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Citation	Diabetes (2013), 62(5): 1500-1504
Issue Date	2013-05
URL	http://hdl.handle.net/2433/174106
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Type	Journal Article
Textversion	publisher

Intracerebroventricular Administration of C-Type Natriuretic Peptide Suppresses Food Intake via Activation of the Melanocortin System in Mice

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C-type natriuretic peptide (CNP) and its receptor are abundantly distributed in the brain, especially in the arcuate nucleus (ARC) of the hypothalamus associated with regulating energy homeostasis. To elucidate the possible involvement of CNP in energy regulation, we examined the effects of intracerebroventricular administration of CNP on food intake in mice. The intracerebroventricular administration of CNP-22 and CNP-53 significantly suppressed food intake on 4-h refeeding after 48-h fasting. Next, intracerebroventricular administration of CNP-22 and CNP-53 significantly decreased nocturnal food intake. The increment of food intake induced by neuropeptide Y and ghrelin was markedly suppressed by intracerebroventricular administration of CNP-22 and CNP-53. When SHU9119, an antagonist for melanocortin-3 and melanocortin-4 receptors, was coadministered with CNP-53, the suppressive effect of CNP-53 on refeeding after 48-h fasting was significantly attenuated by SHU9119. Immunohistochemical analysis revealed that intracerebroventricular administration of CNP-53 markedly increased the number of c-Fos-positive cells in the ARC, paraventricular nucleus, dorsomedial hypothalamus, ventromedial hypothalamic nucleus, and lateral hypothalamus. In particular, c-Fos-positive cells in the ARC after intracerebroventricular administration of CNP-53 were coexpressed with α -melanocyte-stimulating hormone immunoreactivity. These results indicated that intracerebroventricular administration of CNP induces an anorexigenic action, in part, via activation of the melanocortin system. *Diabetes* 62:1500–1504, 2013

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family and has been demonstrated to be abundantly present in the brain, interestingly in discrete hypothalamic areas, such as the arcuate nucleus (ARC) of the hypothalamus, that play pivotal roles in energy regulation (1–3). Two predominant molecular forms of CNP in the porcine brain were reported to be a 22-residue peptide (CNP-22) and its N-terminally elongated 53-residue peptide (CNP-53) (1). Moreover, natriuretic peptide receptor-B (NPR-B), a CNP receptor, is also widely distributed in the brain and is reported to be abundantly expressed in the ARC of the

hypothalamus (4,5). These findings indicate the possibility that the brain CNP/NPR-B system may regulate energy homeostasis.

In the current study, we examined the effects of intracerebroventricular administration of CNP on food intake induced by refeeding after fasting and by orexigenic peptides, such as neuropeptide Y (NPY) and ghrelin. Also, we examined the involvement of the melanocortin system in the CNP actions.

RESEARCH DESIGN AND METHODS

Animals and diets. Male C57BL/6J mice (6 weeks old) obtained from Japan SLC (Shizuoka, Japan) were housed in plastic cages in a room kept at a room temperature of $23 \pm 1^\circ\text{C}$ and a 12:12-h light–dark cycle (lights turned on at 9:00 A.M.). The mice had ad libitum access to water and food (CE-2; CLEA Japan, Tokyo, Japan). All experiments were performed at 10 weeks of age in accordance with the guidelines established by the Institutional Animal Investigation Committee at Kyoto University and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to optimize comfort and to minimize the use of animals.

Peptides. CNP-22, CNP-53, ghrelin, and NPY were purchased from Peptide Institute (Osaka, Japan). SHU9119 was purchased from Bachem AG (Bubendorf, Switzerland).

Intracerebroventricular injection. Intracerebroventricular injection was performed according to our previous report (6).

Measurement of food intake

Fasting-refeeding. Mice were fasted for 48 h and then refed for 4 h. Water was available ad libitum during the experiments. The intracerebroventricular or intraperitoneal administration of CNP-22 or CNP-53 was performed just before refeeding. Food intake was measured for 4 h of refeeding. At the end of experiments, the hypothalamus was collected for examination of the expressions of mRNA for neuropeptides (7).

Nocturnal food intake. To assess the effect of intracerebroventricular administration of CNP-22 or CNP-53 on nocturnal food intake, peptides were injected intracerebroventricularly 1 h before the beginning of the dark phase. Food intake was measured for 15 h after intracerebroventricular injection. Water was available ad libitum during the experiments.

