C. 研究結果

5 週間の高脂肪食負荷を行った C57BL/6J マウスにレプチン 500ng/g/day を 2 週間持続皮下投与しても生食投与群と比べ明らかな摂食量および体重に明らかな変化を認めなかった。一方、exendin-4 20ng/g/day の 2 週間持続皮下投与では、有意な摂食量の減少とともに明らかな体重の減少が観察された。さらにレプチン 500ng/g/day と exendin-4 20ng/g/day の共投与では、exendin-4 20ng/g/day よりさらに有意な摂食量と体重の減少が観察された。これらのことから exendin-4 にはレプチン抵抗性改善作用があるものと考えられた。また糖代謝についてもレプチン単独投与では生食投与群と比べ明らかな血糖値の改善は認められず、exendin-4 単独投与では有意な血糖低下が認められ、レプチンと exendin-4 の共投与ではさらなる血糖の低下が観察された。この時、レプチン単独投与では生食投与群と比べ明らかなインスリン感受性の改善は認められず、exendin-4 単独投与では有意なインスリン感受性の改善が認められ、レプチンと exendin-4 の共投与ではさらなるインスリン感受性の改善が観察された。一方、exendin-4 は膵β細胞に発現する GLP-1 受容体に作用することによりグルコース応答性インスリン分泌を増強することが知られている。そこで次の、STZ/HFD マウスを用いて膵β細胞機能に対するレプチンと exendin-4 共投与の治療効果を検討した。2 週間のレプチン単独投与によりインスリン分泌能の改善が認められたが、レプチンと exendin-4 の共投与によりさらなるインスリン分泌能の増強が観察された。

D. 考察

レプチン単独では摂食抑制や体重減少が認められない高脂肪食負荷マウスにおいて、exendin-4 との共投与により摂食抑制および体重減少が増強されたことから、exendin-4 にはレプチン抵抗性改善作用があることが示唆された。またインスリン分泌能についてもレプチンと exendin-4 の共投与でさらなる増強効果が認められ、糖尿病治療におけるレプチンと exendin-4 共投与の有用性が示された。レプチンは膵β細胞において脂肪毒性の解除を介したインスリン分泌能の改善作用が報告されており、GLP-1 製剤は膵β細胞に発現する GLP-1 受容体に作用することによりグルコース応答性インスリン分泌を増強することが知られている。今後、膵β細胞におけるレプチンと GLP-1 製剤の相互作用についてさらなる検討が望まれる。

E. 結論

高脂肪食負荷マウスやストレプトゾトシン／高脂肪食負荷マウスの検討から、レプチン抵抗性を伴う 2 型糖尿病におけるレプチンと GLP-1 製剤の併用治療の有用性が示された。今後、相互作用におけるメカニズムのさらなる検討が期待される。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

2. 学会発表

国際学会

1. Takeru Sakai, Toru Kusakabe, Ken Ebihara, Daisuke Aotani, Sachiko Yamamoto-Kataoka, Takeshi Fujisawa, Yuji Yamamoto, Megumi Aizawa-Abe, Licht Miyamoto, Takashi Miyazawa, Kiminori Hosoda and Kazuwa Nakao. Gene analysis of congential generalized lipodystrophy in Japan. 11th International Congress on Obesity, Stockholm in Sweden, July 11- 15, 2010.
2. Toru Kusakabe, Ken Ebihara, Licht Miyamoto, Takeru Sakai, Daisuke Aotani, Yuji Yamamoto, Sachiko Yamamoto-Kataoka, Megumi Aizawa-Abe, Junji Fujikura, Kiminori Hosoda, Kazuwa Nakao. Amylin enhances the effect of leptin on AMPK activity and energy metabolism in diet-induced obese mice. KEYSTONE SYMPOSIA, Whistler in Canada, April 12-17, 2010.

