by measuring 24-h U-NE excretion using high-performance liquid chromatography.[18,20]

2.5 ICV infusion

Under sodium pentobarbital anaesthesia (25–40 mg/kg, i.p.), mice were placed in a stereotaxic frame, and the skin overlying the midline of the skull was incised. A small hole was made with a dental drill at 0.3 mm posterior and 1 mm lateral to bregma.[18,20] An infusion cannula (Alzet® brain infusion kit 3; DURECT Corporation, CA, USA) was inserted and fixed to the skull surface with tissue adhesive (the tip of the cannula located 3 mm below the skull surface).[18,20] In acute experiments, the infusion cannula was connected to a syringe filled with PRE084, a specific S1R agonist (1 mM), and the agent was infused using a microsyringe pump (infusion rate 1.0 μL/min for 10 min) while measuring BP and the heart rate. In chronic experiments, the infusion cannula was connected to an osmotic minipump (Alzet model 1004; DURECT) inserted subcutaneously into the back for infusion of PRE084 into AB-H (2 mM; infusion rate 0.11 μL/h for 4 weeks initiated concomitantly with HS intake) or BD1063, a specific S1R antagonist, into Sham (2 mM; infusion rate 0.11 μL/h for 4 weeks initiated from 4 weeks after sham operation).

2.6 Oral drug administration

Fluvoxamine maleate (Sigma Aldrich Co., St Louis, MO, USA) mixed in powdered chow was administered orally to AB-H for 4 weeks initiated concomitantly with HS intake for an estimated oral administered fluvoxamine dose of ~1.0 mg/kg/day.[19]

2.7 Measurement of organ weight

After completion of the experiments, mice were killed with an overdose of sodium pentobarbital, the heart and lungs were removed, and organ weight was measured.

2.8 Evaluation of S1R expression in the brain

2.8.1 *Western blot analysis*

Animals were killed with an overdose of sodium pentobarbital. Tissues obtained from the circumventricular tissues including the hypothalamus were homogenized in lysis buffer containing 40 mmol/L HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 1% Triton® X-100, 10% glycerol, 1 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA). A 15 μg protein aliquot from each sample was separated on a polyacrylamide gel with 10% sodium dodecyl sulfate. The proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon®-P membranes; Millipore, Billerica, MA, USA). A rabbit immunoglobulin G (IgG) polyclonal antibody against S1R (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as primary antibody.[26] Membranes were then incubated with horseradish peroxidase-

conjugated horse anti-rabbit IgG antibody (1:10 000). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for the brain tissues. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL™ western blotting detection kit; Amersham Pharmacia Biotech, Uppsala, Sweden), and the film was analysed using the public domain software NIH Image (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.8.2 *Reverse transcriptase PCR*

Total RNA was prepared from the circumventricular tissues including hypothalamus using RNAlater solution (Ambion, Austin, TX, USA). Complementary DNAs were synthesized by standard techniques using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time PCR was performed, recorded, and analysed using a thermal cycler dice real-time system (Takara Bio, Shiga, Japan) with SYBR Green I detection. cDNA was amplified using a SYBR Premix Ex Taq (Perfect Real Time) PCR kit (Takara Bio) with specific primers (Sigma R1, forward: 5′-CTC GCT GTC TGA GTA CGT G-3′; reverse: 5′-AAG AAA GTG TCG GCT AGT GCA A-3′; HPRT1, forward: 5′-GCG TCG TGA TTA GCG ATG ATG AAC-3′; reverse: 5′-CCT CCC ATC TCC TCC ATG ACA TCT-3′). HPRT1 was used as a reference to normalize the amount of total RNA amplified in each reaction. Relative gene expression data were analysed using the 2 ($-\triangle\triangle$CT) method.[27]

2.9 Evaluation of the depression-like behaviour index

Depression was evaluated by the tail suspension test, which uses increased immobility time as an index of depression-like behaviour in mice.[28] Tail suspension was conducted in a force transducer, and strain amplitude data were also recorded using the Powerlab system as a marker of struggle activity. The test was performed in the afternoon, and immobility time and struggle activity were determined during 6-min recordings. Additionally, locomotor activity was evaluated by a digital actophotometer.[29] Two mice were placed in the actophotometer apparatus cage, and the total number of ambulatory movements was scored over 24 h. The daily variation of locomotor activity was also evaluated by the ratio of movement scores from night to day.