Food intake induced by NPY and ghrelin. The experiments were performed from 11:00 A.M. to 3:00 P.M. CNP-22 or CNP-53 was intracerebroventricularly administered just before intracerebroventricular injection of NPY (5 nmol/mouse) or intraperitoneal injection of ghrelin (100 nmol/kg). Food intake was measured for 4 h after peptide injection. In these experiments, food and water were available ad libitum.

PCR. The extraction of mRNA and quantitative real-time RT-PCR were performed according to our previous report (8). Primers for prepro-melanocortin, cocaine and amphetamine-related peptide, NPY, agouti gene-related peptide (*AgRP*) and glyceraldehyde 3-phosphate dehydrogenase are shown in Supplementary Table 1.

Immunohistochemistry for c-Fos and α -MSH in the hypothalamus. The immunohistochemical methods and the stereotaxic coordinates for the hypothalamic nuclei were based on our previous report (6). Briefly, mice were anesthetized with pentobarbital at 1 h after intracerebroventricular injection of CNP-53 (1.5 nmol/mouse) and perfused with 50 mL 0.1 mol/L PBS, followed by 50 mL ice-cold 4% paraformaldehyde in 0.1 mol/L PBS. Sections of 30- μm thickness were cut with a cryostat. According to the mouse brain atlas (9), cross-sections were selected in correspondence to -1.70 mm [ARC, lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), ventromedial hypothalamic

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This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0718/-/DC1>.

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See accompanying commentary, p. 1379.

nucleus (VMH) and to -0.82 mm [paraventricular nucleus (PVN)], relative to bregma. For c-Fos and α -melanocyte-stimulating hormone (α -MSH) protein staining, the sections were incubated with anti-c-Fos rabbit antibody (Ab-5; 1:5,000; Oncogene Science, Cambridge, MA) and anti- α -MSH sheep antibody (AB5087; 1:10,000; EMD Millipore, Billerica, MA), respectively. The antibody was detected using the Vectastain ABC Elite kit (PK-6101; Vector Laboratories, Burlingame, CA) and a diaminobenzidine substrate kit (SK-4100; Vector Laboratories) was used for visualization. The second antibodies for fluorescence visualization used were goat anti-rabbit488 (A11008; 1:200; Life Technologies, Carlsbad, CA) for anti-c-Fos rabbit antibody and goat anti-sheep546 (A21098; 1:200; Life Technologies) for anti- α -MSH sheep antibody.

Data analysis. All values are given as the mean \pm SEM. Statistical analysis of the data were performed by ANOVA, followed by the Tukey-Kramer test. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of intracerebroventricular administration of CNP-22 and CNP-53 on food intake at refeeding after fasting. The intracerebroventricular administration of CNP-22 (1.5 and 4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) significantly suppressed food intake during 4-h refeeding after 48-h fasting in comparison with data from saline-treated mice (Fig. 1A). In this experiment, CNP-53 (1.5 nmol), but not other treatments, induced significant reduction of body weight compared with saline treatment (Supplementary Table 2). The mRNA expressions of pre-*opiomelanocortin* and cocaine and amphetamine-related peptide significantly decreased, and the mRNA expressions of *NPY* and *AgRP* significantly increased after refeeding compared with control animals (Supplementary Fig. 1). The intracerebroventricular administration of CNP-53 did not influence the mRNA expressions of these neuropeptides in the hypothalamus (Supplementary Fig. 1). Next, the peripheral action of CNP on food intake was examined when a 10-fold greater dose than intracerebroventricular injection of each CNP was intraperitoneally administered. The intraperitoneal administrations of CNP-22 (1.5 μ mol/kg) and CNP-53 (0.5 μ mol/kg) did not change the food intake during 4-h refeeding after 48-h fasting (Fig. 1B), nor were there changes in body weight (Supplementary Table 3).

The intracerebroventricular administrations of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) at 1 h before the start of the dark phase significantly suppressed nocturnal food intake compared with saline treatment (Fig. 1C).

Effect of intracerebroventricular administration of CNP-22 and CNP-53 on NPY-induced and ghrelin-induced food intake. When CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) were concomitantly administered intracerebroventricularly with NPY, they significantly suppressed the food intake induced by NPY compared with that of saline treatment (Fig. 2A). When CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) were administered intracerebroventricularly with ghrelin, they significantly suppressed the food intake induced by ghrelin compared with that of saline treatment (Fig. 2B).

