ABSTRACT: In a recent study, we examined the glycemic and autonomic nervous responses to probiotic strain Lactobacillus johnsonii La1, and presented evidence that intraduodenal (ID) injection of this bacteria affected autonomic nerves and lowered blood pressure (BP) in anesthetized rats. In the study presented here, we examined the feeding, autonomic and cardiovascular effects of the lactobacillus strain Lactobacillus paracasei ST11 (NCC2461) in rats, and found that it suppressed food intake. Moreover, ID injection of NCC2461 suppressed gastric vagal nerve activity (GVNA) and accelerated renal sympathetic nerve activity (RSNA) and BP in a dose-dependent manner. Pretreatment with the histaminergic H1-receptor antagonist diphenhydramine eliminated the effects of NCC2461 on RSNA and BP. Furthermore, bilateral subdiaphragmatic vagotomy did not affect elevating effects of NCC2461, suggesting that vagal afferents are not involved in the pathway of NCC2461 effects. These evidences thus suggest that NCC2461 may exert its hypophagic and hypertensive actions through changes in autonomic neurotransmission via the central histaminergic nerves.

KEY WORDS: Afferent vagal nerve, Cardiovascular function, Food intake, Histaminergic nerve, Kidney, Lactobacillus paracasei ST11

INTRODUCTION

Because we observed in a previously published study that intraduodenal (ID) injection of the probiotic strain Lactobacillus johnsonii La1 (NCC533) suppressed RSNA and BP in urethane-anesthetized rats (Tanida et al. 2005c), we speculated that this viable strain could be useful for the management of hypertension. On the other hand, the probiotic strain Lactobacillus paracasei ST11 (NCC2461), another well documented strain, has been found to efficiently strengthen the host defense system by secretion of various cytokines (Ibuno-Zekri et al. 2003, von der Weild et al. 2001). Previous studies have observed that response of cytokines secretion to NCC2461 was different from NCC533 (Ibuno-Zekri et al. 2003, von der Weild et al. 2002). It is well known that some cytokines affect autonomic nerves (Hori et al. 1995, Niijima et al. 1995), which play an important role in the regulations of blood pressure (BP) and glucose metabolism (Buijs et al. 2001, Nagai et al. 1996, Tanida et al. 2005a). With respect to BP regulation or feeding regulation, some evidences that renal sympathetic nerve or gastric parasympathetic nerve plays an important role in them, respectively, has been shown (Handa and Johns 1985, Shen et al. 2005). Thus, we therefore hypothesized that ID injection of NCC2461 might cause a change in BP or feeding behavior by altering renal sympathetic nerve activity (RSNA) or gastric vagal nerve activity (GVNA), respectively.

The central histaminergic system is thought to modulate cardiovascular functions via histaminergic neurotransmission (Jochem 2000, Tanida et al. 2007a). We previously found that intra-cerebral ventricular (ICV) injection of high-dose histamine increased RSNA and BP, and that this effect was eliminated by pretreatment with diphenhydramine, a histamine H1 blocker (Tanida et al. 2005a). We also found that sectioning of afferent vagal nerves eliminated sympathetic and cardiovascular responses to duodenal stimulation with NCC533 (Tanida et al. 2005c), suggesting that the afferent neural pathway might be involved in autonomic changes due to duodenal stimulation. We therefore hypothesized that the histaminergic system or afferent neural pathway may be involved in the effects of NCC2461 on RSNA.
and BP. To understand the mechanisms of the autonomic and cardiovascular actions of NCC2461, we investigated the effect of diphenhydramine or subdiaphragmatic vagotomy on the changes in RSNA and BP resulting from ID injection of NCC2461.

MATERIALS AND METHODS

Animals
Male Wistar rats, weighing 300-350 g, were used. Rats were housed in a room maintained at 24 ± 1°C and illuminated for 12 h (07:00 to 19:00) everyday. Food and water were freely available. Rats were adapted to the environment for at least 1 week prior to the experiment. The Institutional Animal Care and Use Committee of Osaka University approved all animal care and handling procedures.