2.10 Measurement of serum and brain DHEAS

Under anaesthesia with an overdose of sodium pentobarbital, a blood sample was collected from the right ventricle, and the mice were perfused with distilled H_2O. After adequate perfusion to remove blood, the brain circumventricular tissues and hypothalamus were dissected out. The tissues (0.10 g) were homogenized in 200 μL distilled H_2O, rapidly centrifuged, and the supernatant was collected.[18] DHEAS concentration was measured by enzyme-linked immunosorbent assay (ELISA).

2.11 Statistical analysis

All values are expressed as mean ± SE. Analysis of variance was used to compare U-NE, organ weight, LVDD, LVSD, LVWT, %FS, immobility time, strain amplitude, DHEAS concentration, mRNA levels, and protein levels between groups. An unpaired *t*-test was used to compare locomotor activity between Sham and AB-H and changes in protein levels between mice treated with and without S1R ligands. Differences were considered significant at $P < 0.05$.

3. Results

3.1 Characteristics of AB-H

Both relative heart weight (heart weight/body weight) and absolute heart weight were greater in AB-H than Sham (relative heart weight: Sham, 4.87 ± 0.05 mg/g; AB-H, 6.45 ± 0.08 mg/g, absolute heart weight: Sham, 0.23 ± 0.02 g; AB-H, 0.26 ± 0.05 g; $P < 0.05$, $n = 8$ per group). Relative lung weight (lung weight/body weight) tended to increase in AB-H compared with Sham (Sham, 5.81 ± 0.15mg/g; AB-H, 6.20 ± 0.09 mg/g; $n = 8$ per group). The body weight of AB-H was significantly lower than that of Sham (Sham, 47.4 ± 0.8 g; AB-H, 40.6 ± 1.1 g; $P < 0.05$, $n = 8$ per group). Echocardiography revealed that LV dimensions and LVWT were greater in AB-H than Sham, and %FS was significantly lower in AB-H than Sham (*Figure 1*). Sympathetic activity evaluated by U-NE excretion was increased in AB-H compared with Sham (Sham, 350 ± 44 ng/day; AB-H, 731 ± 26 ng/day; $P < 0.05$, $n = 8$ per group). In Sham, HS intake did not alter body weight, organ weight, cardiac function, or sympathetic activity (Sham vs. Sham-H). Mean BP was lower and the heart rate was higher in AB-H than Sham (mean BP: Sham, 90 ± 2 mmHg; AB-H, 78 ± 1 mmHg, heart rate: Sham, 422 ± 16 b.p.m.; AB-H, 477 ± 14 b.p.m.; $P < 0.05$, $n = 5$ per group). Tail suspension test revealed increased immobility time and decreased strain amplitude in AB-H compared with Sham (*Figure 2A–C*). Locomotor activity (24 h) was lower in AB-H than Sham (Sham, 7387 ± 459 counts; AB-H, 3877 ± 864 counts; $P < 0.05$, $n = 6$ per group), and the ratio of locomotor activity during night to day, a marker of daily variation, was smaller in AB-H than Sham (Sham, 3.2 ± 0.6; AB-H, 1.6 ± 0.1; $P < 0.05$, $n = 6$ per group).

3.2 S1R expression in the brain

The protein levels of brain S1R were decreased in AB-H compared with Sham. In Sham, HS intake did not alter those levels (*Figure 3*). The mRNA levels of brain S1R did not significantly alter between Sham and AB-H (Sham, 1.2 ± 0.1; AB-H, 0.9 ± 0.1; $n = 5$ per group).