Effect of melanocortin receptor antagonist, SHU9119, on the anorectic effect of CNP. To examine its involvement in the anorectic effect of CNP, SHU9119 was administered intracerebroventricularly together with CNP-53 (1.5 nmol/mouse). SHU9119 (1 nmol/mouse) significantly attenuated the suppressive action of CNP-53 on the food intake during 4-h refeeding after 48-h fasting, whereas SHU9119 itself significantly enhanced the increase of food intake in comparison with mice administered saline treatment (Fig. 3).

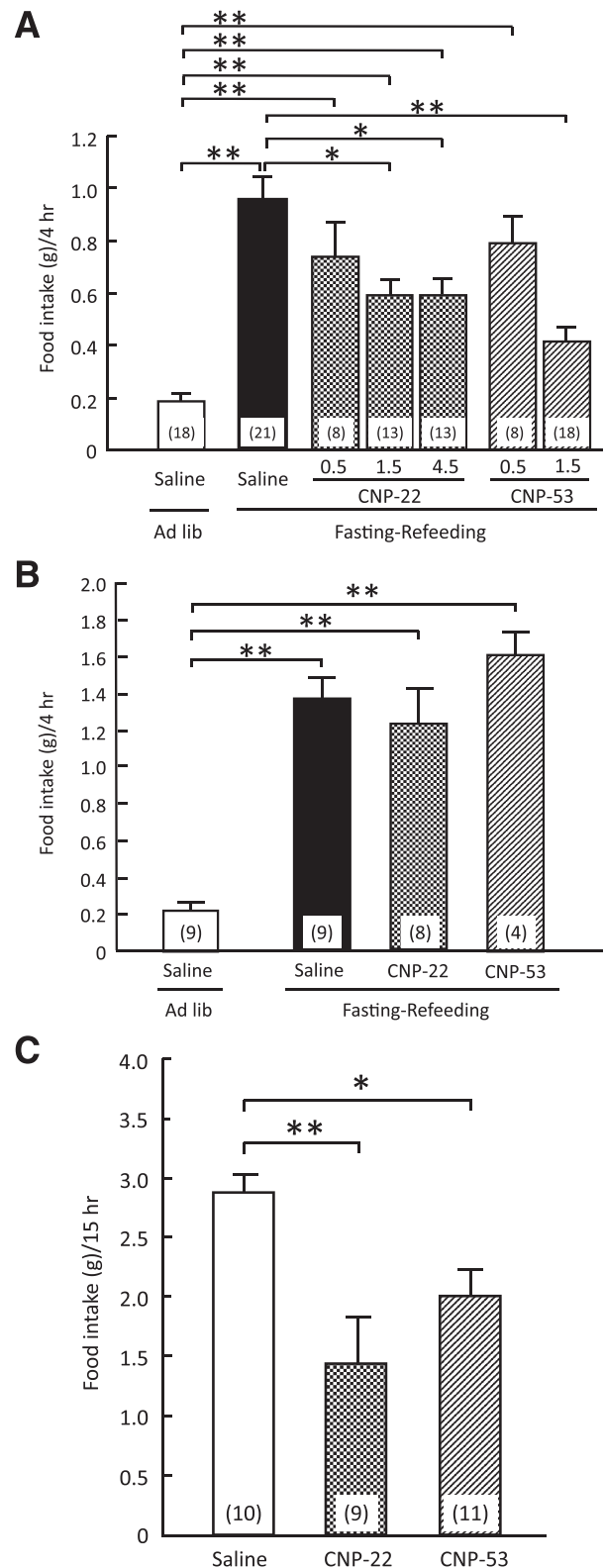


FIG. 1. Effects of CNP on refeeding after fasting. **A:** Effects of intracerebroventricular administration of CNP-22 (0.5, 1.5, and 4.5 nmol/mouse) and CNP-53 (0.5 and 1.5 nmol/mouse) on 4-h refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. **B:** Effects of intraperitoneal administration of CNP-22 (1.5 μ mol/kg) and CNP-53 (0.5 μ mol/kg) on 4-h refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. **C:** Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on nocturnal food intake in mice. Food intake was observed for 15 h after intracerebroventricular injection. Data represent mean \pm SEM. The number of mice is given in parentheses. Significant differences: * $P < 0.05$, ** $P < 0.01$.