Measurement of Food Intake
On the day of testing, the rats were confirmed to have had normal food intake and have normal body weight. Twelve rats, matched on the basis of body weight, were divided into three groups (n=4 for each) and were presented water, NCC2461 (0.5×10^9 cfu/ ml water) or NCC533 (0.5×10^9 cfu/ ml water). These solutions were provided at 1700 hr to unrestrained, unanesthetized rats using the water supply bottle. Food and water were freely available. Cumulative food and water consumptions were measured for 24 hours.

General Surgery
On the experimental day, food was removed 3-4 h prior to surgery. General preparation was performed as described previously (Tanida et al. 2005a, Tanida et al. 2005b). Briefly, a polyethylene catheter was inserted into the left femoral vein and the duodenal cavity for intravenous (IV) and intraduodenal (ID) injections respectively, and another catheter was inserted into the left femoral artery for BP determination under anesthesia induced by intraperitoneal (IP) injection of 1g/kg urethane. The rats were then cannulated intratracheally, fixed in a stereotaxic apparatus, and maintained at 37.0-37.5°C. We monitored the depth of anesthesia with paw pinch tests (Mutoh 2000). For recording RSNA, the left renal nerve was exposed retroperitoneally through a left flank incision. For recording gastric vagal nerve activity (GVNA), the gastric branch of the ventral subdiaphragmatic vagal nerve was identified and exposed on the esophagus after midline abdominal incision. The distal end of nerve was ligated, and then hooked to a pair of silver wire electrodes for recording efferent nerve activity. The recording electrodes were immersed in a pool of liquid paraffin oil to prevent dehydration and for electrical insulation. The rat was allowed to stabilize for 30-60 min after placement of the recording electrodes. Electrical changes in the RSNA and GVNA were amplified, filtered and monitored by an oscilloscope. Raw data of the nerve activity was converted to standard pulses by a window discriminator. Data were obtained as described previously (Tanida et al. 2005b). The catheter in the left femoral artery was connected to a BP transducer, whose output signal was amplified in a BP amplifier and averaged to produce mean arterial pressure (MAP). The BP was monitored with an oscilloscope, sampled with the Power-Lab and stored on a hard disk for off-line analysis.

Experimental Procedure
Baseline measurements of the RSNA, GVNA and MAP were made for 5 min before ID injection of NCC2461 (1×10^9 cfu/2 ml water) or water (2 ml). Lyophilized culture of NCC2461 was used in the experiments after dissolving into water. The indicated parameters were recorded for 60 min after the injection. To investigate the effect of diphenhydramine, a histaminergic H1-receptor antagonist, a brain cannula made of polyethylene tubing was inserted into the left lateral cerebral ventricle under pentobarbital anesthesia 1 week before the experiment (Tanida et al. 2005a, Tanida et al. 2006). Diphenhydramine hydrochloride (5 µg/10 µl aCSF, ICV) was administered 15 min prior to the NCC2461 injection using the brain cannula. At the end of the experiment, hexamethonium chloride (10 mg/kg, IV) was administered to block action potentials of post-ganglionic neural activity in order to determine the noise level of the recording. Then, animals were sacrificed. In some rats (n=4), vagotomy of afferent nerves was performed prior to NCC2461 injection. For cutting the subdiaphragmatic vagus nerve, stomach was retracted through a midline abdominal incision, and the nerve bundles of anterior and posterior vagi were dissected from the esophagus and sectioned using an ophthalmic clip. Following vagotomy treatment, experiment was carried out. Control rats (n=4) received a sham-operation without the application of cutting. Following vagotomy treatment, experiment was carried out.

FIGURE 1. Effects of ingestion of water, Lactobacillus paracasei ST11 (NCC2461) or Lactobacillus johnsonii La1 (NCC533) on cumulative (24 hrs) food intake. All data are expressed as means ± SEM. Numbers of animals used are shown in the parentheses. * P<0.05 versus water-group.
Statistical Analysis

RSNA, GVNA and MAP were measured during each 5 min period after NCC2461 injection and analyzed by digital signal processing analyses. All data were expressed as means ± S.E.M. Mann-Whitney U-test was used to compare basal levels in the each groups. Because of inter-individual variability in the pre-injection state, percent changes from the baseline values were calculated for the RSNA and GVNA. Absolute value changes from the baseline were calculated for MAP. Two-way ANOVA and was applied to compare group responses of the RSNA, GVNA and MAP. With respect to analysis of food intake data, multiple comparisons after ANOVA were performed by Fisher’s LSD post hoc test. *P*<0.05 was considered statistically significant.

