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 neurotensin 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.

CNP-22 and CNP-53 are coexpressed with melanocortin-1 receptors in the hypothalamus, that play pivotal roles in energy regulation (1). Moreover, 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.

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 ± 1°C and a 12:12-h light–dark cycle (lights 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 expression 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.

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 preproenalocortin, 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
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 antic-Fos rabbit antibody (Ab-5; 1:5,000; Oncogene Science, Cambridge, MA) and ant α-MSH sheep antibody (AB5872A; 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 antic-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 ± 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 (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 pro-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 μmol/kg) and CNP-53 (0.5 μmol/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).
whether c-Fos immunoreactivity coexisted with diabetes. To understand the neuronal pathway involved in the anorexigenic 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) 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 ± 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 anorexigenic 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 prepromelanocortin, 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
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 and CNP-53 (1.5 nmol/mouse). A: 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, 100 μm.

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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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