C-Type Natriuretic Peptide as a New Regulator of Food Intake and Energy Expenditure

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The physiological implication of C-type natriuretic peptide (CNP) including energy metabolism has not been elucidated, because of markedly short stature in CNP-null mice. In the present study we analyzed food intake and energy expenditure of CNP-null mice with chondrocyte-targeted CNP expression (CNP-Tg/*Nppc*$^{-/-}$ mice), in which marked skeletal dysplasia was rescued, to investigate the significance of CNP under minimal influences of skeletal phenotypes. In CNP-Tg/*Nppc*$^{-/-}$ mice, body weight and body fat ratio were reduced by 24% and 32%, respectively, at 20 wk of age, and decreases of blood glucose levels during insulin tolerance tests were 2-fold exaggerated at 17 wk of age, as compared with CNP-Tg/*Nppc*$^{+/+}$ mice. Urinary noradrenalin excretion of CNP-Tg/*Nppc*$^{-/-}$ mice was greater than that of CNP-Tg/*Nppc*$^{+/+}$ mice by 28%. In CNP-Tg/*Nppc*$^{-/-}$ mice, rectal temperature at 1600 h was higher by 1.1 C, and uncoupling protein-1 mRNA expression in the brown adipose tissue was 2-fold increased, which was canceled by propranolol administration, as compared with CNP-Tg/*Nppc*$^{+/+}$ mice. Oxygen consumption was significantly increased in CNP-Tg/*Nppc*$^{-/-}$ mice compared with that in CNP-Tg/*Nppc*$^{+/+}$ mice. Food intake of CNP-Tg/*Nppc*$^{-/-}$ mice upon *ad libitum* feeding and refeeding after 48 h starvation were reduced by 21% and 61%, respectively, as compared with CNP-Tg/*Nppc*$^{+/+}$ mice. This study unveiled a new aspect of CNP as a molecule regulating food intake and energy expenditure. Further analyses on precise mechanisms of CNP actions would lead to the better understanding of the significance of the CNP/guanylyl cyclase-B system in food intake and energy expenditure. **(*Endocrinology* 151: 3633–3642, 2010)**

C-type natriuretic peptide (CNP) exerts its biological actions using a single-transmembrane guanylyl cyclase (GC), GC-B, which is also known as the natriuretic peptide receptor (NPR)-B, as a receptor (1, 2). CNP was first isolated from porcine brain and expected to be a neuropeptide (3), but the physiological significance of the CNP/GC-B system has been established in the vascular and skeletal systems (1, 4–8). It was reported that CNP and GC-B are expressed in the central and peripheral nervous systems (1, 7, 9–11). The physiological significance of CNP in the nervous system, however, has not been elucidated well. It has been proven that the hypothalamus is an important center to control food intake and energy expenditure (reviewed in Ref. 12). CNP mRNA was detected in the rat hypothalamus, especially in the arcuate nucleus (ARC) and paraventricular nucleus (13), and GC-B mRNA was reportedly expressed in neurons of the magnocellular and parvocellular paraventricular nuclei, the ARC, and the supraoptic nucleus of the rat hypothalamus (14). It is, therefore, speculated that CNP might par-

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Abbreviations: AgRP, Agouti-related protein; ARC, arcuate nucleus; BAT, brown adipose tissue; CART, cocaine- and amphetamine-regulated transcript; CNP, C-type natriuretic peptide; CPT, carnitine palmitoyltransferase; D2, type II iodothyronine deiodinase; FFA, free fatty acid; GC, guanylyl cyclase; GTT, glucose tolerance test; icv, intracerebroventricular; ITT, insulin tolerance test; MCH, melanin concentrating hormone; mtTFA, mitochondrial transcription factor A; NPY, neuropeptide Y; PGC, PPARγ-coactivator; POMC, proopiomelanocortin; PPAR, peroxisome proliferator-activated receptor; SNS, sympathetic nervous system; Tg, transgenic; UCP, uncoupling protein; WAT, white adipose tissues; WT, wild type.

ticipate in the regulation of food intake and energy expenditure.

We have shown that the disruption of CNP or GC-B gene (*Nppc* or *Npr2*, respectively) results in dwarfism and early death due to impaired endochondral ossification (7, 8). Although body weight of $Nppc^{-/-}$ or $Npr2^{-/-}$ mice was less than that of wild-type (WT) littermates (7, 8), it was speculated that major causes of their leanness were brain compression and difficulty to eat due to misalignment of tooth between upper and lower jaws, both of which were caused by deformity in the skull and cervical spine (8). Therefore, we generated $Nppc^{-/-}$ mice with chondrocyte-specific CNP expression by crossing $Nppc^{-/-}$ mice and CNP transgenic (Tg) mice that express CNP under the control of the promoter and enhancer of the mouse pro-α1(II) collagen gene (7) and investigated the physiological significance of CNP in the body weight control and metabolic homeostasis with the influence of skeletal problems being minimized.

Materials and Methods

Animals

Male $Nppc^{+/+}$ and $Nppc^{-/-}$ mice with the C57BL/6 genetic background and the transgene that expresses CNP in chondrocytes under the control of the promoter and enhancer of the mouse pro-α1(II) collagen gene (CNP-Tg/$Nppc^{+/+}$ and CNP-Tg/$Nppc^{-/-}$, respectively) were used in this study (7). Intending to completely rescue the dwarf phenotype of $Nppc^{-/-}$ mice, we used mice homozygous for the CNP transgene in this study. Male WT (Non-Tg/$Nppc^{+/+}$) C57BL/6 mice were purchased from Oriental BioService, Kyoto, Japan. Mice were housed in the specified pathogen-free mouse facility of Kyoto University Graduate School of Medicine with unrestricted access to food (F-2, Funabashi Farms, Japan) and water under a 14-h light, 10-h dark cycle (the light cycle is from 0700 h to 2100 h) at 23 C. The genotype of each mouse was determined by PCR using genomic DNA isolated from a tail tip as template, as described previously (7). Primers used are listed in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The PCR conditions were the initial denaturation at 95 C for 2 min, followed by 35 cycles of 95 C for 10 sec, 57 C for 10 sec, and 72 C for 30 sec, and the final extension at 72 C for 10 min. Naso-anal length and body weight were measured weekly from 7 d after birth. The experimental protocol of this study was approved by the Animal Research Committee, Kyoto University.