3.3 Effects of acute PRE084 ICV infusion on cardiovascular regulation

ICV infusion of PRE084 lowered heart rate in both AB-H and Sham. However, changes in the heart rate were significantly smaller in AB-H than in Sham (*Figure 4A*). Furthermore, sympathetic activity evaluated by power spectral analysis of SBP was decreased significantly only in Sham (*Figure 4B*).

3.4 Effects of chronic PRE084 ICV infusion

Chronic ICV infusion of PRE084 (PRE) increased protein levels of brain S1R in AB-H compared with no treatment (*Figure 5A*). mRNA levels of brain S1R slightly increased after chronic PRE084 ICV infusion (AB-H, 0.9 ± 0.1; AB-H with PRE, 1.3 ± 0.1; $P < 0.05$, $n = 5$ per group). Chronic PRE084 ICV infusion lowered the enhanced sympathetic activity (AB-H, 731 ± 26 ng/day; AB-H with PRE, 571 ± 43 ng/day; $P < 0.05$, $n = 8$ and 5, respectively) and improved cardiac function in AB-H compared with no treatment (*Figure 5B*). Furthermore, PRE084 also decreased both relative heart weight (heart weight/body weight) and absolute heart weight compared with no treatment (relative heart weight: AB-H, 6.45 ± 0.08 mg/g; AB-H with PRE, 4.50 ± 0.15 mg/g, absolute heart weight: AB-H, 0.26 ± 0.05 g; AB-H with PRE, 0.19 ± 0.07 g; $P < 0.05$, $n = 8$ and 5, respectively). Chronic ICV infusion of PRE084 decreased immobility time and increased strain amplitude in AB-H compared with no treatment (*Figure 5C*).

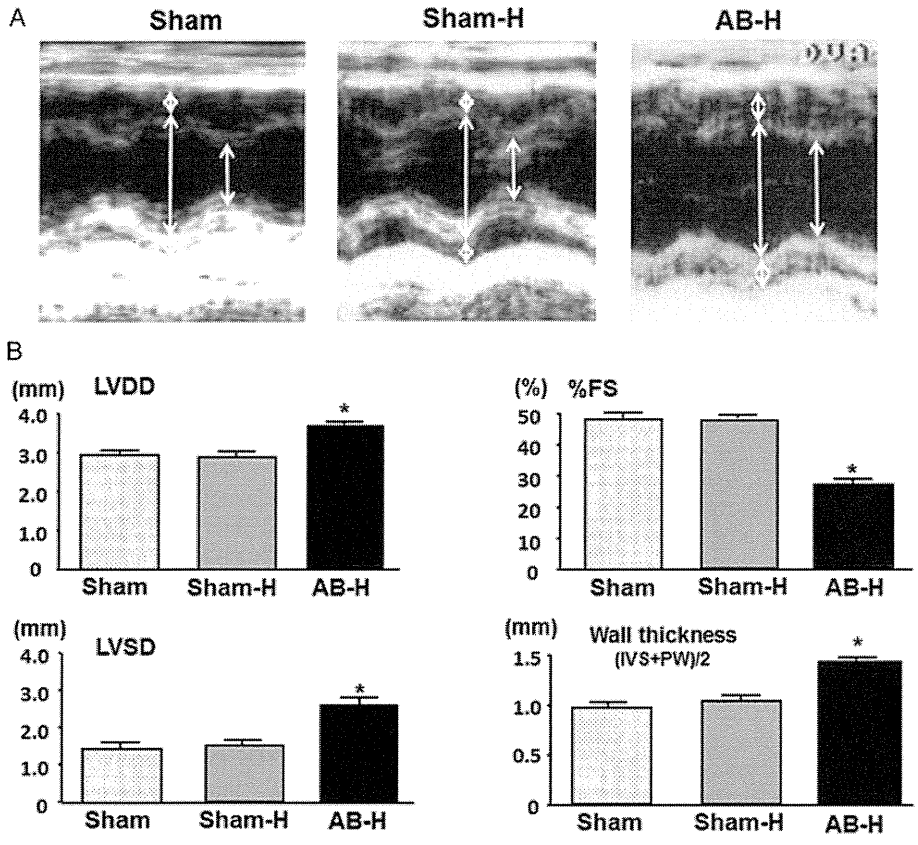

Figure 1 (*A*) Representative M-mode echocardiography. (*B*) Cardiac function evaluation by echocardiography. *$P < 0.05$ vs. Sham and Sham-H ($n = 8$ per group). LVDD, left ventricular end-diastolic diameter; LVSD, left ventricular end-systolic diameter; %FS, per cent fractional shortening; IVS, interventricular septum; PW, posterior wall.