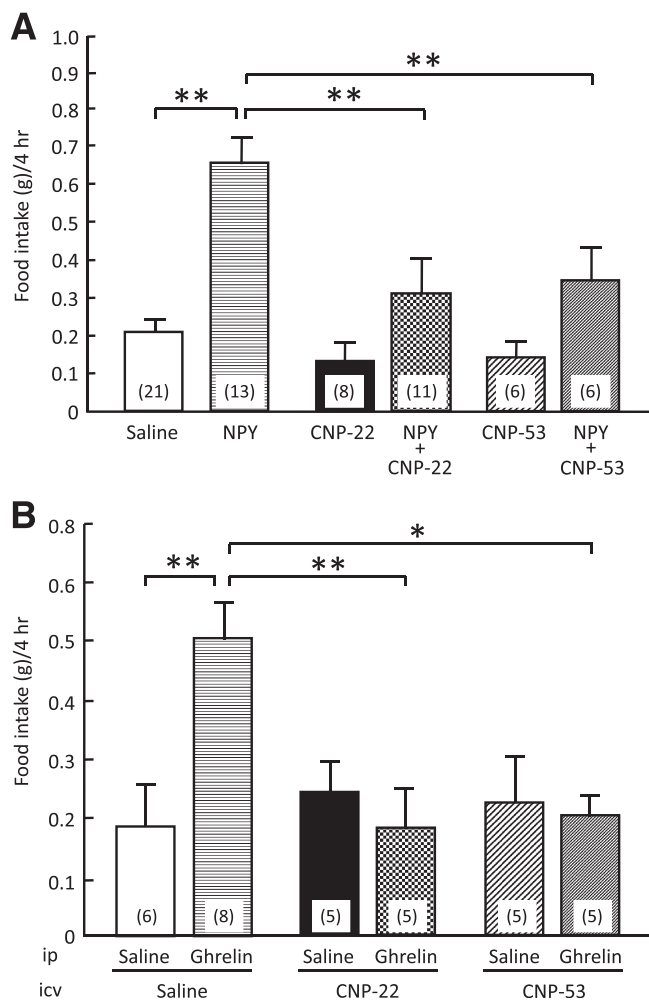


FIG. 2. Effects of CNP-22 and CNP-53 on food intake induced by NPY and ghrelin. **A:** Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on NPY-induced (5 nmol/mouse, intracerebroventricular) food intake in mice. Food intake was observed for 4 h after coadministration of NPY and CNP. **B:** Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on ghrelin-induced (100 nmol/kg, intraperitoneal) food intake in mice. Food intake was observed for 4 h after coadministration of ghrelin and CNP. Data represent mean \pm SEM. The number of mice is given in parentheses. Significant differences: * P < 0.05, ** P < 0.01.

c-Fos-immunoreactive cells in the hypothalamus after intracerebroventricular administration of CNP.

To understand the neuronal pathway involved in the anorectic actions of CNP, the expression of c-Fos, one of the markers of neuronal activation, was monitored by immunohistochemical examination at 1 h after intracerebroventricular injection of CNP-53 (1.5 nmol/mouse). The numbers of c-Fos-immunoreactive cells in the ARC, PVN, and DMH were predominantly increased after intracerebroventricular injection of CNP-53 in comparison with saline treatment (Fig. 4A). The c-Fos-positive cells were also moderately increased in the VMH and LH (Fig. 4A). Next, we examined whether c-Fos immunoreactivity coexisted with α -MSH-containing cells. In the ARC of saline-treated mice, only a few α -MSH-immunoreactive cells showed weak c-Fos immunoreactivity (Fig. 4B). However, c-Fos-immunoreactive cells that increased with intracerebroventricular administration of CNP-53 in the ARC expressed a large amount of α -MSH immunoreactivity (Fig. 4B).