Body fat accumulation and blood and urinary parameters

At 20 wk of age, mice were anesthetized with the ip injection of 50-mg/kg pentobarbital, and epididymal, perirenal, mesenteric, sc white adipose tissues (WAT), and interscapular brown adipose tissue (BAT) were dissected to measure weight. Blood was collected from cardiac ventricles of mice *ad libitum* fed or overnight (1900 h to 1000 h) fasted at 18–20 wk of age under the anesthesia with the ip injection of 50-mg/kg pentobarbital. Blood glucose and serum free fatty acids (FFA) concentrations were measured by a portable glucose meter Glutest Neo (Sanwa Kagaku Kenkyusho, Nagoya, Japan) and a NEFA C-test (Wako Pure Chemical Industries, Osaka, Japan), respectively. Serum concentrations of insulin, leptin, and free T_4 were measured by an ultrasensitive rat insulin ELISA kit with a mouse insulin standard (Morinaga Institute of Biological Science, Kanagawa, Japan), a mouse Leptin ELISA kit (Millipore, Billerica, MA), and an Enzaplate N-FT4 (Bayer Medical, Tokyo, Japan) (15), respectively. Plasma ghrelin concentrations were estimated using an active ghrelin ELISA kit that recognizes n-octanoylated ghrelin (Mitsubishi Kagaku Iatron, Tokyo, Japan) as described previously (16). Mice were individually placed in metabolic cages (Shinano Manufacturing, Tokyo, Japan), and urine samples were collected for 24 h. Urinary noradrenalin concentrations were measured by HPLC in SRL, Tokyo, Japan. The data divided by urinary creatinine concentrations were used to estimate the whole body sympathetic nervous system (SNS) activity.

Glucose and insulin tolerance tests

The glucose tolerance test (GTT) and the insulin tolerance test (ITT) were performed at 17 wk of age. After 4 h fasting (1000 h to 1400 h), mice were ip injected with glucose (0.5 g/kg body weight) or human regular insulin (0.5 U/kg body weight). Blood was collected from a tail vein before the injection, and 30, 60, 90, and 120 min after the injection.

Blood pressure, heart rate, and core body temperature

Blood pressure and heart rate of male mice were measured by a tail-cuff method with a model MK-2000ST (Muromachi Kikai, Tokyo, Japan) at 1000 h to 1200 h. Mice were acclimated to the measurement by experiencing a series of 10 readings once a day for 2 d, and 10 consecutive readings were averaged for each mouse. Rectal temperature was measured as core body temperature with a digital thermometer 02PT (Shibaura Electronics, Saitama, Japan) at 1000, 1600, and 2200 h. A sensor was inserted 1 cm from the anus.

Food intake

Mice were individually housed in ordinary cages, and food intake was assessed by decreases in the weight of chow pellets on hoppers, accounting for spilled crumbs. Mice were acclimated to individual housing for 4 d, and food intake was measured for 3 d. To assess food intake at refeeding after starvation, mice were deprived of food for 48 h from 0900 h and returned to *ad libitum* feeding, and the food intake was measured for 2 h. To see an acute response to ghrelin in food intake, 360 μg/kg of rat ghrelin (Peptide Institute, Osaka, Japan) or saline was sc injected to each mouse at 1100 h on *ad libitum* feeding, and food intake after the injection was measured for 2 h, as described previously (16).

Analyses of mRNA expression

Mice were killed by cervical dislocation at 20 wk of age. Immediately after decapitation, whole hypothalami were dissected out using the fornix and chiasma opticum as landmarks, and interscapular BAT and sc WAT were obtained. Tissues were homogenized by a glass-Teflon homogenizer in a QIAzol reagent (QIAGEN, Hilden, Germany). Total RNA was extracted with an

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RNeasy mini kit (QIAGEN), according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent with an oligo deoxythymidine primer (Takara Bio, Otsu, Japan); the reaction was carried out at 42 C for 15 min and terminated by heating at 70 C for 2 min.

CNP and GC-B mRNA expression in tissues was assessed by RT-PCR, in which cDNA corresponding to 50 ng total RNA was used in the PCR step as template. Expression levels of neuropeptide Y (NPY), agouti-related protein (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), melanin-concentrating hormone (MCH), orexin, uncoupling protein (UCP)-1, mitochondrial transcription factor A (mtTFA), peroxisome proliferator-activated receptor (PPAR) γ, PPARγ-coactivator (PGC)-1α, type II iodothyronine deiodinase (D2), muscle-type carnitine palmitoyltransferase (CPT)-1 mRNA were quantified by a real-time RT-PCR with an ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Carlsbad, CA) and a SYBR Premix ExTaq kit (Takara Bio, Otsu, Japan) using a dilution series of the pooled cDNA as the standard. The PCR conditions were the initial denaturation at 95 C for 10 min, followed by 40 cycles of 95 C for 10 sec and 60 C for 32 sec. The mRNA level of each gene was normalized with that of a housekeeping gene, β-actin. Gene-specific primers used are shown in Supplemental Table 1.

Propranolol administration

CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ male mice at 20 wk of age were ip injected with propranolol (Sigma, St. Louis, MO) of 20 mg/kg body weight or vehicle (saline) once a day for 3 d, as described in Ref. 17. The interscapular BAT was obtained 2 h after the final injection of propranolol or vehicle, and UCP-1 mRNA expression in the BAT was analyzed, as described above.

Intracerebroventricular injection of CNP

RNA was extracted from the interscapular BAT of C57BL/6J male mice that received a single intracerebroventricular (icv) injection of 10-μg CNP (CNP-22 human, Peptide Institute, Osaka, Japan; dissolved in 2 μl of saline) or vehicle through a 27-gauge microsyringe placed in an appropriate position 4 h before they were killed, as described previously (18). UCP-1, PGC-1α, and D2 mRNA expression in the BAT was analyzed as described above.

