

**Figure 4.** The serum brain natriuretic peptide (BNP) concentration and New York Heart Association (NYHA) functional class before and 6 to 12 months after the radiofrequency catheter ablation (RFCA). The serum BNP level was elevated before the RFCA, however, it significantly decreased 6 to 12 months after the RFCA ( $p < 0.05$ ) (panel A). Although the NYHA functional class was demonstrated to be significantly worse before the RFCA (panel B), the RFCA significantly improved the NYHA functional class as compared to that before the intervention ( $p < 0.01$ ).

**Table 2.** Patient Symptoms and Medications before and after Radiofrequency Catheter Ablation

	Before	After
<b>Symptoms</b>		
palpitations	100%	0%
general fatigue	83%	0%
depression	67%	0%
fainting	33%	0%
<b>Medications</b>		
beta-blockers	100%	33%
calcium-channel blockers	33%	0%
digitalis	17%	0%

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## ORIGINAL ARTICLE

# Activation of mineralocorticoid receptors in the rostral ventrolateral medulla is involved in hypertensive mechanisms in stroke-prone spontaneously hypertensive rats

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Mineralocorticoid receptor (MR) is recognized as a target for therapeutic intervention in hypertension and heart failure. MRs in the central nervous system are thought to have an important role in blood pressure regulation. Thus, we examined whether activation of the MR pathway in the rostral ventrolateral medulla (RVLM) of the brainstem contributes to the neural mechanism of hypertension in stroke-prone spontaneously hypertensive rats (SHRSPs). We microinjected eplerenone, aldosterone or Na<sup>+</sup>-rich artificial cerebrospinal fluid (aCSF) into the RVLM of anesthetized Wistar–Kyoto (WKY) rats and SHRSPs. Arterial pressure (AP), heart rate (HR) and renal sympathetic nerve activity (RSNA) were recorded. The expressions of the MR protein and the serum- and glucocorticoid-regulated kinase protein (Sgk1), which is a marker of MR activity, in the RVLM were measured by western blot analysis. Bilateral microinjection of eplerenone into the RVLM decreased AP and RSNA in WKY rats and SHRSPs, and the decreases in those variables were significantly greater in SHRSPs than WKY rats. Microinjection of aldosterone or Na<sup>+</sup>-rich aCSF into the RVLM increased AP and RSNA dose-dependently. The increases in those variables were significantly greater in SHRSPs than in WKY rats. The pressor responses of aldosterone or Na<sup>+</sup>-rich aCSF were attenuated by the prior injection of eplerenone in SHRSPs. Sgk1 expression levels in the RVLM were significantly greater in SHRSPs than in WKY rats. These findings suggest that activation of MRs in the RVLM enhances sympathetic activity, thereby contributing to the neural mechanism of hypertension in the SHRSP.

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**Keywords:** blood pressure; mineralocorticoid receptor; rostral ventrolateral medulla; sympathetic nervous system

## INTRODUCTION

Accumulating evidence indicates that the sympathetic nervous system has an important role in the pathogenesis of hypertension.<sup>1–3</sup> Mineralocorticoid receptor (MR) is recognized as a target for therapeutic intervention in hypertension and heart failure.<sup>4,5</sup> The beneficial effects of the MR antagonist spironolactone or the more specific antagonist eplerenone on end-organ damage have been studied in animal models.<sup>6–10</sup> It has been reported that MRs regulate epithelial Na channels (ENaCs) that are important for the regulation of sodium transport and the maintenance of extracellular fluid volume and arterial pressure (AP) in the kidney.<sup>11</sup> Recent studies have reported the distribution of MRs or ENaCs in the choroid plexus, ependyma and neurons, such as those in the supraoptic nucleus, paraventricular nucleus and nucleus tractus solitarius.<sup>12,13</sup> In addition, the expression

of MR mRNA has been reported in the brainstem,<sup>14</sup> including the rostral ventrolateral medulla (RVLM) and caudal ventrolateral medulla.<sup>15,16</sup> These findings suggest that MRs in the central nervous system, especially in neurons, regulate Na<sup>+</sup> influx via ENaCs, leading to hypertension and sympathoexcitation. However, it remains to be determined whether activation of MRs in the brainstem contributes to blood pressure regulation via the sympathetic nervous system.

The RVLM of the brainstem is one of the most important vasomotor centers that determines sympathetic nervous system activity, and it is essential for the maintenance of basal vasomotor tone.<sup>3,17,18</sup> There is a evidence that increased activity of the RVLM leads to sympathetic hyperactivity in various model of hypertension.<sup>18–20</sup> For example, both the pressor response to the microinjection of angiotensin II into the RVLM and the stimulation of AT<sub>1</sub>

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receptors were enhanced in spontaneously hypertensive rats (SHRs).<sup>21</sup> The activation of endogenous AT<sub>1</sub> receptors has also been demonstrated in SHRs and stroke-prone spontaneously hypertensive rats (SHRSPs).<sup>22,23</sup> It has been reported that microinjection of aldosterone into the RVLM increases AP, and that the MR blocker spironolactone decreases AP.<sup>24</sup> The purpose of the present study was thus to determine whether the MR pathway in the RVLM contributes to the neural regulation of blood pressure through the sympathetic nervous system, and whether the activation of endogenous MRs in the RVLM leads to sympathetic hyperactivity and hypertension in SHRSPs. Furthermore, we examined neuronal sensitivity to Na<sup>+</sup> in the RVLM, because activation of MRs would induce ENaC upregulation.<sup>11</sup> SHRSPs are widely used as a model of hypertension, because they develop exaggerated blood pressure elevation with high sympathetic nervous system activity, thereby causing stroke and death.<sup>23</sup> In addition, oral administration of eplerenone prevented salt-induced cardiac fibrosis in SHRSPs.<sup>6</sup> For this purpose, we investigated the effects of aldosterone, Na<sup>+</sup>-rich artificial cerebrospinal fluid (aCSF) and the MR blocker eplerenone administered into the RVLM on arterial blood pressure, heart rate (HR) and renal sympathetic nerve activity (RSNA). The expression of MRs and serum- and glucocorticoid-regulated kinase (Sgk1) expression levels, which indicate MR activity, were also evaluated.

## METHODS

This study was reviewed and approved by the Animal Experiments Ethics Committee, Kyushu University Graduate School of Medical Sciences, and was conducted according to the Guidelines for Animal Experiments of Kyushu University.