FIGURE 2. Effects of intraduodenal injection of *Lactobacillus paracasei* ST11 (NCC2461) on GVNA. (A) Data of typical recordings of the GVNA of a rat injected a water or NCC2461. Arrows indicate injection points. Lower bars represent 20 min, vertical scale bars to the right of the recordings represent neural discharge rates of 100 spikes/5sec. GVNA (B) after intraduodenal injection of water or NCC2461 are expressed as means ± SEM of percentages of values at 0 min. Numbers of animals used are shown in the parentheses. Significant differences (* P<0.05) between values from 5-60 min after intraduodenal injection of water or NCC2461 are analyzed by ANOVA

<table>
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<tr>
<th>Group</th>
<th>RSNA (spikes/5sec)</th>
<th>BP (mmHg)</th>
<th>GVNA (spikes/5sec)</th>
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<tr>
<td><strong>Experiment (1)</strong></td>
<td></td>
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<tr>
<td>water</td>
<td>174.6±16.5 (4)</td>
<td>84.8±7.7 (4)</td>
<td>86.5±12.3 (4)</td>
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<td>NCC2461 (1x10^8 cfu)</td>
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<td>NCC2461 (1x10^9 cfu)</td>
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<td>81.1±3.2 (4)</td>
<td>99.3±20.7 (4)</td>
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<td><strong>Experiment (2)</strong></td>
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<td></td>
</tr>
<tr>
<td>aCSF + water</td>
<td>111.9±17.3 (4)</td>
<td>79.8±8.7 (4)</td>
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<tr>
<td>aCSF + NCC2461</td>
<td>125.2±13.4 (4)</td>
<td>81.8±15.2 (4)</td>
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<tr>
<td>diphen + NCC2461</td>
<td>163.8±47.5 (4)</td>
<td>82.3±7.9 (4)</td>
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<tr>
<td><strong>Experiment (3)</strong></td>
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<td>136.5±7.9 (4)</td>
<td>84.7±6.8 (4)</td>
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<tr>
<td>vagotomy, NCC2461</td>
<td>127.9±16.4 (4)</td>
<td>83.6±9.1 (4)</td>
<td></td>
</tr>
</tbody>
</table>

RSNA, renal sympathetic nerve activity; MAP, mean arterial pressure; GVNA, gastric vagal nerve activity. Numbers of animal used are shown in the parentheses. Data are shown as means ±SEM.
Typical recordings of RSNA and BP in the 60 min following an intestinal injection of NCC2461 are shown in Fig. 3A. Water injection did not affect RSNA or BP, but NCC2461 injection (1×10⁹ cfu) gradually elevated RSNA, which reached a maximum of 190.3 ± 25.9% at 50 min (Fig. 3B), and MAP, which ascend to 8.0 ± 4.2mmHg at 60 min the last time examined (Fig. 3 C). In contrast, water injection did not significantly affect levels of RSNA and MAP, at least up to 60 min after the injection. These effects appeared to be dose-dependent, because 1×10⁸ cfu NCC2461 affected RSNA and MAP to a lesser extent than 1×10⁹ cfu NCC2461. The significance of differences between groups analyzed by ANOVA for values obtained from 5-60 min after the injection were RSNA: water vs. NCC2461 (1×10⁹ cfu), not significant (NS) (F = 0.14); water vs. NCC2461 (1×10⁸ cfu), P < 0.0005 (F = 3.97); NCC2461 (1×10⁸ cfu) vs. NCC2461 (1×10⁹ cfu), P < 0.005 (F = 3.34); MAP: water vs. NCC2461 (1×10⁹ cfu), NS (F = 0.93); water vs. NCC2461 (1×10⁸ cfu), P < 0.05 (F = 2.0); NCC2461 (1×10⁸ cfu) vs. NCC2461 (1×10⁹ cfu), P < 0.005 (F = 2.63). No significant differences were detected among the 3 groups in the respective basal values at 0 min (Table 1).