Oxygen consumption and locomotor activity

Male mice at 15 wk of age were individually placed in air-tight $15 \times 15 \times 15$ cm plexiglass cages, and oxygen consumption was measured for 24 h by indirect calorimetry with a model MK-5000RQ with an analysis software MMS-2 (Muromachi Kikai, Tokyo, Japan) (19). Spontaneous locomotor activity was measured in a SUPERMEX apparatus with an analysis software CompACT AMS (Muromachi Kikai, Tokyo, Japan) (20). Mice were acclimated to the monitoring for 1 h once a day for 3 d before the 24-h recording.

Statistics

All data are shown as means ± SEM. Statistical differences between two groups and those among more than three groups were assessed by an unpaired *t* test and an ANOVA, respectively. Differences in naso-anal length, body weight, oxygen consumption, and locomotor activity between genotypes were assessed by a repeated measures ANOVA. The level of significance was set at $P < 0.05$.

Results

Growth and metabolic parameters

The growth of male Non-Tg/*Nppc*$^{-/-}$ mice was impaired in both naso-anal length and body weight as compared with male WT (Non-Tg/*Nppc*$^{+/+}$) mice (Fig. 1, A and B), as previously reported (7). The chondrocyte-targeted CNP expression increased the naso-anal length and body weight of male CNP-Tg/*Nppc*$^{-/-}$ mice as compared with those of male Non-Tg/*Nppc*$^{-/-}$ mice (Fig. 1, A and B). The naso-anal length of male CNP-Tg/*Nppc*$^{-/-}$ mice was still significantly less than that of male CNP-Tg/*Nppc*$^{+/+}$ mice, but it surpassed that of male Non-Tg/*Nppc*$^{+/+}$ mice (Fig. 1A). The body weight of male CNP-Tg/*Nppc*$^{-/-}$ mice was significantly reduced as compared with not only that of male CNP-Tg/*Nppc*$^{+/+}$ mice but also that of male Non-Tg/*Nppc*$^{+/+}$ mice (Fig. 1B). At 20 wk of age, the body weight of male CNP-Tg/*Nppc*$^{-/-}$ mice was less than that of male CNP-Tg/*Nppc*$^{+/+}$ mice by 24%. Male CNP-Tg/*Nppc*$^{-/-}$ mice appeared lean and had less abdominal fat as compared with male CNP-Tg/*Nppc*$^{+/+}$ mice (Supplemental Fig. 1, A and B), and epididymal fat pads of male CNP-Tg/*Nppc*$^{-/-}$ mice were less than those of CNP-Tg/*Nppc*$^{+/+}$ mice (Supplemental Fig. 1, C–F). The body fat of male CNP-Tg/*Nppc*$^{-/-}$ mice was significantly less than that of male CNP-Tg/*Nppc*$^{+/+}$ mice: by 50% in absolute values and by 32% in ratios to body weight (Fig. 1, C and D). Upon *ad libitum* feeding, blood glucose, serum insulin, and serum FFA concentrations of CNP-Tg/*Nppc*$^{-/-}$ mice tended to be lower than those of CNP-Tg/*Nppc*$^{+/+}$ mice (Table 1). After overnight fast, blood glucose and serum insulin concentrations decreased in both CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice, and blood glucose concentrations were significantly lower and serum insulin concentrations tended to be lower in CNP-Tg/*Nppc*$^{-/-}$ mice than those in CNP-Tg/*Nppc*$^{+/+}$ mice (Table 1). After overnight fast, serum FFA concentrations significantly increased in both CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice, and the increases in CNP-Tg/*Nppc*$^{-/-}$ mice were greater than those in CNP-Tg/*Nppc*$^{+/+}$ mice (Table 1). There were no significant differences in serum free T_4 concentrations between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice upon *ad libitum* feeding (Table 1). Serum leptin concentrations in CNP-Tg/*Nppc*$^{-/-}$ mice were about one fifth of those in CNP-Tg/*Nppc*$^{+/+}$ mice upon *ad libitum* feeding (Table 1). Plasma ghrelin concentrations in CNP-Tg/*Nppc*$^{-/-}$ mice tended to be higher than those in CNP-Tg/*Nppc*$^{+/+}$ mice after overnight fast (Table 1).

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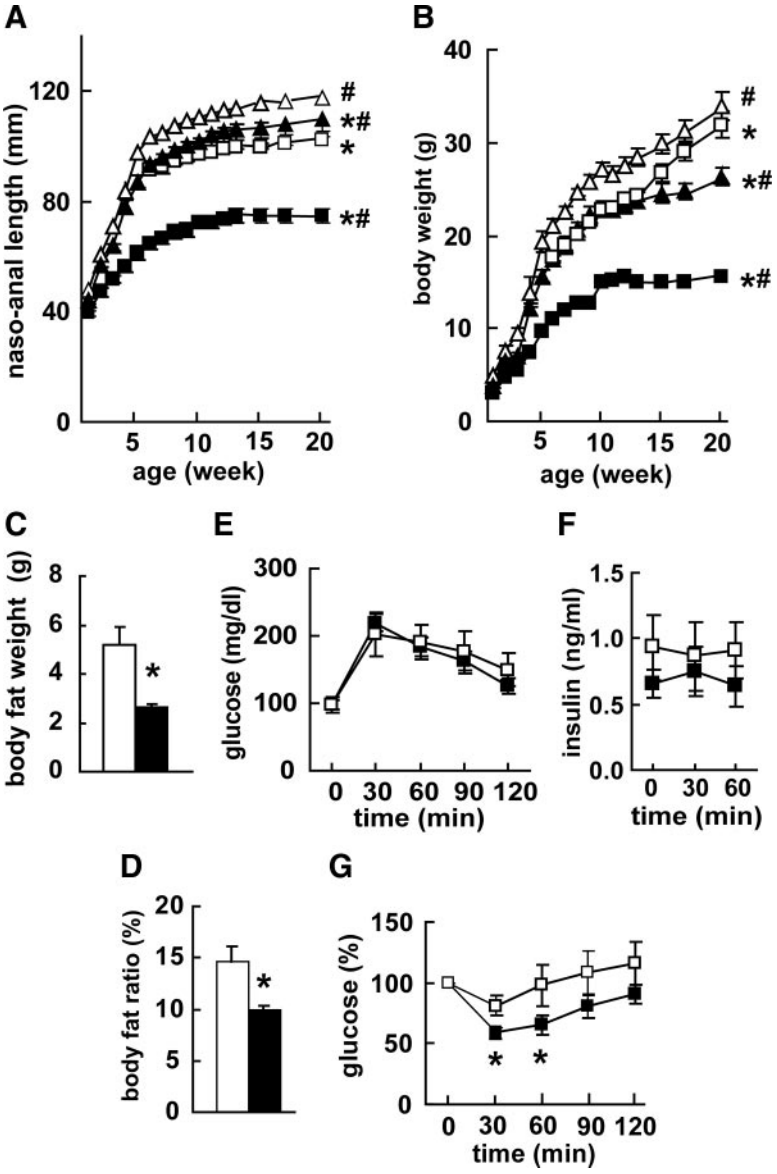