### Animals and general procedures

Male Wistar-Kyoto rats (WKY/Izm) and SHRSP/Izm (12–16 weeks old; SLC Japan, Hamamatsu, Japan) were used. Food and tap water were available *ad libitum* throughout the study. The rats were kept in a temperature- and humidity-controlled room with a 12-h light period between 0800 hours to 2000 hours.

### Microinjection into the RVLM and recording of blood pressure, HR and RSNA

SHRSPs and WKY rats were initially anesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup> *i.p.*, followed by a maintenance dosage of 20 mg kg<sup>-1</sup> per h *i.v.*). A catheter was inserted into the femoral artery to record AP and HR. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. Body temperature was monitored with a rectal thermometer and maintained in the range of 36.5–37.5 °C with a heating pad. The left renal nerve was exposed using a left retroperitoneal flank incision. A pair of stainless steel bipolar electrodes was placed beneath the renal nerve to record multifiber RSNA.<sup>25</sup> All signals were recorded on a computer using a PowerLab system (AD Instruments, Nagoya, Japan). The signal from the electrodes was amplified, passed through a band pass filter and then rectified and integrated (resetting every 0.1 s). The rats were placed in a stereotaxic frame, and the dorsal surface of the medulla was surgically exposed to allow for positioning of the microinjection pipettes in the RVLM (with the pipette angled rostrally 18 °C, 1.8 mm lateral, 3.5 mm below the calamus scriptorius), as previously described.<sup>26</sup> The microinjections (all microinjections were in a volume of 50–100 nl unless otherwise indicated) into the RVLM were made according to the following protocols: (1) bilateral microinjections of the MR blocker eplerenone (100 pmol each); (2) unilateral microinjection of aldosterone (10 pmol-1 nmol); (3) unilateral microinjection of aldosterone (100 pmol) 30 min after bilateral microinjections of eplerenone (100 pmol each); (4) unilateral microinjection of Na<sup>+</sup>-rich aCSF (0.15–0.2 M); and (5) unilateral microinjection of Na<sup>+</sup>-rich aCSF (0.2 M in 50 nl) 30 min after bilateral microinjections of eplerenone (100 pmol each).

aCSF (which contained (in mmol l<sup>-1</sup>) 121 NaCl, 3.4 KCl, 1.2 MgCl<sub>2</sub>, 0.6 NaH<sub>2</sub>PO<sub>4</sub>, 29 NaHCO<sub>3</sub> and 3.4 glucose) was used as a vehicle, and Na<sup>+</sup>-rich

aCSF was prepared by adjusting the [Na<sup>+</sup>] of aCSF with additional NaCl.<sup>27</sup> Aldosterone was obtained from Sigma-Aldrich (St Louis, MO, USA). The MR blocker eplerenone was a gift from the Pfizer Pharmaceutical Company, (New York, NY, USA). Drug doses were based on previous reports<sup>24,27</sup> or our preliminary experiments. Before microinjection of the drugs, the RVLM was identified by monitoring the mean arterial pressure (MAP) after injecting a small dose (1 nmol) of L-glutamate. For bilateral injections, injections were first made on one side, and then the pipette was moved to the contralateral side; the two injections were made ~3 min apart.

### Western blot analysis for the MR and Sgk1 in the RVLM

To obtain RVLM tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg kg<sup>-1</sup> *i.p.*) and transcardially perfused with phosphate-buffered saline. The brain was quickly removed. The RVLM tissue was homogenized and then sonicated in a lysing buffer containing 40 mmol l<sup>-1</sup> of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% Triton X-100, 10% glycerol, 1 mmol l<sup>-1</sup> phenylmethanesulfonyl fluoride and 1 protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA). The tissue lysate was centrifuged at 6000 r.p.m. for 5 min at 4 °C in a microcentrifuge. The lysate was collected, and the protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Aliquots of protein (50 µg) from each sample were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. Subsequently, the separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore, Billerica, MA, USA). The membranes were incubated with goat IgG polyclonal antibody against MR (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), with rabbit IgG polyclonal antibody against SGK1 (Abcam, Tokyo, Japan) and with rabbit IgG polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (1:1000; Santa Cruz Biotechnology) for 24 to 48 h. The membranes were then washed and incubated with horseradish peroxidase-conjugated horse anti-goat IgG or anti-rabbit antibody (1:10000; Santa Cruz Biotechnology) for 40 min. Immunoreactivity was detected by autoradiography using enhanced chemiluminescence and a western blotting detection kit (Amersham, Piscataway, NJ, USA).

### Statistical analysis

All values are expressed as the mean ± s.e.m. The changes in MAP, HR and RSNA values during the eplerenone microinjection studies and the MR and Sgk1 receptor expression were compared between SHRSPs and WKY rats using an unpaired *t*-test. Intergroup differences in MAP and RSNA after aldosterone and Na<sup>+</sup>-rich aCSF microinjection were compared using two-way analysis of variance. *P* values of <0.05 were considered statistically significant.