Since the suppressive effects of high dose histamine on RSNA and BP were eliminated by intracranial injection of diphenhydramine in urethane-anesthetized rats (Tanida et al., 2007a), we examined the effect of ICV pre-injection of diphenhydramine on changes in RSNA and MAP caused by NCC2461 injection (Fig. 4). NCC2461 significantly elevated RSNA and MAP in rats compared to aCSF-injected rats pre-injected with aCSF (Fig. 4 A and B). However, the renal sympathetic and cardiovascular responses to NCC2461 were attenuated by ICV pre-injection of diphenhydramine. The significance of the differences between values from 5-60 min as a group was analyzed by ANOVA. The following comparisons were made: RSNA: aCSF-water vs. aCSF-NCC2461, P < 0.0005 (F = 3.99); aCSF-water vs. diphen-NCC2461, NS (F = 0.48); aCSF-NCC2461 vs. diphen-NCC2461, P < 0.05 (F = 1.74), MAP: aCSF-water vs. aCSF-NCC2461, P < 0.05 (F = 1.89); aCSF-water vs. diphen-NCC2461, NS (F = 0.52); aCSF-NCC2461 vs. diphen-NCC2461, P < 0.005 (F = 2.68). Absolute basal (0 min) RSNA and MAP values for the experiments shown in Fig. 4 are

FIGURE 3. Effects of intraduodenal injection of Lactobacillus paracasei ST11 (NCC2461) on RSNA and BP. (A) Data of typical recordings of the RSNA and BP of a rat injected a water or NCC2461. Arrows indicate injection points. Lower bars represent 20 min, vertical scale bars to the right of the recordings represent neural discharge rates of 100 spikes/5sec or BP values of 200 mmHg. RSNA (B) and MAP (C) after intraduodenal injection of water or NCC2461 are expressed as means ± SEM of the percentage of the value at 0 min (RSNA) or of absolute value change from the value at 0 min (MAP). Numbers of animals used are shown in the parentheses. Significant differences (* P<0.05) between values from 5-60 min after intraduodenal injection of water or NCC2461 are analyzed by ANOVA
summarized in Table 1. Differences in respective basal values were not statistically significant (Mann-Whitney U test).

FIGURE 4. Effects of diphenhydramine on changes in RSNA and MAP after intraduodenal injection of *Lactobacillus paracasei* ST11 (NCC2461). (A) Data of typical recordings of the RSNA and BP of a rat pre-injected an aCSF or diphenhydramine (dphen) centrally and injected NCC2461 intestinally. Arrows indicate injection points. Lower bars represent 20 min, vertical scale bars to the right of the recordings represent neural discharge rates of 100 spikes/5sec or BP values of 200 mmHg. RSNA (B) and MAP (C) after intraduodenal injection of NCC2461 are expressed as means + SEM of the percentage of the value at 0 min (RSNA) or of absolute value change from the value at 0 min (MAP). ICV injection of aCSF or diphenhydramine (dphen) was given 15 min before intraduodenal injection of NCC2461. Significant differences (*P<0.05) between values from 5-60 min after injection in groups are analyzed by ANOVA.

FIGURE 5. Effects of denervation of vagus nerves on changes in RSNA and MAP after intraduodenal injection of *Lactobacillus paracasei* ST11 (NCC2461). Data of typical recordings of the RSNA and BP of a sham-operated rat (sham) and a vagotomized rat (vagotomy) injected NCC2461. Arrows indicate injection points. Lower bars represent 20 min, vertical scale bars to the right of the recordings represent neural discharge rates of 100 spikes/5sec or BP values of 200 mmHg. RSNA (B) and MAP (C) after intraduodenal injection of NCC2461 are expressed as means + SEM of the percentage of the value at 0 min (RSNA) or of absolute value change from the value at 0 min (MAP). Data from sham-operated (sham) and vagotomized (vagotomy) rats are shown. Significant differences (*P<0.05) between values from 5-60 min after intraduodenal injection of NCC2461 are analyzed as groups by ANOVA.