FIG. 1. Growth, body fat accumulation, and glucose and insulin tolerance. A and B, Growth curves of male wild-type (Non-Tg/*Nppc*$^{+/+}$, *open squares*, n = 6), CNP-Tg/*Nppc*$^{+/+}$ (*open triangles*, n = 11), Non-Tg/*Nppc*$^{-/-}$ (*closed squares*, n = 5), and CNP-Tg/*Nppc*$^{-/-}$ (*closed triangles*, n = 7) mice are shown in naso-anal length (A) and body weight (B). *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice; #, $P < 0.05$ *vs.* Non-Tg/*Nppc*$^{+/+}$ mice by a repeated measures ANOVA. C and D, The body fat weight of male CNP-Tg/*Nppc*$^{+/+}$ (*open columns*, n = 11) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed columns*, n = 7) at 20 wk of age are shown in absolute values (C) or ratios to body weight (D). *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice by an unpaired *t* test. E and F, GTT in 17-wk-old male mice. Curves of blood glucose (E) and serum insulin concentrations (F) are shown. G, ITT in 17-wk-old male mice. Curves of blood glucose concentrations are shown in percentages to the value before the injection of insulin. In E–G, *open* and *closed squares* represent data of CNP-Tg/*Nppc*$^{+/+}$ (n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (n = 14), respectively. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice at each time point by an unpaired *t* test.

Glucose tolerance did not differ between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice (Fig. 1E), whereas serum insulin concentrations in CNP-Tg/*Nppc*$^{-/-}$ mice tended to be lower than those in CNP-Tg/*Nppc*$^{+/+}$ mice during the GTT (Fig. 1F). The insulin sensitivity of CNP-Tg/*Nppc*$^{-/-}$ mice assessed by the ITT was significantly better than that of CNP-Tg/*Nppc*$^{+/+}$ mice; decreases of blood glucose levels were 2-fold augmented (Fig. 1G).

Tissue-specific expression of CNP and GC-B mRNA

In CNP-Tg/*Nppc*$^{+/+}$ mice, CNP mRNA was detected in the hypothalamus, but it was not detectable in the interscapular BAT and the sc WAT (Supplemental Fig. 2). This was the case also in WT C57BL/6 mice (data not shown). In CNP-Tg/*Nppc*$^{-/-}$ mice, CNP mRNA was not detectable in these tissues at all (Supplemental Fig. 2). GC-B mRNA was detectable in the hypothalamus, the BAT, and the WAT, and its expression levels were similar among tissues and between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice (Supplemental Fig. 2).

Energy expenditure, core body temperature, and sympathetic nervous system activity

The body weight loss during 48-h starvation was significantly greater in male CNP-Tg/*Nppc*$^{-/-}$ mice than that in male CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 2A). In male CNP-Tg/*Nppc*$^{+/+}$ mice, rectal temperature, which was measured as core body temperature, slightly decreased from 1000 h to 1600 h in the light cycle, and the temperature at 2200 h in the dark cycle was higher than that at 1000 h and 1600 h (Fig. 2B). The diurnal change in rectal temperature appeared to reflect the fact that oxygen consumption and locomotor activity in the dark cycle were greater than those in the light cycle (Fig. 2, C and D). Rectal temperature in CNP-Tg/*Nppc*$^{-/-}$ mice was significantly higher than that in CNP-Tg/*Nppc*$^{+/+}$ mice with the diurnal change being maintained (Fig. 2B). Oxygen consumption in male CNP-Tg/*Nppc*$^{-/-}$ mice was greater than that in male CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 2C), whereas respiratory quotient did not significantly differ between the genotypes (data not shown). There were no significant differences in locomotor activity between the genotypes (Fig. 2D). At 10–12 wk of age, systolic blood pressure and heart rate tended to be higher, and urinary noradrenalin excretion was significantly greater in male CNP-Tg/*Nppc*$^{-/-}$ mice than those in male CNP-Tg/*Nppc*$^{+/+}$ mice, respectively (Table 1).

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TABLE 1. Parameters of metabolism and sympathetic nervous system activity

Genotypes	CNP-Tg/*Nppc*$^{+/+}$ (n = 10)	CNP-Tg/*Nppc*$^{-/-}$ (n = 7)
Blood glucose (mg/dl)		
Ad libitum fed	124 ± 15	92 ± 7
Overnight fast	53 ± 3[b]	42 ± 4[a,b]
Serum insulin (ng/ml)		
Ad libitum fed	3.46 ± 0.76	2.12 ± 0.32
Overnight fast	0.68 ± 0.13[b]	0.39 ± 0.11[b]
Serum FFA (mg/dl)		
Ad libitum fed	0.60 ± 0.05	0.45 ± 0.09
Overnight fast	1.24 ± 0.09[b]	1.30 ± 0.11[b]
Serum free T_4 (ng/dl)		
Ad libitum fed	1.46 ± 0.16	1.28 ± 0.14
Serum leptin (ng/ml)		
Ad libitum fed	9.97 ± 2.12	2.58 ± 0.62[a]
Plasma ghrelin (fmol/ml)		
Overnight fast	36.1 ± 5.7	52.0 ± 7.6
Urinary NA (ng/mgCr)	111 ± 11	142 ± 14[a]
Systolic blood pressure (mm Hg)	95 ± 5	101 ± 5
Heart rate (beat per min)	730 ± 11	756 ± 10

CNP, C-type natriuretic peptide; Tg, transgenic; FFA, free fatty acids; NA, noradrenalin; Cr, creatinine; n, number of mice.