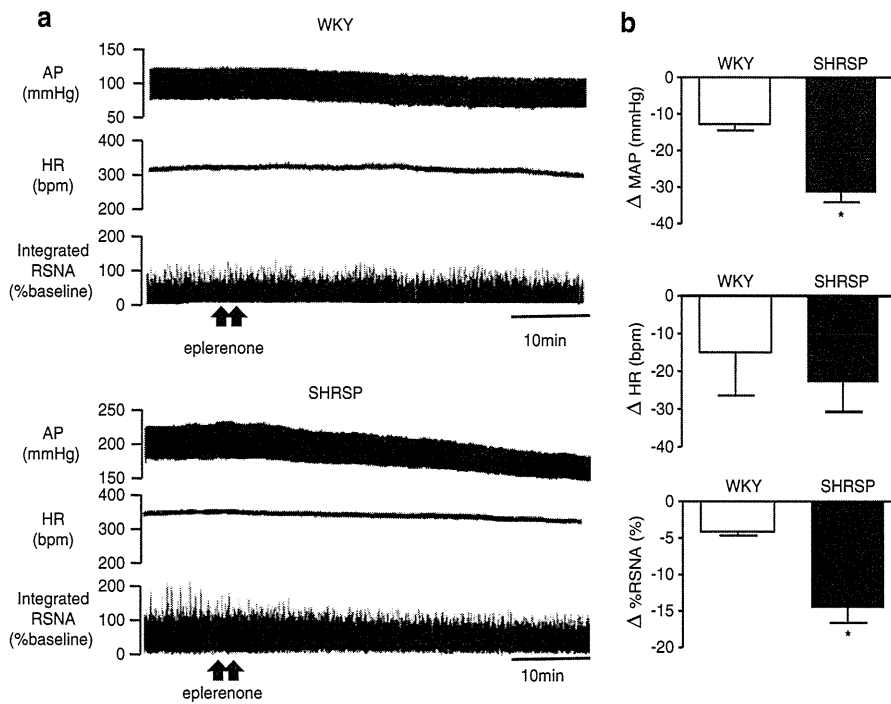
## RESULTS

### Effect of blockade of MR in the RVLM on arterial pressure, HR and RSNA

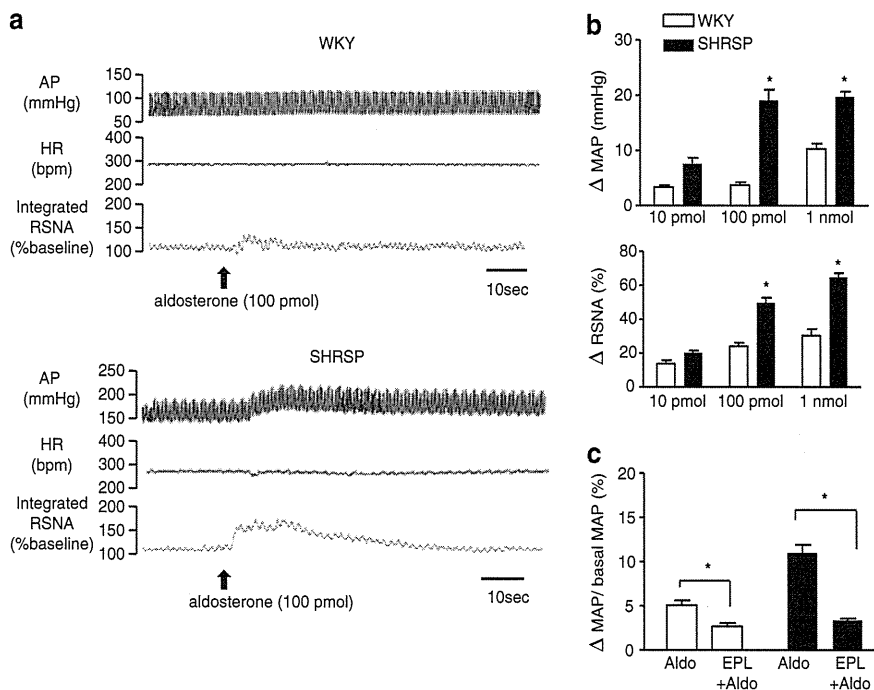
The basal MAP and HR were significantly higher in SHRSPs than in WKY rats (183.1 ± 4.1 *vs.* 103.2 ± 2.7 mm Hg, 355.7 ± 3.1 *vs.* 318.1 ± 3.3 b.p.m., *P* < 0.01, *n* = 5 for each). Bilateral microinjection of the MR blocker eplerenone into the RVLM induced a significant decrease in MAP, HR and RSNA in both SHRSPs and WKY rats. The magnitudes of the decreases in AP and RSNA were significantly greater in SHRSPs than in WKY rats (ΔMAP, -31.5 ± 2.7 *vs.* -12.0 ± 1.2 mm Hg, *P* < 0.01; RSNA Δbaseline, -14.6 ± 2.1 *vs.* -4.3 ± 0.5%, *P* < 0.01, *n* = 5 for each; Figures 1a and b). In contrast, the decrease in HR was not significantly different between SHRSPs and WKY rats (ΔHR, -22.9 ± 7.8 *vs.* -17.4 ± 9.2 b.p.m.; NS, *n* = 5). These changes occurred several minutes after injection, peaked at 40–60 min, and gradually recovered over time, but they lasted more than 2 h.

### Effect of microinjection of aldosterone into the RVLM on arterial pressure, HR and RSNA

The basal MAP and HR were significantly higher in SHRSPs than in WKY rats (185.7 ± 5.6 *vs.* 91.9 ± 2.7 mm Hg, 356.5 ± 4.2 *vs.*



**Figure 1** The responses of arterial pressure (AP), heart rate (HR) and renal sympathetic nerve activity (RSNA) to bilateral microinjection of the mineralocorticoid receptor (MR) blocker eplerenone into the RVLM of Wistar–Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSPs). (a) Raw data of the changes in AP, HR and RSNA evoked by bilateral microinjection of eplerenone in WKY rats and SHRSPs. (b) Group data of the changes in mean arterial pressure (MAP), HR and RSNA in response to bilateral microinjection of eplerenone (100 pmol). Values are expressed as the mean  $\pm$  s.e.m. \* $P < 0.05$  ( $n = 5$  for each).



**Figure 2** The responses of arterial pressure (AP), heart rate (HR) and renal sympathetic nerve activity (RSNA) to microinjection of aldosterone into the rostral ventrolateral medulla (RVLM). (a) Raw data of the changes in AP, HR and RSNA after unilateral injection of aldosterone (10 pmol–1 nmol) into the RVLM in Wistar–Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSPs). (b) Group data of the changes in mean arterial pressure (MAP) and HR evoked by unilateral microinjection of aldosterone (10 pmol–1 nmol) into the RVLM in WKY rats and SHRSPs. (c) Group data of the changes in MAP in response to microinjection of aldosterone (100 pmol) without pretreatment (Aldo) and microinjection of aldosterone with pretreatment of eplerenone (100 pmol) (Aldo + EPL) in WKY rats and SHRSPs. Values are expressed as the mean  $\pm$  s.e.m. \* $P < 0.05$  ( $n = 4$  for each).

$302 \pm 5.1$  b.p.m.,  $P < 0.01$ ,  $n = 4$  for each). Unilateral microinjection of aldosterone into the RVLM significantly increased MAP and RSNA in both SHRSPs and WKY rats. The pressor response induced by aldosterone occurred in a dose-dependent manner (Figures 2a and b). No significant changes in HR were observed in either strain of rats (data not shown). The magnitude of the increases in these variables was significantly greater in SHRSPs than in WKY rats ( $P < 0.05$ ,  $n = 4$  for each; Figure 2b). Pretreatment with eplerenone nearly prevented the aldosterone-induced pressor responses in SHRSPs and WKY rats. However, the blocking effect of MRs was significantly greater in SHRSPs ( $P < 0.05$ ,  $n = 5$  for each; Figure 2c).