We also examined the effects of vagotomy of afferent nerves on the sympathetic and cardiovascular changes elicited by ID injection of NCC2461 (Fig. 5). In both groups, RSNA and MAP were markedly elevated by NCC2461 injection (Fig. 5A and B). The significance of differences between values from 5-60 min analyzed as a group by ANOVA is as follows: RSNA: sham-NCC2461 vs. vagotomy-NCC2461, NS (F=1.42), MAP: sham-NCC2461 vs. vagotomy-NCC2461, NS (F=0.34). In sham-operated or vagotomized rats, basal (0 min) values of RSNA and MAP were shown in Table 1. Respective basal did not differ significantly by Mann-Whitney U-test.

DISCUSSION

In a recent study we found that the NCC533 affected autonomic nerves and suppressed 2-DG-induced hyperglycemia and BP in rats (Tanida et al. 2005c, Yamano et al. 2006). In the experiment reported here, we first examined the effects of ingestion of NCC533 or NCC2461, another probiotic strain, on feeding response in conscious rats, and found that NCC533 or NCC2461 stimulated or suppressed food intake, respectively (Fig. 1). In addition, to confirm autonomic responses to NCC2461, we
second investigated the effects of ID injection of NCC2461 on RSNA, GVNA and BP in urethane-anesthetized rats, and found that NCC2461 caused elevation of RSNA and BP and suppression of GVNA in dose-dependent manner (Fig. 2 and 3). Finally, we obtained data that effects of NCC2461 on RSNA and BP were eliminated by diphenhydramine, a histamine H1-receptor antagonist (Fig. 4). These findings therefore suggest that NCC2461 may affect autonomic nerves and modulate feeding behavior or cardiovascular function through mediation by central histaminergic neurotransmission.

It is well known that there is close relation between autonomic function and appetite modulation (Elmqquist 2001, Sakurai 2007), but it was not determined whether ingestion of lactobacillus such as NCC533 or NCC2461, affects feeding behavior. In this study, our finding that ad libitum consumption of beverages containing NCC533 or NCC2461 increases or decreases 24 hrs cumulative food intakes, respectively, suggests that lactobacillus might influence feeding regulation. Moreover, since it has been confirmed that suppressions of food intake and parasympathetic nerve discharge are caused simultaneously (Shen et al. 2005), we examined effects on GVNA and found that ID injection of NCC2461 decreases GVNA. With respect to feeding and autonomic regulations, central or peripheral peptides such as leptin, orexin and ghrelin etc. play an important role in regulating the above functions via respective receptors expressed in the hypothalamus (Elmqquist 2001, Sakurai 2007, Yoshihara et al. 2002). Our preliminary experiment confirmed that c-Fos induction of paraventricular nucleus, one of some hypothalamic nuclei, was activated by ID injection of NCC2461 (unpublished data). Thus, it is suggested that NCC2461 might affect food intake and autonomic nerves through the hypothalamic control. However, to understand detail mechanism in effects of NCC2461, it will be needed to investigate these peptides changes.

It is well known that histaminergic neurons play an important role in the regulation of sympathetic and cardiovascular functions (Jochem 2000, Tanida et al. 2007a). Recently we showed that central injection of low-dose histamine lowered, and that of high-dose histamine elevated RSNA and BP (Tanida et al. 2007a). In addition, the effects of low- or high-dose histamine were blocked by thioperamide or diphenhydramine, respectively (Tanida et al. 2007a). These findings suggest that central histamine may affect RSNA and BP through the various histaminergic receptors. In support of this notion, a previous study provided evidence that the inhibitory effects of NCC533 were attenuated by thioperamide (Tanida et al. 2005c). In the study presented here, we found new evidence, namely, that the elevation of RSNA and BP due to NCC2461 was undone by diphenhydramine (Fig. 4). It is thus possible that NCC2461 may elevate RSNA and BP through the central histaminergic H1-receptors. On the other hand, With respect to the role of histaminergic receptors in synaptic neurotransmission, it is known that H1-receptors localized in the post-synaptic cleft are affected by large histamine releases from the pre-synaptic cleft in the histamine neurons (Arrang et al. 1983). The effect of NCC2461 via H1-receptors may therefore trigger a large release of histamine. However, we could not determine the validity of this hypothesis within the constraints of this study, so that further examination will be needed.