国内学会

1. 酒井 建、日下部徹、海老原健、宮本理人、青谷大介、片岡祥子、山本祐二、阿部 恵、藤倉純二、細田公則、中尾一和. アミリンによるレプチンの糖脂質代謝亢進作用の増強効果に関する検討. 第 53 日本糖尿病学会年次学術集会、2010/5/27-29、岡山
2. 日下部徹、海老原健、五百川仁見、青谷大介、

片岡祥子、酒井　建、山本祐二、阿部　恵、藤倉純二、細田公則、中尾一和. LMNA 遺伝子異常を伴った部分性脂肪萎縮症に対するレプチン治療の効果. 第 53 日本糖尿病学会年次学術集会、2010/5/27-29、岡山

3. 日下部徹、海老原健、中尾一和. レプチンと膵β細胞由来ホルモン、アミリンの共投与による肥満治療の検討. 第 31 回日本肥満学会、2010/10/1-2、群馬
4. 伊賀那津子、日下部徹、海老原健、青谷大介、片岡祥子、酒井　建、五百川仁見、海老原千尋、阿部　恵、山本祐二、藤倉純二、細田公則、中尾一和. 第 31 回日本肥満学会、第 20 回臨床内分泌代謝 Update、2011/1/28-29、北海道

E. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌（中尾一和）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
G. Yamada, H. Ariyasu, H. Iwakura, H. Hosoda, T. Akamizu, K. Nakao, K. Kangawa.	Generation of Transgenic Mice Overexpressing a Ghrelin Analog.	Endocrinology	151	5935-5940	2010
M. Inuzuka, N. Tamura, N. Yamada, G. Katsuura, N. Oyamada, D. Taura, T. Sonoyama, Y. Fukunaga, K. Ohinata, M. Sone, K. Nakao.	C-type natriuretic peptide as a new regulator of food intake and energy expenditure.	Endocrinology	151	3633-3642	2010
H. Ariyasu, H. Iwakura, G. Yamada, N. Kanamoto, M. Bando, K. Kohno, T. Sato, M. Kojima, K. Nakao, K. Kangawa, T. Akamizu.	A postweaning reduction in circulating ghrelin temporarily alters growth hormone (GH) responsiveness to GH-releasing hormone in male mice but does not affect somatic growth.	Endocrinology	151	1743-1750	2010

雑誌（海老原健）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
X. Rong, Y. Li, K. Ebihara, M. Zhao, T. Kusakabe, T. Tomita, M. Murray, K. Nakao.	Irbesartan treatment up-regulates hepatic expression of PPARalpha and its target genes in obese Koletsky (fa(k)/fa(k)) rats: a link to amelioration of hypertriglyceridaemia.	Br J Pharmacol.	160	1796-1807	2010
X. Rong, Y. Li, K. Ebihara, M. Zhao, J. Naowaboot, T. Kusakabe, K. Kuwahara, M. Murray, K. Nakao.	Angiotensin II type 1 receptor-independent beneficial effects of telmisartan on dietary-induced obesity, insulin resistance and fatty liver in mice.	Diabetologia	450	In press	2010