### MR and Sgk1 expression levels in the RVLM

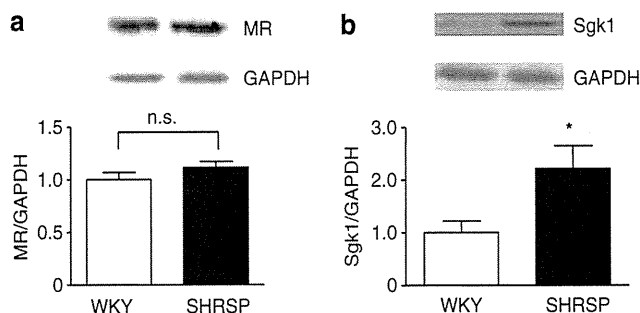
The protein expression levels of MRs in the RVLM did not differ between SHRSPs and WKY rats. However, the levels of Sgk1, which is induced by MRs and is a marker of the activity of MRs, were significantly greater in SHRSPs than in WKY rats ( $P < 0.05$ ,  $n = 4$  for each; Figure 3).

### Effect of microinjection of $\text{Na}^+$ -rich aCSF into the RVLM on arterial pressure, HR and RSNA

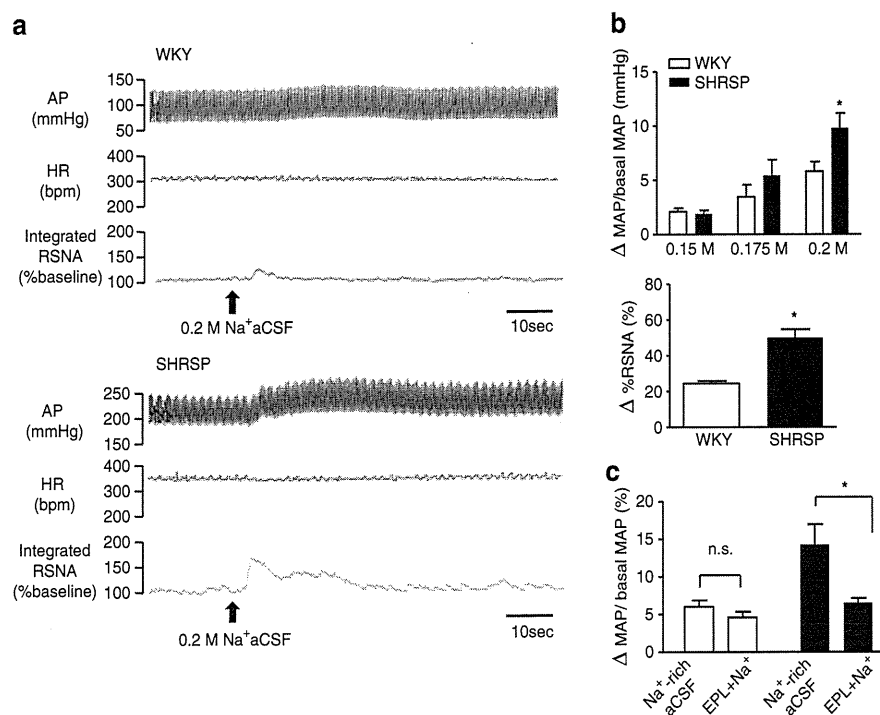
Similarly, the basal MAP and HR were significantly higher in SHRSPs than in WKY rats ( $180.8 \pm 4.1$  vs.  $87.1 \pm 9.9$  mmHg,  $357.9 \pm 5.6$  vs.  $312.5 \pm 6.4$  b.p.m.,  $P < 0.01$ ,  $n = 5$  for each). Microinjection of  $\text{Na}^+$ -rich aCSF into the RVLM increased both MAP and RSNA in a dose-dependent manner (Figures 4a and b), whereas microinjection of 0.15 M aCSF into the RVLM caused no significant changes in MAP, HR or RSNA. The magnitudes of the increases in these variables were significantly greater in SHRSPs than in WKY rats ( $P < 0.05$ ,  $n = 5$  for each; Figure 4b). In addition, pretreatment with bilateral microinjection of eplerenone into the RVLM significantly attenuated the  $\text{Na}^+$ -rich aCSF-induced pressor responses in SHRSPs, but did not significantly change them in WKY rats ( $P < 0.05$ ,  $n = 5$  for each; Figure 4c).

### DISCUSSION

The findings of this study were as follows: (1) blockade of MRs in the RVLM decreased MAP and RSNA in both SHRSPs and WKY rats, but the decreases were apparently greater in SHRSPs than in WKY rats; (2) microinjection of aldosterone or  $\text{Na}^+$ -rich aCSF into the RVLM increased MAP via sympathetic nerve activity in both SHRSPs and WKY rats, but the increases were greater in SHRSPs than in WKY rats;



**Figure 3** Western blot analysis demonstrating (a) mineralocorticoid receptor (MR) and (b) serum- and glucocorticoid-regulated kinase (Sgk1) expression in the rostral ventrolateral medulla (RVLM) in 12-week-old Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSPs). The densitometric average was normalized to the values obtained from the analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Expressions are shown relative to those seen in WKY rats, which were assigned a value of 1. Values are expressed as the mean  $\pm$  s.e.m. \* $P < 0.05$  ( $n = 4$  for each).



**Figure 4** The response to unilateral microinjection of  $\text{Na}^+$ -rich artificial cerebrospinal fluid (aCSF) (0.15–0.2 M) into the rostral ventrolateral medulla (RVLM). (a) Raw data of the changes in mean arterial pressure (AP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSPs). (b) Group data of the changes in mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) evoked by unilateral microinjection of  $\text{Na}^+$ -aCSF (0.2 M) into the RVLM in WKY rats and SHRSPs. (c) Group data of the changes in mean MAP in response to microinjection of  $\text{Na}^+$ -rich-aCSF (0.2 M) without pretreatment ( $\text{Na}^+$ -rich aCSF) and microinjection of  $\text{Na}^+$ -rich-aCSF (0.2 M) with pretreatment of eplerenone (100 pmol) (EPL + Na) in WKY rats and SHRSPs. Values are expressed as the mean  $\pm$  s.e.m. \* $P < 0.05$  ( $n = 5$  for each).

(3) in SHRSPs, the prior injection of an MR blocker completely prevented the aldosterone-induced pressor response, but it only partially prevented the  $\text{Na}^+$ -rich aCSF-induced pressor response; and (4) the protein expression levels of MRs in the RVLM did not differ between SHRSPs and WKY rats. However, the Sgk1 expression levels were significantly greater in SHRSPs than in WKY rats. Taken together, our findings indicate that activation of MRs in the RVLM contributes to the neural mechanisms of hypertension in SHRSPs.