[a] $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice.

[b] $P < 0.05$ *vs. ad libitum* fed in the same genotype.

mRNA expression in BAT

In the interscapular BAT, UCP-1 mRNA levels were significantly higher in CNP-Tg/*Nppc*$^{-/-}$ mice than those in CNP-Tg/*Nppc*$^{+/+}$ mice upon *ad libitum* feeding (Fig. 3A). Levels of mtTFA, PPARγ, PGC-1α, D2, and CPT-1 mRNA in the BAT of CNP-Tg/*Nppc*$^{-/-}$ mice were also significantly higher than those in the BAT of CNP-Tg/*Nppc*$^{+/+}$ mice upon *ad libitum* feeding, respectively (Fig. 3, B–F). The ip injection of propranolol at a dose that did not suppress UCP-1 mRNA levels in the BAT in CNP-Tg/*Nppc*$^{+/+}$ mice significantly suppressed UCP-1 mRNA levels in the BAT in CNP-Tg/*Nppc*$^{-/-}$ mice to levels almost equal to those in the BAT of saline-injected CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 3G).

The icv injection of CNP to wild-type C57BL/6J male mice failed to suppress UCP-1 mRNA levels in the BAT, but it suppressed PGC-1α and D2 mRNA levels in the BAT, although it did not reach significance, as compared with saline injection (Supplemental Fig. 3).

Food intake

At 14 wk of age, food intake of male CNP-Tg/*Nppc*$^{-/-}$ mice was significantly less by 21% than that of male CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4A). When mice were refed after 48-h starvation, food intake of male CNP-Tg/*Nppc*$^{-/-}$ mice was reduced by 61% of that of male CNP-Tg/*Nppc*$^{+/+}$ mice during the first 2 h of refeeding (Fig. 4B). The sc injection of ghrelin augmented food intake in both CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ male mice, and increases of food intake were more exaggerated in CNP-Tg/*Nppc*$^{-/-}$ mice than those in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4C).

Expression of mRNA species in hypothalamus

Upon *ad libitum* feeding, mRNA levels of NPY and AgRP in the hypothalamus did not differ between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice (Fig. 4, D and E). In hypothalami of both CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice, NPY and AgRP mRNA levels significantly increased after 48-h fasting as compared with those upon *ad libitum* feeding, and their fold-increases were greater in CNP-Tg/*Nppc*$^{-/-}$ mice than those in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4, D and E). Hypothalamic mRNA levels of POMC, CART, MCH, and orexin were not significantly different between the genotypes either upon *ad libitum* feeding or after the 48-h starvation (data not shown).

Discussion

The chondrocyte-specific CNP expression in CNP-Tg/*Nppc*$^{-/-}$ mice that homozygously harbored the CNP transgene almost completely rescued the dwarf phenotype of *Nppc*$^{-/-}$ mice in naso-anal length (Fig. 1A), which enabled us to investigate roles of CNP in body weight control with minimal influences of skeletal problems in this study. Using Tg mice that homozygously harbor the transgene has a caveat that the transgene might disrupt an unrelated gene, which would participate in the phenotype in which we are interested. Comparisons among mice, all of which homozygously harbor the CNP transgene on C57BL/6 genetic background, can minimize the possibility to take phenotypes due to the disruption of an unrelated gene as those due to the elimination of CNP. Here we showed that the loss of CNP in the whole body except chondrocytes

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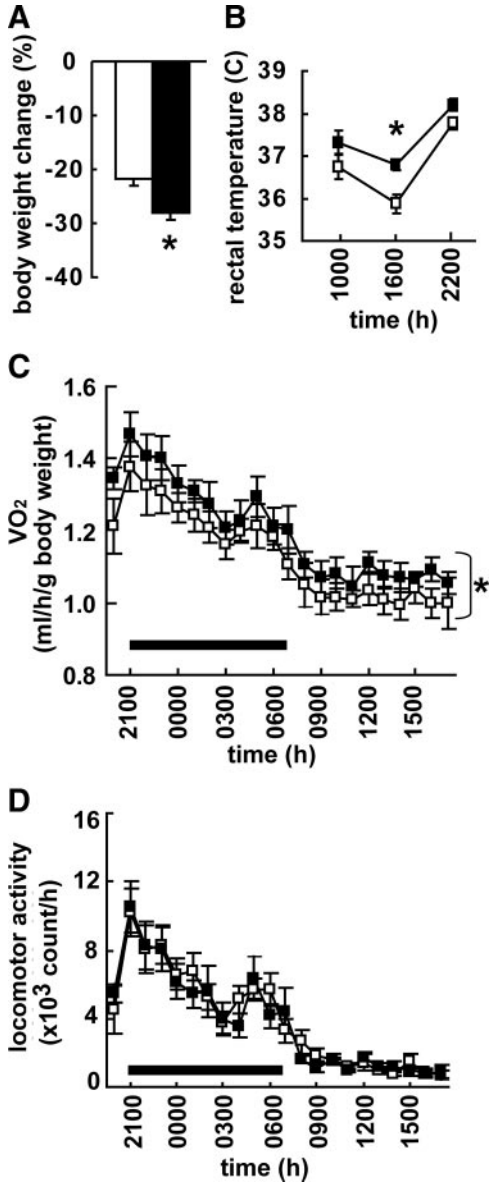

FIG. 2. Parameters of energy expenditure. A, Body weight changes in CNP-Tg/*Nppc*$^{+/+}$ (*open column*, n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed column*, n = 6) during 48-h starvation are shown in percentages to body weight before the starvation. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice by an unpaired *t* test. B, Diurnal changes of rectal temperature in CNP-Tg/*Nppc*$^{+/+}$ (*open squares*, n = 14) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed squares*, n = 7). *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice at each time point by an unpaired *t* test. C and D, Oxygen consumption (VO_2, C) and locomotor activity (D) were recorded for 24 h upon *ad libitum* feeding. *Open* and *closed symbols* represent CNP-Tg/*Nppc*$^{+/+}$ (n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (n = 6), respectively. A *horizontal black bar* in each panel indicates a dark phase. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice by a repeated measures ANOVA.