雑誌（細田公則）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
H. Iwakura, Y. Li, H. Ariyasu, H. Hosoda, N. Kanamoto, M. Bando, G. Yamada, K. Hosoda, K. Nakao, K. Kangawa, T. Akamizu.	Establishment of a Novel Ghrelin-Producing Cell Line.	Endocrinology.	151	695-705	2010
K. Tomiyama, R. Maeda, I. Urakawa, Y. Yamazaki, T. Tanaka, S. Ito, Y. Nabeshima, T. Tomita, S. Odori, K. Hosoda, K. Nakao, A. Imura, Y. Nabeshima	Relevant use of Klotho in FGF19 subfamily signaling system in vivo.	Proc Natl Acad Sci U S A.	107	1666-1671	2010
S. Yasue, H. Masuzaki, S. Okada, T. Ishii, C. Kozuka, T. Tanaka, J. Fujikura, K. Ebihara, K. Hosoda, A. Katsurada, N. Ohashi, M. Urushihara , H. Kobori, N. Morimoto, T. Kawazoe, M. Naitoh, M. Okada, H. Sakaue, S. Suzuki, K. Nakao.	Adipose Tissue-Specific Regulation of Angiotensinogen in Obese Humans and Mice: Impact of Nutritional Status and Adipocyte Hypertrophy.	Am J Hypertens.	23	425-431	2010
S. Okada, C. Kozuka, H. Masuzaki, S. Yasue, T. Ishii-Yonemoto, T. Tanaka, Y. Yamamoto, M. Noguchi, T. Kusakabe, T. Tomita, J. Fujikura, K. Ebihara, K. Hosoda, H. Sakaue, H. Kobori, M. Ham, YS. Lee, JB. Kim, Y. Saito, K. Nakao.	Adipose tissue-specific dysregulation of angiotensinogen by oxidative stress in obesity.	Metabolism.	59	1241-1251	2010
T. Ishii-Yonemoto, H. Masuzaki, S. Yasue, S. Okada, C. Kozuka, T. Tanaka, M. Noguchi, T. Tomita, J. Fujikura, Y. Yamamoto, K. Ebihara, K. Hosoda, K. Nakao	Glucocorticoid reamplification within cells intensifies NFB and MAPK signaling and reinforces inflammation in activated preadipocytes	Am J Physiol Endocrinol Metab	299	E142	2010

Generation of Transgenic Mice Overexpressing a Ghrelin Analog

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After the discovery of ghrelin, we attempted to generate ghrelin gene transgenic (Tg) mice. These animals, however, produced only des-acyl ghrelin, which lacked the n-octanoyl modification at Ser^3 necessary to manifest ghrelin activity. Because the mechanism for acyl-modification of ghrelin had been unclear until the recent identification of GOAT (ghrelin O-acyltransferase), it had been difficult to generate Tg mice overexpressing ghrelin using standard procedures. Therefore, we planned to generate Tg mice overexpressing a ghrelin analog, which possessed ghrelin-like activity in the absence of acylation at Ser^3 and could be synthesized *in vivo*. As the replacement of Ser^3 of ghrelin with Trp^3 (Trp^3-ghrelin) preserves a low level of ghrelin activity and Trp^3-ghrelin can be synthesized *in vivo*, we generated mice overexpressing Trp^3-ghrelin by using the hSAP (human serum-amyloid-P) promoter. Plasma Trp^3-ghrelin concentrations in the Tg mice were approximately 85-fold higher than plasma ghrelin concentrations in non-Tg littermates. Because Trp^3-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo*, plasma Trp^3-ghrelin concentrations in Tg mice were calculated to have an activity approximately 6-fold greater than that of acylated ghrelin seen in non-Tg mice (85-fold x 1/10–1/20). Tg mice exhibited a normal growth and glucose metabolism in their early life stage. However, 1-yr-old Tg mice demonstrated impaired glucose tolerance and reduced insulin sensitivity. This model will be useful to evaluate the long-term effects of ghrelin or ghrelin analogs. In addition, this technique may be a useful method to generate gain-of-activity models for hormones that require posttranscriptional modifications. (***Endocrinology* 151: 5935–5940, 2010**)

Ghrelin, an endogenous ligand for the GH secretagogue receptor (GHS-R) (or ghrelin receptor), is a stomach-derived 28-amino acid peptide hormone modified by n-ontanoylic acid at the third Ser residue (Ser^3) (1). This modification is essential for ghrelin activity (1).

Since ghrelin was discovered, several groups, including us, have been trying to generate transgenic (Tg) mice overexpressing ghrelin under the control of different promoters (2–7). All of these animals, with the exception of two lines created by Reed *et al.* (5) and Bewick *et al.* (8), produced des-acyl ghrelin only. This form lacks the n-octanoyl modification at Ser^3 and is devoid of ghrelin activity. The mechanism for ghrelin acylation had been unclear until the recent identification of ghrelin O-acyltransferase (GOAT) (9). Because GOAT had not yet been identified when we initiated this study and it had proved to be difficult to generate the Tg mice overexpressing ghrelin by standard procedures, we planned to generate Tg mice overexpressing a ghrelin analog possessing ghrelin-like activity without Ser^3 acylation that could be synthesized *in vivo*.