The most important finding of this study was that microinjection of the MR-specific blocker eplerenone into the RVLM induced decreases in MAP and RSNA in WKY rats and SHRSPs, and these decreases were greater in SHRSPs. MRs in the brain have been shown to be involved in sympathetic nerve activation in salt-sensitive hypertensive rats,<sup>28</sup> in a rat model of myocardial infarction<sup>29</sup> and in other salt-sensitive models.<sup>30</sup> The blockade of MRs in the brain has been suggested to decrease sympathetic nerve activation and result in a decrease in the blood pressure rise from high salt intake in salt-sensitive rats.<sup>28</sup> This decrease has also been associated with an improvement in the cardiac function of mice with pressure overload.<sup>30</sup> In the present study, we confirmed that endogenous blockade of MRs in the RVLM decreased blood pressure via sympathoinhibition, with greater decreases in SHRSPs. However, the decreases in HR were not significantly different, because it is difficult to assess HR in an acute experiment with anesthetics. To investigate the long-term effects of endogenous MR blockade on blood pressure or RSNA, we microinjected eplerenone into the RVLM bilaterally. It has been reported that Sgk1 is induced by aldosterone and upregulates ENaC levels and activity in the kidney,<sup>11,31</sup> and that this aldosterone-induced upregulation is dependent on MRs.<sup>32</sup> Therefore, these studies suggest that Sgk1 can be classified as an aldosterone-effector kinase and a marker of MR signaling. In the RVLM, the protein expression of MRs did not differ between the two strains, but Sgk1 was significantly higher in SHRSPs than in WKY rats. The enhanced protein expression of Sgk1 in SHRSPs in the present study might indicate an enhancement of MR stimulation in the RVLM. We observed that endogenous blockade of MRs by eplerenone induced a greater depressor response through sympathoinhibition in SHRSPs than in WKY rats, and exogenous stimulation of MR by aldosterone elicited a greater pressor response through sympathoexcitation in SHRSPs than in WKY rats. Altogether, these findings suggest that activation of MRs in the RVLM is enhanced and contributes to elevation of the AP in SHRSPs.

The effects of aldosterone have been ascribed to a genomic mechanism of binding to its receptors, followed by translocation of the steroid receptor complex to the nucleus, where it acts as a transcriptional regulator. However, recent studies suggest that effects may be because of non-genomic actions of aldosterone, which occur more rapidly after binding MRs.<sup>33,34</sup> For example, the rapid action of aldosterone in the kidney<sup>33</sup> or vasculature<sup>34</sup> has been demonstrated within a few minutes. Therefore, it is possible that acute inhibition of MR activation might be caused by non-genomic mechanism in the RVLM. However, we cannot exclude the possibility that genomic action is also involved in our observation, because the depressor response evoked by eplerenone lasted more than 2 h. In addition, it should be noted that the depressor and sympathoinhibitory responses occurred several minutes after the bilateral microinjection of eplerenone into the RVLM, and that the pressor and sympathoexcitatory responses occurred within seconds after the microinjection of aldosterone. In general, exogenously administered neurotransmitter/neuromodulators into the RVLM have been shown to elicit rapid action of blood pressure and sympathetic nerve activity. In contrast, the blockade of endogenous receptors in the RVLM gradually evokes the

responses of blood pressure and sympathetic nerve activity.<sup>35</sup> In this context, the time course of the responses is not surprising, although we still do not have a clear explanation for this phenomenon. Further study is needed to clarify whether endogenous activation of MR is involved in hypertensive mechanisms both in the genomic and non-genomic action of aldosterone.

Our observations are consistent with previous reports that microinjection of aldosterone into the RVLM increases MAP.<sup>24</sup> It has been reported that intracerebroventricular (ICV) infusion of aldosterone increased MAP<sup>36</sup> and that these sympathoexcitatory and central pressor effects of aldosterone can be blocked by ICV infusion of an MR blocker. In the present study, microinjection of the MR blocker eplerenone into the RVLM also prevented an aldosterone-induced pressor response. These results suggest that aldosterone is likely to be an endogenous ligand of MRs in the RVLM. It has also been reported that central administration of aldosterone appears to depend on the MR-ENaC-ouabain pathway and, ultimately,  $\text{AT}_1$  receptor stimulation.<sup>37</sup> The sympathoexcitatory and pressor responses to central infusion of aldosterone and  $\text{Na}^+$ -rich aCSF can be prevented by central infusion of the sodium channel blocker benzamil or an ouabain blocker,<sup>27,38</sup> and the pressor responses elicited by central infusion of aldosterone or ouabain can be blocked by an  $\text{AT}_1$  blocker.<sup>27,39</sup> In the RVLM, microinjection of an ouabain-like compound<sup>40</sup> or angiotensin II<sup>21</sup> elicits a pressor response and sympathoexcitation. Collectively, these data suggest that aldosterone in the RVLM might activate central mechanism(s) involving the MR-ENaC-ouabain pathway, thereby causing sympathetic hyperactivity and hypertension.

Several studies have reported that aldosterone shows poor penetration of the blood-brain barrier compared with other steroid hormones.<sup>12</sup> However, the enzymes for steroid biosynthesis are present in the central nervous system<sup>41</sup> and RVLM,<sup>15</sup> and aldosterone can be detected in the tissues of various brain regions *in vitro*<sup>41</sup> and *in vivo*.<sup>42</sup> In particular, it should be noted that Gomez-Sanchez *et al.* reported that aldosterone was detectable in the whole brain of adrenalectomized rats, despite the fact that plasma aldosterone was undetectable.<sup>41</sup> These findings suggest that aldosterone is produced locally in the RVLM. In the present study, such locally produced aldosterone might have been enhanced and might have activated MRs in SHRSPs. Alternatively, there might be an aldosterone-independent MR activation pathway in the RVLM. It has been reported that MRs were activated by Rac1, which was independent of aldosterone in the kidney.<sup>43</sup> Further studies are needed to clarify the precise mechanisms involved in the activation of MR in the RVLM.