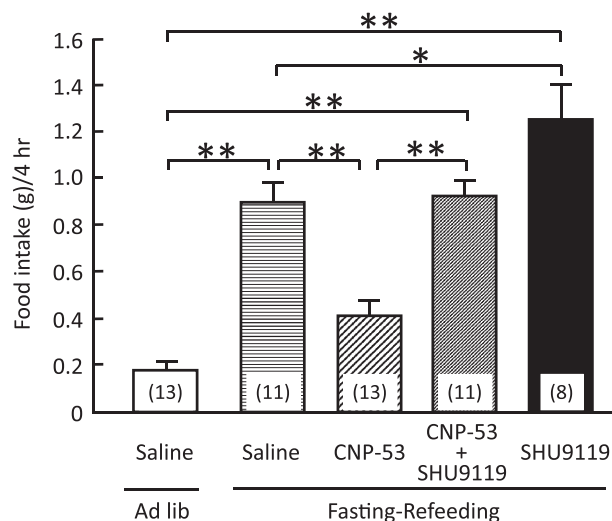


FIG. 3. Effects of intracerebroventricular administration of CNP-53 (1.5 nmol/mouse) and SHU9119 (1 nmol/mouse) on refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. Data represent mean \pm SEM. The number of mice is given in parentheses. Significant differences: * P < 0.05, ** P < 0.01.

DISCUSSION

The current study demonstrated that intracerebroventricular administration of CNP-22 and CNP-53, but not intraperitoneal injection, led to significant reduction of food intake induced by fasting-refeeding. This reduction was inhibited by the melanocortin-3 receptor (MC3R)/melanocortin-4 receptor (MC4R) antagonist SHU9119. In addition, CNP significantly suppressed nocturnal food intake and orexigenic actions induced by NPY and ghrelin. The immunohistochemical study revealed that intracerebroventricular administration of CNP-53 increased the number of c-Fos-expressing cells containing α -MSH in the hypothalamus. These findings indicated that the intracerebroventricular administration of CNP exhibits anorexigenic actions partially via activation of the melanocortin system, although the doses of CNP used in the current study could be pharmacological doses.

The hypothalamus is considered to be an important region in regulating energy homeostasis. In particular, the ARC in the hypothalamus contains both an orexigenic peptide, NPY, and an anorexigenic peptide, α -MSH, and is postulated to be involved in the first-order regulation of food intake. Synthetic MC3R/MC4R agonists, melanotan II, and [Nle⁴-D-Phe⁷]- α -MSH completely blocked food deprivation-induced increase in food intake as well as the food intake stimulated by intracerebroventricular administration of NPY (10,11). Regarding the reciprocal interactions of α -MSH and NPY, melanocortin neurons in the ARC project to the PVN (12). In the current study, intracerebroventricular administration of CNP significantly suppressed food intake after fasting, which was antagonized by SHU9119. Our results also showed that CNP suppressed NPY-induced food intake. Taken together, these findings indicate that CNP exhibits anorexigenic actions via activation of MC3R/MC4R downstream signaling. However, mRNA expressions of preproimelanocortin, cocaine and amphetamine-related peptide, NPY, and AgRP in the hypothalamus after the intracerebroventricular injection of CNP-53 in fasting-refeeding experiment did not change compared with those after saline. The reason for this