3.5 Effects of chronic oral administration of fluvoxamine

Chronic oral administration of fluvoxamine lowered enhanced sympathetic activity (AB-H, 731 ± 26 ng/day; AB-H with fluvoxamine, 561 ± 20 ng/day; $P < 0.05$, $n = 8$ and 5, respectively) and improved cardiac function compared with no treatment (%FS: AB-H, 27 ± 2%; AB-H with fluvoxamine 37 ± 3%; $P < 0.05$, $n = 8$ and 5, respectively). Chronic oral administration of fluvoxamine decreased immobility time and increased strain amplitude compared with no treatment (immobility time: AB-H, 162 ± 9 s; AB-H with fluvoxamine, 93 ± 10 s; strain amplitude: AB-H, 0.12 ± 0.01; AB-H with fluvoxamine, 0.19 ± 0.02; both $P < 0.05$, $n = 15$ and 5, respectively).

3.6 Effects of chronic BD1063 ICV infusion on characteristics of Sham mice

Chronic ICV infusion of BD1063 (BD)-enhanced sympathetic activity (24-h U-NE excretion: Sham, 350 ± 44 ng/day; Sham with BD, 576 ± 25 ng/day; $P < 0.05$, $n = 8$ and 5, respectively) and tended to impair cardiac function compared with no treatment (%FS: Sham, 47 ± 2%; Sham with BD, 40 ± 3%; $P < 0.05$, $n = 8$ and 5, respectively).

3.7 DHEAS concentration

Serum DHEAS concentrations were lower in AB-H compared with Sham or Sham-H (Sham, 0.039 ± 0.009 μg/mL; Sham-H, 0.040 ± 0.006 μg/mL; AB-H, 0.013 ± 0.008 μg/mL; $P < 0.05$, $n = 5$ per group). Brain DHEAS concentrations were also lower in AB-H compared with Sham or Sham-H (Sham, 0.047 ± 0.001 μg/mL; Sham-H, 0.046 ± 0.002 μg/mL; AB-H, 0.033 ± 0.005 μg/mL; $P < 0.05$, $n = 5$ per group).

4. Discussion

The present study demonstrates that in AB-H (i) cardiac function decreased with enhanced sympathetic activity; (ii) brain S1R expression decreased; (iii) the index of depression-like behaviour was higher compared with Sham; (iv) the decrease in sympathetic activity and the heart rate in response to acute ICV infusion of PRE084, a selective S1R agonist, was smaller compared with Sham; and (v) chronic ICV infusion of PRE084 increased brain S1R expression, lowered enhanced sympathetic activity, and improved cardiac function and the index of depression-like behaviour. These findings indicate that the reduction in brain S1R expression in AB-H contributed to both the exacerbation of cardiac dysfunction via enhanced sympathetic activity and the worsening of depression.