In this study, microinjection of  $\text{Na}^+$ -rich aCSF into the RVLM caused concentration-related increases in MAP and RSNA, whereas microinjection of 0.15 M aCSF at the same volume generated no significant increases. It has been reported that acute ICV infusion<sup>44</sup> or microinjection into the paraventricular nucleus of  $\text{Na}^+$ -rich aCSF<sup>27</sup> causes sympathetic hyperactivity and hypertension in normotensive rats, and pressor responses are enhanced in salt-sensitive rats compared with salt-resistant rats.<sup>44</sup> Abrams *et al.*<sup>45</sup> hypothesized that when MRs bind ligand, there is a subsequent upregulation of ENaCs, which would increase the membrane permeability to sodium in the brain. This response, in the face of transient increases in sodium levels, would lead to membrane depolarization and an increase in neural activity, driving sympathetic outflow and increasing MAP. It has also been reported that in the paraventricular nucleus, it is possible for an increase in intracellular  $\text{Na}^+$  caused by a larger extra-/intracellular gradient to increase intracellular  $\text{Ca}^{2+}$  through the  $\text{Na}^+/\text{Ca}^{2+}$  channel exchanger, and thereby increase Ang II release.<sup>27</sup> Considering these



observations, it is possible that Na<sup>+</sup>-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<sup>+</sup>-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<sup>+</sup> might be mediated by MR activation. ICV infusion of the MR blockers, spironolactone<sup>46</sup> or benzamil,<sup>47</sup> prevented Na<sup>+</sup>-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.<sup>30</sup> Taken together, these findings suggest that MRs mediate Na<sup>+</sup> via ENaCs or transporters on the cell surface of neurons in the RVLM.

### Study limitations

MRs are largely occupied by the glucocorticoid corticosterone,<sup>48</sup> which is present in a higher concentration than aldosterone in the brain.<sup>41</sup> The enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2), which is distributed throughout the brainstem, including the nucleus tractus solitarius,<sup>49</sup> rapidly converts corticosterone to an inactive metabolite. Thus, the coexpression of 11 $\beta$ -HSD2 with MRs may identify brain regions that are particularly sensitive to aldosterone. Although the precise expression of 11 $\beta$ -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<sup>+</sup>-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.

### ACKNOWLEDGEMENTS

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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  $\gamma$ -amino butyric acid (GABA) receptor blocker, into the RVLM were enhanced in *AdMnSOD*-transfected SHRs compared with adenovirus vectors encoding the  $\beta$ -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.

## 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)

**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  $\beta$ -galactosidase gene; *AdMnSOD*, adenovirus vectors encoding the manganese superoxide dismutase gene; AT1, angiotensin type 1; GABA,  $\gamma$ -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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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

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  $\gamma$ -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  $\beta$ -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- $\mu$ m 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  $\beta$ -tubulin as the loading control. The densitometric average was normalized to the values obtained from the analysis of  $\beta$ -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 SHR [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 $\gamma$ -amino butyric acid receptor blockade in the paraventricular nucleus of spontaneously hypertensive rats

*AdMnSOD* gene transfer into the RVLM in SHR chronically decreased BP in awake rats. Therefore, to elucidate the BP-lowering effect on the GABAergic mechanisms within the PVN in SHR, 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 SHR, 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  $\pm$  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

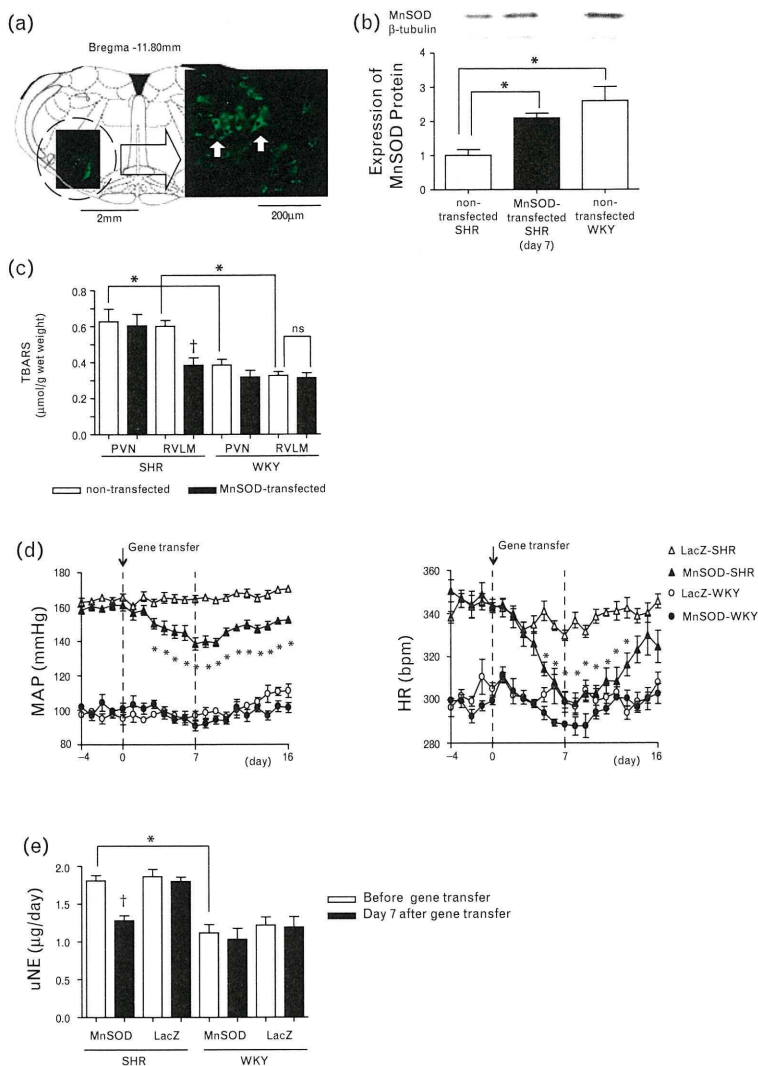
### Overexpression of manganese superoxide dismutase in rostral ventrolateral medulla *in vivo*

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 SHR compared with that of WKY rats, but MnSOD expression in the RVLM tissue of *AdMnSOD*-transfected SHR was significantly increased compared with that of nontransfected SHR 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 SHR 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 SHR 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 SHR were significantly decreased compared with those in *AdLacZ*-transfected SHR, but not those in WKY rats (Fig. 1d). The uNE excretion was significantly higher in SHR compared with WKY rats. At day 7 after the gene transfer, uNE excretion was significantly decreased in *AdMnSOD*-transfected SHR, but not in *AdLacZ*-transfected SHR (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 SHR than in *AdLacZ*-transfected SHR in each experiment. Baseline MAP and HR did not differ between the *AdLacZ*-transfected SHR and the nontreated SHR. 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 SHR, the maximal changes in MAP are expressed as the percentage change from baseline.