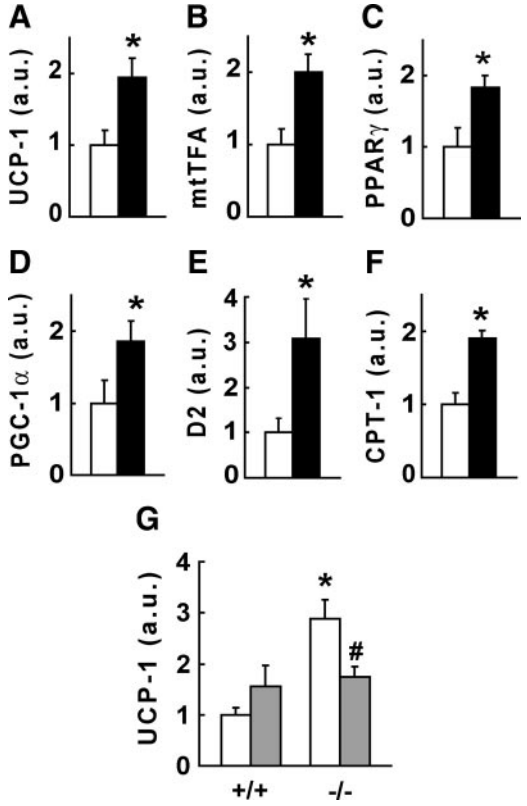

FIG. 3. mRNA expression levels of genes participating in thermogenesis and energy expenditure in brown adipose tissue. Levels of UCP-1 (A), mtTFA (B), PPAR-γ (C), PGC-1α (D), D2 (E), and muscle-type CPT-1 (F) mRNA in CNP-Tg/*Nppc*$^{+/+}$ (*open columns*, n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed columns*, n = 6) are shown. Levels of mRNA are normalized with those of mRNA for a house-keeping gene, β-actin. The mean of mRNA levels in CNP-Tg/*Nppc*$^{+/+}$ mice is set as 1.0 arbitrary unit (a.u.). *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice by an unpaired *t* test. G, The effect of β-adrenergic blockade by the ip injection of propranolol on UCP-1 mRNA expression in the brown adipose tissue. +/+ and −/− indicate CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice, respectively. *Open* and *gray columns* represent saline- (n = 4 for each genotype) and propranolol-injected groups (n = 5 for each genotype), respectively. The mean of mRNA levels in the saline-injected group of CNP-Tg/*Nppc*$^{+/+}$ mice is set as 1.0 a.u. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice with the same treatment, #, $P < 0.05$ *vs.* the saline-injected group of the same genotype by an unpaired *t* test.

resulted in reduced body fat accumulation and better insulin sensitivity (Fig. 1, B–G, and Supplemental Fig. 1), indicating that CNP might participate in body fat accumulation and the occurrence of insulin resistance. Two mechanisms could be speculated on how CNP controls energy balance: inhibiting energy expenditure and regulating food intake.

First, we investigated effects of the CNP elimination on energy expenditure. CNP-Tg/*Nppc*$^{-/-}$ mice lost more body weight during 48 h starvation than CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 2A), indicating that energy expenditure was augmented by the elimination of CNP except chondrocytes. Actually, oxygen consumption in CNP-Tg/*Nppc*$^{-/-}$ mice was greater than that in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 2C). The sympathetic nerve input and exogenously administered β-adrenoceptor agonists, including adrenalin and noradrenalin, stimulate thermogenesis in the BAT (reviewed in Ref. 21). FFA and glucose are oxidized in mitochondria to generate proton gradient across the mitochondrial inner membrane, which is used to synthesize ATP by F0/F1-ATPase (21). β-Adrenoceptor stimulation increases the mRNA expression of UCP-1, which

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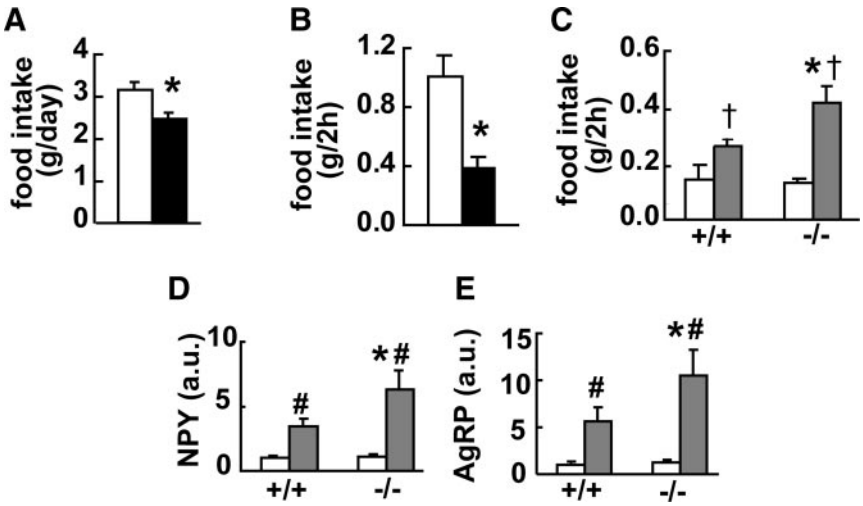