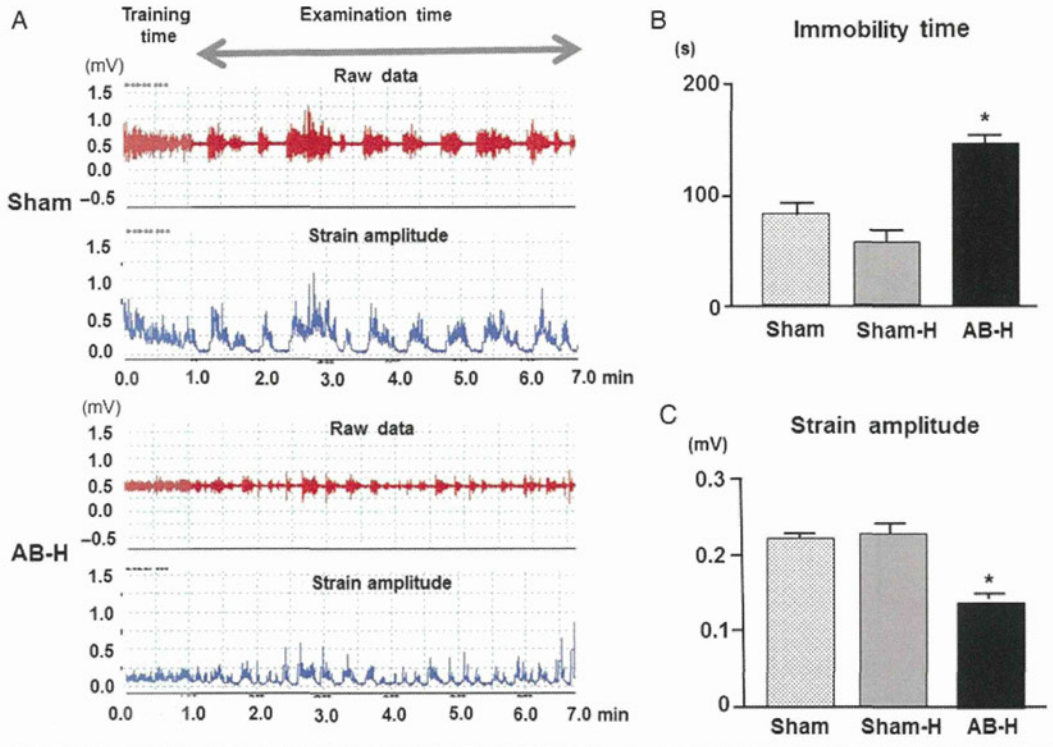

Figure 2 (*A*) Representative recordings of strain amplitude in the tail suspension test from Sham (upper) and AB-H (lower). (*B* and *C*) Grouped data of immobility time (*B*) and strain amplitude (*C*) in Sham and AB-H. *$P < 0.05$ vs. Sham (Sham and Sham-H, $n = 13$; AB-H, $n = 15$).

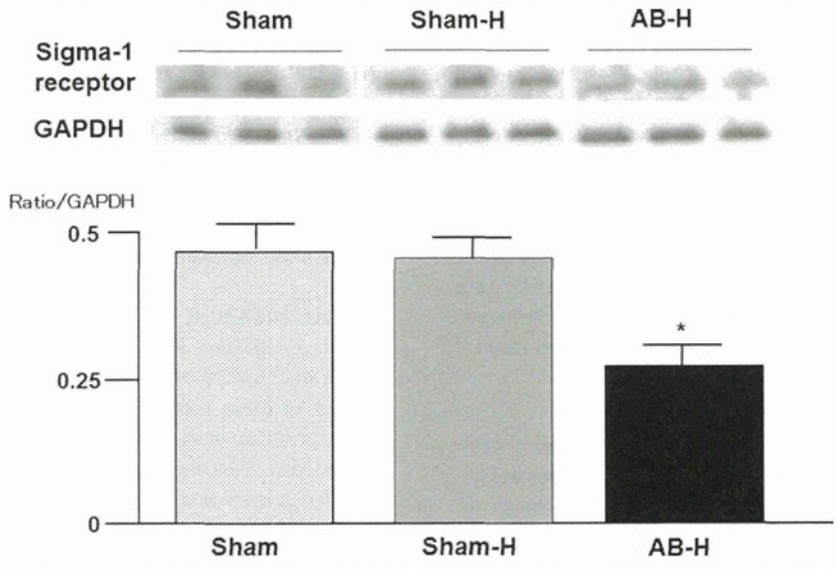

Figure 3 Representative western blots demonstrating the expression of S1R in the brain. The graph shows the means for the quantification of three separate experiments. Data are expressed as the relative ratio of GAPDH expression. *$P < 0.05$ vs. Sham and Sham-H. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