Fig. 1



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

Group	Compound	Baseline	
		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

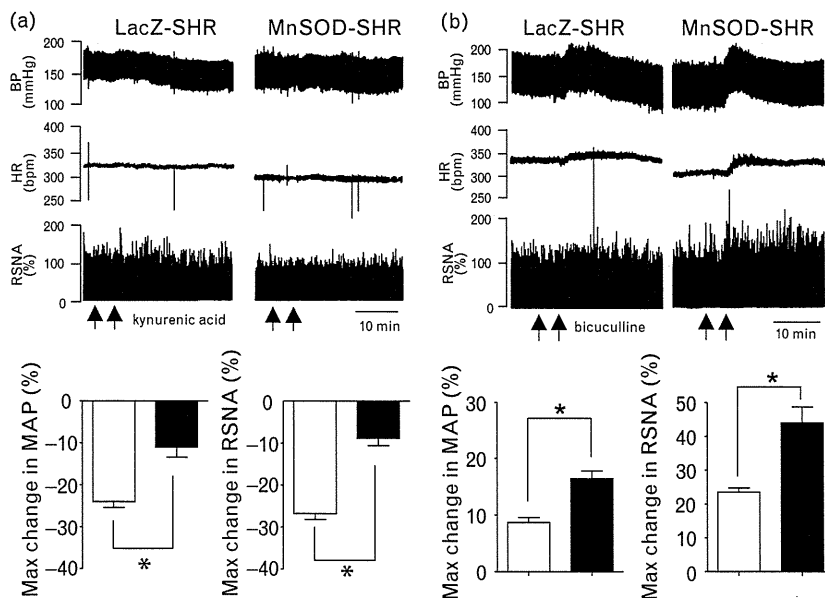
Values are the mean ± SEM and represent mean arterial pressure (MAP) and heart rate (HR) before injection of each drug in each group. Values in parentheses indicate the number of animals. LacZ,  $\beta$ -galactosidase; MnSOD, manganese superoxide dismutase; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto. \* $P < 0.05$  versus baseline values of adenovirus vectors encoding the  $\beta$ -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  $\gamma$ -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 [ $\Delta$ MAP/baseline MAP (%)  $-7.0 \pm 1.6$  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  *$\beta$ -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)  $-5.5 \pm 0.9$  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 SHR (Fig. 2b). In *AdMnSOD*-transfected SHR, however, the pressor response and the change in RSNA evoked by microinjection of bicuculline were significantly greater than those in *AdLacZ*-transfected SHR. In WKY rats, these responses did not differ between the *AdLacZ* and *AdMnSOD*-transfected groups [ $\Delta$ MAP/baseline MAP (%)  $47.2 \pm 2.8$  versus  $46.4 \pm 3.0\%$ ,  $P > 0.05$ ,  $n = 4$  for each; change in RSNA (%baseline)  $112.7 \pm 2.8$  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 SHR [ $\Delta$ MAP  $-25.5 \pm 2.4$  mmHg;  $\Delta$ HR  $-40.8 \pm 3.0$  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 [ $\Delta$ MAP/baseline MAP (%)  $-5.9 \pm 0.4$  versus  $-24.4 \pm 1.1\%$ ,  $P < 0.001$ ,  $n = 5$  for each; change in RSNA (%baseline)  $-11.1 \pm 1.7$  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 [ $\Delta$ MAP/baseline MAP (%)  $39.2 \pm 5.5$  versus  $9.7 \pm 1.2\%$ ,  $P < 0.001$ ; Mito-tempo injection group  $n = 6$ , control group  $n = 5$ ; change in RSNA (%baseline):  $83.3 \pm 8.0$  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 SHR, the pressor responses and the changes in RSNA were significantly smaller in *AdMnSOD*-transfected SHR compared with *AdLacZ*-transfected SHR (Fig. 3a). The adenovirus itself did not affect bicuculline-elicited responses because the changes in MAP and RSNA did not differ between *AdLacZ*-transfected SHR and nontransfected SHR (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 $\gamma$ -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 SHR (MAP  $165 \pm 2$ – $142 \pm 3$  mmHg,  $P < 0.001$ ,  $n = 5$  for each; change in MAP  $-23 \pm 4$  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 SHR and nontreated SHR (baseline cardiovascular values: Table 1, Fig. 3d).