FIG. 4. Food intake and hypothalamic mRNA expression. A, Food intake of male mice at 17 wk of age upon *ad libitum* feeding is shown in absolute values. B, Food intake of male mice at 17 wk of age during the first 2 h at refeeding after 48-h starvation is shown in absolute values. In A and B, *open* and *closed columns* represent data of CNP-Tg/*Nppc*$^{+/+}$ (n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (n = 6), respectively. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice by an unpaired *t* test. C, Response in food intake to sc injection of ghrelin at 360 μg/kg body weight or saline. Food intake during the first 2 h after the injection is shown in absolute values. *Open* and *gray columns* represent data of saline (n = 8) and ghrelin groups (n = 8), respectively. +/+ and −/− indicate CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice, respectively, in panels C–E. *, $P < 0.05$ *vs.* +/+ in each treatment group; †, $P < 0.05$ *vs.* the saline group in each genotype by an ANOVA. D and E, mRNA expression levels of NPY (D) and AgRP (E) in the hypothalamus upon *ad libitum* feeding (*open columns*; +/+, n = 10; −/−, n = 10) and after 48-h starvation (*gray columns*; +/+, n = 10; −/−, n = 8). Expression levels of mRNA are normalized with those of a house-keeping gene, β-actin. The mean of mRNA expression levels in +/+ upon *ad libitum* feeding is set 1.0 arbitrary unit (a.u.). *, $P < 0.05$ *vs.* +/+ at each feeding status; #, $P < 0.05$ *vs. ad libitum* feeding at each genotype by an ANOVA.

causes proton leak back across the mitochondrial inner membrane and generates heat, in the BAT by two molecules: PGC-1α and D2 (21). The increase of the intracellular cAMP concentration augments the mRNA expression of PGC-1α and D2. PGC-1α coactivates the UCP-1 gene transcription with transcription factors, such as PPARγ and T_3-bound thyroid hormone receptor, and D2 converts T_4 to T_3 and increases T_3-bound thyroid hormone receptors (21). PGC-1α also coactivates the gene transcription of transcription factors, which positively regulate mitochondrial biogenesis, such as mtTFA (21). The protein kinase A pathway that is activated by cAMP stimulates lipolysis via the activation of hormone-sensitive lipase and increases FFAs, which activate UCP-1 protein (21). FFAs stimulate the gene transcription of the muscle-type CPT-1 via PPARγ and α and enhance β-oxidation of FFAs (22). The muscle-type CPT-1 is a key regulator of β-oxidation in skeletal and cardiac muscles and the BAT (23). In CNP-Tg/*Nppc*$^{-/-}$ mice, CNP mRNA was not aberrantly expressed in the BAT (Supplemental Fig. 2), which enabled us to investigate effects of the CNP elimination in the BAT in this study. The elimination of CNP except chondrocytes elevated UCP-1 and mtTFA mRNA levels in the BAT (Fig. 3, A and B). It also augmented the mRNA expression of PPARγ, PGC-1α, D2, and CPT-1, which consist of the machinery that augments the gene transcription of UCP-1 and mtTFA or activates UCP-1 protein downstream to the β-adrenoceptor/cAMP cascade (Fig. 3, C–F). Because locomotor activity was similar between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice (Fig. 2D), it is unlikely that energy expenditure was augmented by locomotor activity. Taken together with the observation that core body temperature in CNP-Tg/*Nppc*$^{-/-}$ mice was elevated over that in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 2B), it is suggested that the elimination of CNP except chondrocytes augmented energy expenditure by thermogenesis via the generation and activation of the UCP-1 and the augmentation of mitochondrial biogenesis.

Our observation that urinary noradrenalin excretion in CNP-Tg/*Nppc*$^{-/-}$ mice was higher than that in CNP-Tg/*Nppc*$^{+/+}$ mice (Table 1) suggests that the SNS activity was augmented in CNP-Tg/*Nppc*$^{-/-}$ mice as compared with that in CNP-Tg/*Nppc*$^{+/+}$ mice. It was also reported that the SNS activity was augmented in Tg rats expressing a dominant-negative mutant of GC-B, which inhibited the CNP-dependent activation of authentic GC-B, where the activity was assessed by heart rate and the low-frequency/high-frequency ratio in Fourier transformation of pulse intervals (24). Because the ip administration of propranolol, a nonselective β-adrenoceptor blocking agent, could cancel the augmentation of UCP-1 mRNA expression in the BAT of CNP-Tg/*Nppc*$^{-/-}$ mice (Fig. 3G), the site of CNP action on energy expenditure appears to be located within or upstream to the SNS. In rodents, the SNS governs thermogenesis in the BAT, under the control of neurons in the paraventricular nucleus of the hypothalamus (25) where both CNP and GC-B are expressed (13, 14). It was also reported that neurons that inhibit the SNS activity and thermogenesis in the BAT were present in the preoptic area (26), in which CNP mRNA was abundantly detected (27). In mice, GC-B mRNA could be detected in both the hypothalamus and the BAT, but CNP mRNA was detectable only in the hypothalamus (Supplemental Fig. 2). Considering the nature of CNP as a local regulator (1), a site of CNP actions may be the hypothalamus. In wild-type C57BL/6J mice, the icv administration of CNP tended to

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suppress mRNA levels of PGC-1α and D2, which synergistically activate UCP-1 transcription downstream to the β-adrenoceptor/cAMP cascade stimulated by the SNS (21), in the BAT (Supplemental Fig. 3). Taken together, CNP appears to centrally inhibit the SNS activity and thermogenesis in the BAT, although further analyses will be needed to see the precise cite of CNP actions.

It was reported that atrial natriuretic peptide, which is another member of the natriuretic peptide family and increases intracellular cGMP concentrations via GC-A as CNP does via GC-B, could decrease circulating T_4 and T_3 concentrations via a direct action on the thyroid (28). If this is also true for CNP, the elimination of CNP might increase serum T_4 and T_3 concentrations. Because thyroid hormone is a major regulator of mitochondrial biogenesis and thermogenesis (21), an increase of serum thyroid hormone levels can augment thermogenesis and energy expenditure. However, there were no significant differences in serum free T_4 concentrations between CNP-Tg/*Nppc*$^{+/+}$ and CNP-Tg/*Nppc*$^{-/-}$ mice (Table 1).