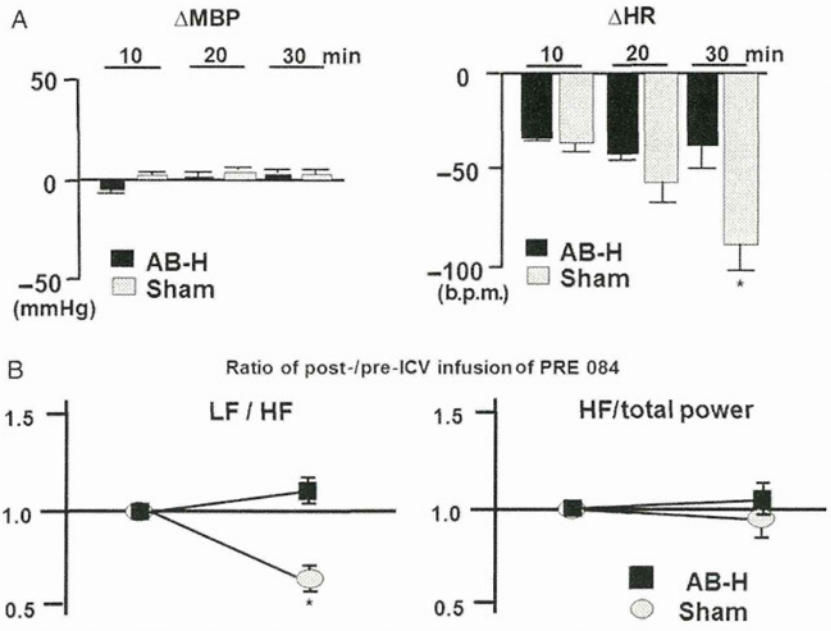

Figure 4 (*A*) Grouped data of MBP and HR response to ICV infusion of PRE084 in Sham and AB-H mice. *X*-axis (10, 20, 30 min) indicates time after initiating PRE084 ICV infusion. $^*P < 0.05$ vs. AB-H ($n = 5$ per group). (*B*) Grouped data of LF/HF, as a marker of sympathetic activity, and HF/total power, as a marker of parasympathetic activity, in response to PRE084 ICV infusion. $^*P < 0.05$ vs. AB-H ($n = 5$ per group). MBP, mean blood pressure; HR, heart rate.

4.1 Reduction of brain S1R in AB-H mice

We demonstrated that the expression of brain S1R was decreased in AB-H and that this reduction was involved in the enhanced sympathetic activity. In AB-H, LV dimensions and LVWT increased, and %FS decreased with the enhanced sympathetic activity. We have previously confirmed the elevated LV end-diastolic pressure observed in this model.[18] Therefore, AB-H was used as a model for pressure-overload-induced heart failure. The protein levels of brain S1R were significantly decreased in AB-H, and ICV infusion of PRE084 decreased sympathetic activity and the heart rate to a greater extent in Sham than in AB-H. These results indicate that the protein levels of brain S1R and the S1R response to the receptor agonist were decreased in AB-H. The mRNA levels of brain S1R were also examined in the present study and found not to differ between Sham and AB-H, suggesting a higher turnover rate of S1R in AB-H.[30]

4.2 Depressive status in AB-H

We demonstrated that immobility time was increased in AB-H compared with Sham. In addition, a decrease in strain amplitude was confirmed as a marker of struggle activity in AB-H. AB-H had lower cardiac function compared with Sham, and the results of the tail suspension test may reflect exercise intolerance caused by cardiac dysfunction. However, short-term momentum in the home cage measured immediately after the test did not differ between AB-H and Sham (data not shown), suggesting that the results of the tail suspension test reflect the greater depressive status in AB-H, rather than exercise intolerance caused by cardiac dysfunction. Furthermore, the decreased 24-h locomotor activity and disappearance of daily variation of locomotor activity were confirmed in AB-H.