The abdominal afferent nerves are implicated in signal transduction from internal organs to brain (Date et al. 2002, Koda et al. 2005, Niijima 1998, Osaka et al. 2002). For example, the hyperthermic response to intestinal osmotic stimulation is attenuated in vagotomized rats (Osaka et al. 2002). Moreover, afferent nerves have been shown to participate in the effects of some peptide hormones, such as leptin (Niijima 1998), ghrelin (Date et al. 2002) and PYY (Koda et al. 2005), derived from internal organs. It may thus be thought that the afferent nerves are implicated in the sympathetic and cardiovascular effects of intestinal stimulation with NCC2461, but effects of NCC2461 were detected in vagotomized rats (Fig. 5). This suggests that such effects may be independent of the afferent nerve pathways. With respect to other pathways, it is possible that hormonal factors containing interleukin or TGF-β mediates the effects of NCC2461. A previous study found that NCC2461 elevated interleukin-10 levels and induced TGF-β production (von der Weid et al. 2001). In addition, peripheral administration of interleukin changed autonomic nerve activities in urethane-anesthetized rats (Hori et al. 1995, Niijima et al. 1995). These findings support the notion mentioned earlier, but to reveal detail mechanism, blood levels of these factors after ID injection of NCC2461 remains to be determined in a future study.

At this point in time, we can present two hypotheses as subjects for future investigations. The first concerns the possible role of the hypothalamic suprachiasmatic nucleus (SCN), a circadian oscillator located in the brain. We previously showed that the SCN is related to regulation of autonomic nerves (Buijs et al. 2001, Nagai et al. 1996), and that the SCN is involved in sympathetic and cardiovascular responses to intestinal stimulation with NCC533 (Tanida et al. 2005c). Moreover, RSNA and BP changes induced by hormonal factors, such as orexin-A (Tanida et al. 2006), adiponectin (Tanida et al. 2007b) and L-carnosine (Tanida et al. 2005a), were undone in SCN-lesioned rats. To determine the involvement of the SCN in the effects of NCC2461, a disruption study of the SCN will be necessary. The second speculation concerns not only the role of RSNA, but also that of sympathetic nerves projecting into the adipose tissue. Since it is well known that the autonomic nerves innervating the brown and white adipose tissues play an important role in modulating energy metabolism or lipolysis (Niijima 1998, Shen et al. 2005), it is hypothesized that NCC2461 may affect sympathetic outflows to the aforementioned adipose tissues and thus cause changes in lipid metabolism. In support of this notion, our preliminary examination confirmed that ID administration of NCC2461 activated the neural activities of sympathetic nerves innervating the brown and white adipose tissues of rats, and that long-term administration of NCC2461 solution as the sole drinking water reduced the weights of body and abdominal fat tissues (unpublished data). Moreover, present study confirmed suppressing effects of NCC2461 on food intake. These findings thus support the idea that NCC2461 may affect autonomic nerves and cause changes in body weight.
The physiological role of the elevations of RSNA and BP induced by NCC2461 is not clear. It is generally known that there is a close relationship between obesity-related hypertension and sympathetic dysfunction (Haynes 2005). Also there is ample evidence to support regulations of energy balance and BP via the autonomic alteration. Some peptide hormones such as leptin, orexin and L-carnosine, affect both energy balance and cardiovascular function via the sympathetic elevation (Haynes 2005, Sakurai 2007, Tanida et al. 2005a, Tanida et al. 2006). In regard to this point, our preliminary study observed that ID injection of NCC2461 elevated temperature of the brown adipose tissue, one of energy production marker, in conscious rats (unpublished data). Then, NCC2461, which is involved in the control of energy balance, may be a nutritional mediator that is responsible for the generation and maintenance of hypertension induced by abnormality of energy balance.

In conclusion, we found that NCC2461 suppressed GVNA and elevated RSNA and BP in a dose-dependent manner, and that histaminergic H1-receptors are involved in the sympathetic and cardiovascular effects of NCC2461. Thus, it is suggested that NCC2461 might affect autonomic and cardiovascular changes in mechanism different from the effects of NCC553 via the histaminergic H3-receptor. Moreover, NCC2461 suppressed food intake during 24 hrs. Thus, it is suggested that NCC2461 plays an important role in autonomic and cardiovascular controls and has an anti-obesity action.

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