Next, we investigated effects of the CNP elimination on food intake. Food intake of CNP-Tg/*Nppc*$^{-/-}$ mice was less than that of CNP-Tg/*Nppc*$^{+/+}$ mice upon *ad libitum* feeding (Fig. 4A), and the 48-h starvation stimulated food intake much less in CNP-Tg/*Nppc*$^{-/-}$ mice than it did in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4B), indicating that CNP might participate in the control of food intake. Neuropeptides expressed in the ARC of the hypothalamus play important roles to control food intake (12). NPY and AgRP expressed in neurons of the ARC stimulate MCH and orexin neurons in the lateral hypothalamic area and increase food intake (reviewed in Ref. 12). POMC and CART expressed in other neurons of the ARC inhibit MCH and orexin neurons in the lateral hypothalamic area and decrease food intake (12). It was shown that CNP mRNA is abundantly expressed in the ARC of the hypothalamus (13). We, therefore, analyzed mRNA expression of these peptides in the hypothalamus. CNP mRNA expression in the hypothalamus was neither impaired in CNP-Tg/*Nppc*$^{+/+}$ mice nor aberrantly induced in CNP-Tg/*Nppc*$^{-/-}$ mice by the presence of the transgene (Supplemental Fig. 2). In hypothalami of CNP-Tg/*Nppc*$^{-/-}$ mice, increases of NPY and AgRP mRNA levels upon starvation were augmented (Fig. 4, D and E) and mRNA expression of POMC, CART, MCH, and orexin was not deteriorated (data not shown), as compared with those in hypothalami of CNP-Tg/*Nppc*$^{+/+}$ mice. Based on these data, we can speculate that there might be AgRP/NPY resistance in CNP-Tg/*Nppc*$^{-/-}$ mice. If CNP is indispensable for the generation or function of a system that links the activation of AgRP/NPY neurons with feeding behavior, the activation of AgRP/NPY neurons cannot increase food intake in the absence of CNP from the beginning of life. It was reported that CNP/GC-B signaling might be important for perinatal brain maturation (29). In another report, it was suggested that the CNP/GC-B system enhances the maturation of olfactory receptor neurons (30). Although we have not yet identified any apparent anatomical abnormalities in the brain (data not shown), there might be functional problems in the neuronal circuit regulating food intake in CNP-Tg/*Nppc*$^{-/-}$ mice. Although skeletal problems were minimized in our CNP-Tg/*Nppc*$^{-/-}$ mice, we cannot completely exclude the possibility that food intake is influenced by minimal skeletal differences between CNP-Tg/*Nppc*$^{-/-}$ and CNP-Tg/*Nppc*$^{+/+}$ mice.

Ghrelin, which was isolated as an endogenous ligand for the growth-hormone secretagogue receptor, is a peptide secreted from the gastric mucosa and stimulates appetite via direct actions on the hypothalamus or signals through the vagal afferent (31–33). Increases of food intake by ghrelin injected sc in CNP-Tg/*Nppc*$^{-/-}$ mice were greater than those in CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4C). This observation indicates that CNP might inhibit ghrelin signaling that augments food intake. This suggests that a physiological action of CNP would be the inhibition of food intake, which does not support our observation that CNP-Tg/*Nppc*$^{-/-}$ mice took less food than CNP-Tg/*Nppc*$^{+/+}$ mice (Fig. 4, A and B). We, therefore, cannot simply conclude that CNP physiologically stimulates food intake.

NPY potently stimulates food intake, at least in part, through the NPY Y5 receptor (reviewed in Ref. 12). In Y5 receptor-null mice, however, food intake, body weight, and total fat pad weight were greater than those in WT mice (34). Five NPY receptors have been identified in mice and play roles different from each other in the regulation of physiological functions such as food intake (35). The complexity of the receptor system might be a cause of the difficult-to-understand phenotype of the Y5-null mice. CNP also has a complicated receptor system. Three GC-B isoforms are generated from the single GC-B gene by alternative splicing in mice: GC-B1, which is the authentic GC-B that increases GC activity upon CNP binding, and two dominant-negative isoforms of GC-B (GC-B2 and GC-B3) (10). The brain is demonstrated to be an organ that expresses mRNA of GC-B2, which has the basal GC activity as GC-B1 does, lacks the CNP-dependent increase of the GC activity, and interferes with the CNP-dependent increase of the GC activity of GC-B1, at a high level (10). Elucidating the nucleus- or cell-specific expression of CNP and GC-B isoforms in the brain would help us understand precise mechanisms of how the CNP/GC-B system controls food intake and energy expenditure.

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It is known that the augmented SNS activity is associated with insulin resistance in the obesity; the hyperactivity of SNS induces insulin resistance by vasoconstriction via α_1-adrenoceptor, and signaling through β-adrenoceptor stimulates the lipolysis of visceral fat and increased serum FFA induces insulin resistance (36). In CNP-Tg/*Nppc*$^{-/-}$ mice, the SNS activity was augmented but insulin sensitivity was better than those in CNP-Tg/*Nppc*$^{+/+}$ mice, which might be contradictory to the above notion. The activation of β_3-adrenoceptor increases energy expenditure by fat oxidation, reduces body weight, and improves insulin sensitivity especially in rodents (37). Because hyperinsulinemia, high FFA levels, and increased adipocytokines such as TNF-α amplify the insulin resistance in the obesity (36), the balance between attenuation and augmentation of insulin sensitivity by the SNS activity in the leanness might be different from that in the obesity. The leptin transgenic skinny mouse is another example that exhibits reduced body weight, increased energy expenditure, augmented SNS activity, and better insulin sensitivity (38–40).

It is proved that leptin decreases food intake, increases energy expenditure, and improves insulin sensitivity and glucose metabolism (38–42). We reported that leptin activates the SNS and increases catecholamine secretion via the ventromedial hypothalamus (43). In our CNP-Tg/*Nppc*$^{-/-}$ mice, however, leptin is not a molecule responsible for the loss of adiposity, because serum leptin concentrations were significantly lower than those in CNP-Tg/*Nppc*$^{+/+}$ mice, reflecting reduced body fat amount.

In conclusion, this study proposed that CNP is a new regulator of food intake and energy expenditure. This study suggested that CNP suppresses energy expenditure in the BAT by attenuating the SNS activity possibly under the control of the hypothalamus. Further analyses on precise mechanisms of CNP actions would lead to the better understanding of the significance of the CNP/GC-B system in food intake and energy expenditure.

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