4.3 Effects of brain S1R stimulation

To clarify the importance of the reduction in brain S1R expression in this heart failure model, chronic ICV infusion of PRE084 in AB-H and BD1063 in Sham was performed. Chronic PRE084 ICV infusion increased brain S1R expression in AB-H. Our findings are compatible with those of previous studies, in which expression of the S1R was reported to increase in response to chronic agonist stimulation.[16] In addition, chronic PRE084 ICV infusion attenuated the enhanced sympathetic activity and improved cardiac function in AB-H. In contrast, chronic BD1063 ICV infusion enhanced sympathetic activity and tended to decrease cardiac function in Sham. ICV infusion of PRE084 also improved the index of depression-like behaviour in AB-H.

To our knowledge, no previous studies have reported chronic PRE084 or BD1063 infusion into the cerebrospinal fluid (CSF). PRE084 has an IC50 of 44 nM in the S1R and IC50 > 10 000 nM in a variety of other receptors.[21] BD1063 has nanomolar affinity for S1R and is 30-fold more selective for S1R than the sigma-2 receptor.[23] In the present study, the estimated concentration of both chemicals in the CSF was considered to be in the nanomolar range.[31] Therefore, the doses of both chemicals in the present study were adequate for use as specific S1R ligands. Using these doses, we were able to successfully show that the brain S1R plays an important role in modulating sympathetic activity and depressive status.

To clarify the effects of systemic treatment with S1R agonist, fluvoxamine, a potent agonist of S1R in addition to its function as a selective serotonin reuptake inhibitor, was orally administered.[19] The dose of fluvoxamine was determined according to a previous report.[19] Systemic administration of S1R agonist also attenuated the

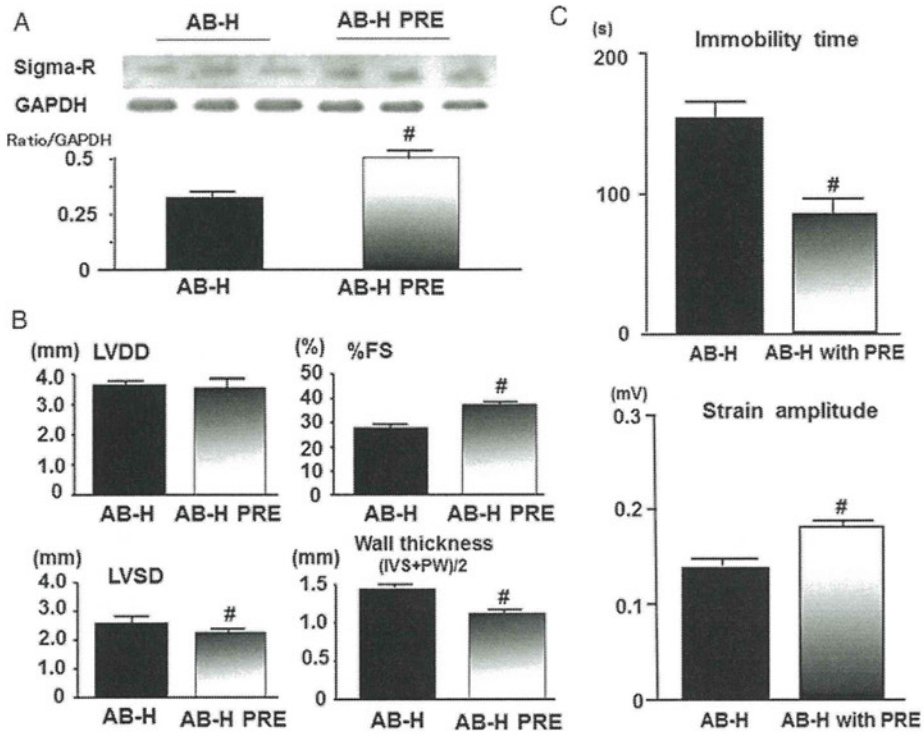

Figure 5 (*A*) Representative western blots demonstrating S1R expression in the brain after PRE084 ICV infusion in AB-H (AB-H with PRE). The graph shows the means for the quantification of three separate experiments. Data are expressed as the relative ratio of GAPDH expression. $^{\#}P < 0.05$ vs. AB-H. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase. (*B*) Cardiac function evaluation by echocardiography after PRE084 ICV infusion in AB-H (AB-H with PRE). $^{\#}P < 0.05$ vs. AB-H (AB-H with PRE, $n = 5$; AB-H, $n = 8$). (*C*) Grouped data of immobility time and strain amplitude after PRE084 ICV infusion in AB-H (AB-H with PRE). $^{\#}P < 0.05$ vs. AB-H (AB-H with PRE, $n = 10$; AB-H, $n = 15$).

sympathetic hyperactivation in addition to improving depression-like behaviour.