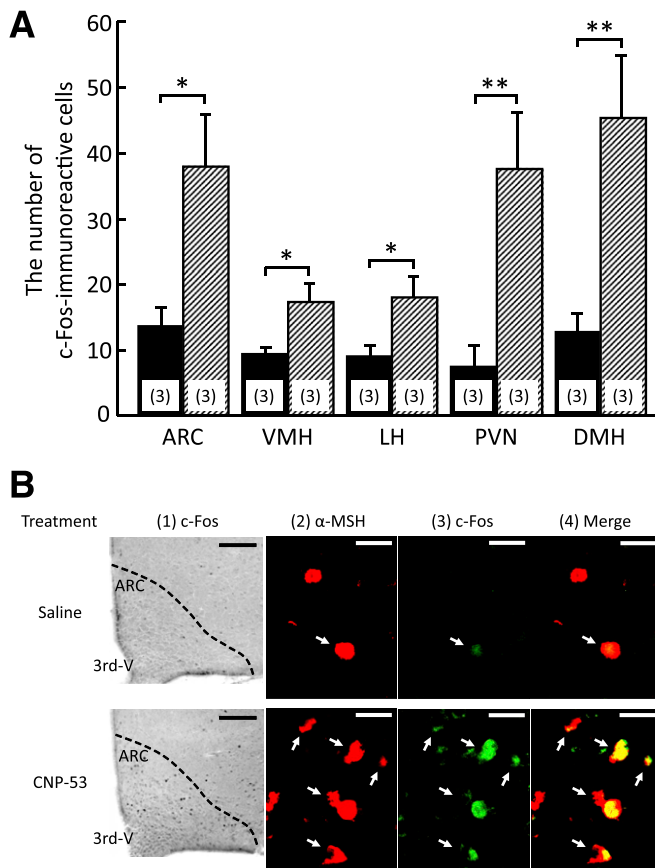


FIG. 4. The c-Fos-immunoreactive cells in the hypothalamus after intracerebroventricular administration of CNP-53 (1.5 nmol/mouse). **A:** Number of c-Fos-immunoreactive cells after saline and CNP-53 treatments. Data represent mean \pm SEM. The number of mice is given in parentheses. Significant differences: * $P < 0.05$, ** $P < 0.01$. **B:** c-Fos-immunoreactive cells induced by intracerebroventricular administration of saline and CNP-53 (1). 3rd-V, the third ventricular. Scale bars, 100 μ m. Coexistence of α -MSH (red) and c-Fos (green) immunoreactivity in the ARC (2–4) after saline (upper) and CNP-53 (1.5 nmol/mouse; lower) treatments. White arrows indicate cells expressing both α -MSH and c-Fos immunoreactivity. 3rd-V, the third ventricular. Scale bars, 20 μ m.

discrepancy may lie in the experimental condition, time course, and regional specificity. To clarify this discrepancy, further examinations will be required.

This study demonstrated that the intracerebroventricular administration of CNP significantly suppressed the nocturnal food intake. Robust feeding during the nocturnal phase of the daily light–dark cycle was demonstrated to be attributed to the upregulation of NPY and its receptors (13). These findings indicate that CNP may decrease food intake in the nocturnal phase via suppression of NPY action.

In the current study, CNP significantly suppressed the increase in food intake induced by ghrelin, an orexigenic hormone secreted by the stomach (14). NPR-B, a CNP receptor, has been identified in appetite-regulating regions, such as the ARC, VMH, PVN, DMH, and LH (15). The systemic administration of ghrelin significantly increased NPY and AgRP expression in the ARC of the hypothalamus in fed and fasted rats (15), resulting in hyperphagia. The intracerebroventricular injection of melanotan II caused a significant decrease in ghrelin-induced food intake (16). These findings suggest that the actions of ghrelin are modulated by α -MSH and NPY systems. Furthermore, plasma ghrelin and hypothalamic ghrelin receptor mRNA

expression are reported to be increased after fasting (17,18). These findings suggest the possibility that intracerebroventricular administration of CNP activates the melanocortin system, which subsequently inhibits the action of NPY, resulting in a reduced increase of food intake induced by ghrelin.

To assess which hypothalamic nucleus is involved in the anorexigenic action of CNP, a marker for neuronal activity, c-Fos expression in the hypothalamus was examined after intracerebroventricular administration of CNP-53. The intracerebroventricular administration of CNP-53 significantly increased the number of c-Fos-expressing cells in several hypothalamic nuclei, such as ARC, PVN, DMH, VMH, and LH, indicating that CNP-53 directly or indirectly stimulates neurons in these hypothalamic nuclei. Especially in the ARC, the result was an increased number of c-Fos-immunoreactive cells containing α -MSH immunoreactivity, indicating that CNP stimulates α -MSH-containing neurons. This possibility is supported by the finding that the suppressive action of CNP-53 on food intake was blocked by concomitant administration of SHU9119, an MC3R/MC4R antagonist.

The current study has demonstrated the anorexigenic action of intracerebroventricular administration of CNP via activation of the melanocortin system. To define the precise effect of CNP in the brain on food intake, further investigation using mice with inducible brain-specific deletion of CNP or NPR-B/NPR-C will be required.

From the present findings, we postulate the possible mechanism for anorexigenic action of exogenous CNP to be as follows: CNP directly or indirectly acts on α -MSH-containing neurons and subsequently stimulates α -MSH release, resulting in suppression of food intake induced by NPY and ghrelin. This possible mechanism may apply to the suppressive effects of CNP on food intake after fasting and in the nocturnal phase. Further work is needed to define the pathophysiological significance of brain CNP in regulation of food intake.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Ministry of Health, Labour, and Welfare of Japan.

No potential conflicts of interest relevant to this article were reported.

N.Y.-G. and G.K. performed experiments, contributed to discussion, and wrote the manuscript. K.E., M.I., Y.O., Y.Y., T.K., A.Y., N.S.-A., H.A., and K.H. contributed to discussion. K.N. contributed to discussion, and reviewed and edited the manuscript. K.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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