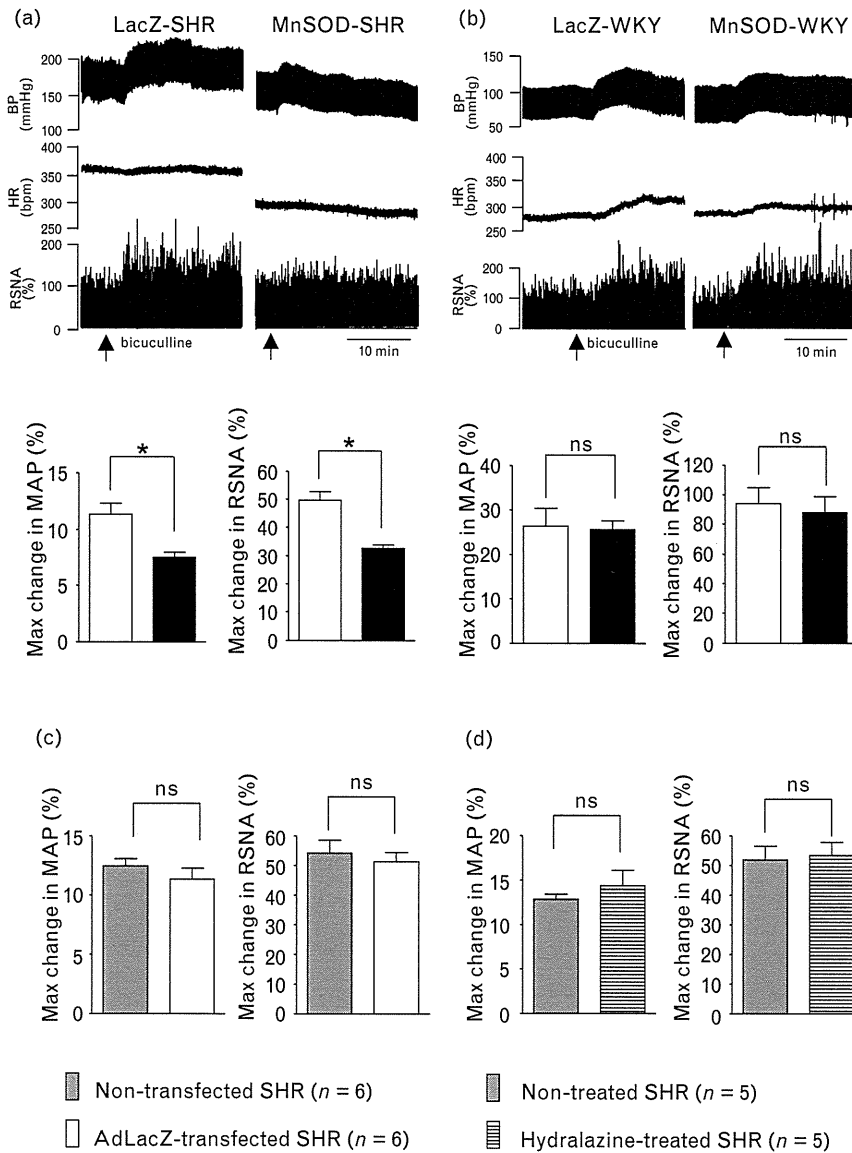
#### Discussion

In the present study, ROS in the RVLM enhanced glutamatergic excitatory inputs and attenuated the GABAergic inhibitory inputs to the RVLM neurons in SHR. 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 SHR 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 SHR 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 SHR 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 SHR in the acute experiments, further supporting our data in MnSOD overexpression in SHR. The excitatory inputs to the RVLM are enhanced and inhibitory inputs are reduced in SHR, thereby increasing the central sympathetic outflow as one of the neural mechanisms



Fig. 3

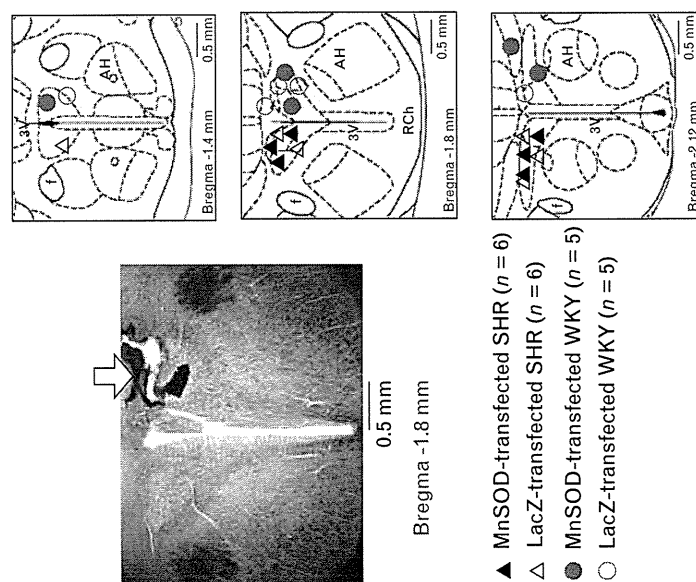


Changes in mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) induced by the microinjection of bicuculline (100 pmol) [(a)  $n = 6$  for each; (b)  $n = 5$  for each; (c)  $n = 6$  for each; and (d),  $n = 5$ ] for each unilaterally into the paraventricular nucleus (PVN) at day 7 after the gene transfer [adenovirus vectors encoding the *manganese superoxide dismutase* (*AdMnSOD*) or  *$\beta$ -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  $\pm$  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; 3V, third ventricle; LacZ,  $\beta$ -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 peroxy-nitrite 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 SHR. 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

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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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<b>Aims</b>	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.
<b>Methods and results</b>	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.
<b>Conclusion</b>	These results indicate that decreased brain S1R contributes to the relationship between heart failure and depression in a mouse model of pressure overload.
<b>Keywords</b>	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.<sup>1,2</sup> Heart failure and depression are both individually associated with poor health outcomes, and depression also adversely affects heart failure outcomes.<sup>3,4</sup> Importantly, depression is associated with the risk of heart failure among patients with hypertension.<sup>5</sup> Furthermore, hypertensive heart disease is well recognized as the major cause of heart failure.<sup>6</sup> One of the physiological mechanisms involved in both depression and heart failure is high sympathetic tone caused by cardiovascular autonomic dysregulation.<sup>7,8</sup> The central nervous system contributes to the worsening of

both heart failure<sup>9</sup> and hypertension.<sup>10</sup> 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.<sup>11</sup> S1R ligands have been reported to have antidepressant activity in behavioural models of depression.<sup>12</sup> The S1R has been shown to modulate neuronal intracellular calcium levels<sup>13</sup> and *N*-methyl-D-aspartate-mediated response.<sup>14,15</sup> 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,<sup>16</sup>

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and DHEAS was reported to decrease in heart failure.<sup>17</sup> 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.<sup>18</sup> The relationship between hypertension and depression has been well recognized, and depression is associated with the risk of heart failure among patients with hypertension.<sup>5</sup> In addition, the pressure overload model was reported to decrease S1R expression in the left ventricle.<sup>16,19</sup> 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.<sup>18</sup> We investigated (i) sympathetic activity by 24-h urinary norepinephrine (U-NE) excretion<sup>18,20</sup> and cardiac function by echocardiography<sup>18,20</sup>; (ii) brain S1R expression; (iii) the index of depression-like behaviour; (iv) the effects of intracerebroventricular (ICV) infusion of PRE084, a selective S1R agonist,<sup>24,25</sup> and BD1063, a selective S1R antagonist,<sup>21,22</sup> 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<sup>18,20</sup> 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).<sup>18,20</sup> 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<sup>18,20</sup> 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.<sup>24,25</sup> 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).<sup>24</sup> 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.<sup>25</sup> In chronic experiments, sympathetic activity was evaluated by measuring 24-h U-NE excretion using high-performance liquid chromatography.<sup>18,20</sup>

### 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.<sup>18,20</sup> An infusion cannula (Alzet<sup>®</sup> 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).<sup>18,20</sup> 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  $\mu$ L/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  $\mu$ L/h for 4 weeks initiated concomitantly with HS intake) or BD1063, a specific S1R antagonist, into Sham (2 mM; infusion rate 0.11  $\mu$ L/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  $\sim$ 1.0 mg/kg/day.<sup>19</sup>

### 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<sup>®</sup> 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  $\mu$ g 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<sup>®</sup>-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.<sup>26</sup> Membranes were then incubated with horseradish peroxidase-