The mechanisms involved in the modulation of sympathetic activity via the brain S1R remain unclear. The S1R is known to modulate neuronal intracellular calcium levels[13] and *N*-methyl-D-aspartate-mediated response.[14,15] Furthermore, S1R was reported to enhance brain plasticity and functional recovery after stroke.[32] These results suggest that the brain S1R affects neuronal activity and that the S1R in the central cardiovascular control region contributes to the regulation of sympathetic activity. Western blotting for brain S1R detection in circumventricular brain tissue including hypothalamus was performed. Wide distribution of the S1R has been reported in the brain, most abundantly in the hippocampus, facial nucleus, thalamus, and hypothalamus.[33] Therefore, the hypothalamus, one of the central cardiovascular control regions, is a promising candidate for the target nucleus involved in modulating sympathetic activity via the S1R. However, the S1R has also been reported in the brainstem, where the other cardiovascular control centre is located. ICV infusion of PRE084 or BD1063 possibly affected the S1R in the brainstem.

In addition, microglia releasing pro-inflammatory mediators such as cytokines and reactive oxygen species[34] also expresses high levels of S1R, and S1R suppresses microglial activation.[35] Such pro-inflammatory mediators in the brain were reported to cause sympathetic hyperactivation in models of heart failure.[36] Therefore, both neuronal and microglial S1R may be involved in the modulation of sympathetic activity.

4.4 Involvement of DHEAS in brain S1R reduction

The mechanisms involved in the reduction in brain S1R expression in this heart failure model also remain unclear. Neurosteroids, such as DHEA and its sulfate conjugate DHEAS, are recognized as endogenous S1R agonists.[14,16] DHEAS is produced mainly in adrenal tissues,[37] and its serum concentration was reported to decrease in heart failure patients.[17] In the present study confirmed that both serum and brain DHEAS concentrations decreased in AB-H compared with Sham mice. Furthermore, a recent study demonstrated that DHEAS deficiencies predicted the severity of depression in heart failure.[38] Therefore, a decrease in serum DHEAS concentration may have been responsible for the reduction in brain S1R expression observed in this heart failure model.

4.5 Limitations

The present study revealed that brain S1R expression decreased in AB-H, and the reduction in brain S1R may be involved in depression-like behaviour in this model. However, depression is a clinical syndrome that may have multi-pathogenetic causes. Therefore, other mechanisms may contribute to depression-like behaviour in this model. In fact, brain angiotensin type 1 receptor (AT1R) was reported to be a novel therapeutic approach for treatment of mood disorders via anti-inflammatory effects.[39] In previous studies, we confirmed that the brain AT1R increased in AB-H.[18] Further studies are needed to

clarify the contribution of AT1R to depression-like behaviour and the interaction between AT1R and S1R.

A strong association with depression has been well known in both heart failure and hypertension. Therefore, the pressure overload model, a mimic of hypertensive heart disease, was used as the experimental heart disease model in the present study. Other experimental heart failure models, such as that induced by myocardial ischaemia, have also been used widely. Recently, patients with coronary disease who screened as positive on depression screening test had a greater risk for adverse cardiovascular outcomes.[40] Therefore, the experimental heart failure model induced by myocardial ischaemia should also be used to test our hypothesis in the future.

4.6 Conclusion

In conclusion, these findings indicate that brain S1R expression was decreased in AB-H, and that this reduction in brain S1R expression contributed to both the exacerbation of cardiac dysfunction via enhanced sympathetic activity and the worsening of depression.

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