Matsumoto *et al.* (10) investigated the effect on ghrelin bioactivities of replacement of the octanoylated Ser at the third position with other amino acids, such as tryptophan

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0635 Received June 4, 2010. Accepted September 20, 2010.
First Published Online October 20, 2010

Abbreviations: C-RIA, RIA recognizing the C-terminal region of ghrelin; GOAT, ghrelin O-acyltransferase; GHS-R, GH secretagogue receptor; hSAP, human serum-amyloid-P; N-RIA, RIA recognizing the N-terminal region of ghrelin; Tg, transgenic; Trp, tryptophan; Trp^3-ghrelin, ghrelin analog with the third amino-acid residue (Ser^3) replaced by Trp.

(Trp), Val, Leu, or Ile. The ghrelin-like activity of these synthetic peptides was evaluated by EC_{50} values, determined by an increase in intracellular calcium concentrations $[Ca^{2+}]_i$ in GHS-R-expressing cells. Replacement of Ser^3 with Trp^3 (Trp^3-ghrelin) preserved ghrelin activity with an EC_{50} of 31 nM in comparison with 1.3 nM for intact ghrelin. Replacement of Ser^3 with Val^3, Leu^3, or Ile^3 led to complete loss of ghrelin potency. Although ghrelin analog, in which the Ser^3 residue was replaced by Trp (Trp^3-ghrelin) is approximately 24-fold less active than native ghrelin *in vitro*, it can be synthesized *in vivo*. Thus, we selected Trp^3-ghrelin as a candidate ghrelin analog.

In this study, we examined whether Trp^3-ghrelin exerts ghrelin-like activity *in vivo*. After confirming this activity, we generated Tg mice overexpressing Trp^3-ghrelin.

Materials and Methods

All animal protocols were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Animals, housed in air-conditioned animal quarters with light between 0800 and 2000 h, were maintained on standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA, Osaka, Japan).

Experiment 1, the *in vivo* effects of Trp^3-ghrelin

Eight-week-old male C57BL/6 mice were purchased from Japan CLEA. Ghrelin was obtained from Peptide Research Institute (Osaka, Japan). Trp^3-ghrelin, in which the Ser^3 residue was replaced by Trp, was synthesized as previously described (10).

Food intake

Mice (n = 8, each group) were injected sc with saline, ghrelin (120 or 360 mcg/kg), or Trp^3-ghrelin (360, 1200, or 3600 mcg/kg) before measuring a 2-h food intake.

GH secretion

Mice (n = 8, each group) were injected with iv saline, ghrelin (4, 12, 40, or 120 mcg/kg), or Trp^3-ghrelin (12, 40, 120, or 360 mcg/kg). Blood samples were collected from the retro-orbital vein 10 min after injection and stored at −20 C until assessed.

Inhibition of glucose stimulated insulin secretion

After a 12-h fast, mice (n = 8, each group) were injected iv with 1.0 g/kg glucose, together with saline, ghrelin (120 or 360 mcg/kg), or Trp^3-ghrelin (1200 or 3600 mcg/kg). Blood samples were collected 1 and 10 min after injection and stored at −20 C until assessed.

Experiment 2, generation of Tg mice overexpressing a ghrelin analog, Trp^3-ghrelin

Plasmid construction and generation of Tg mice

We generated a fusion gene of the human serum-amyloid-P (hSAP) promoter and full-length mouse preproghrelin cDNA (1, 11). Plasmid hSAP-ghrelin was constructed by inserting mouse preproghrelin cDNA into the unique *Eco*RI site between the hSAP promoter and the 3′-flanking sequence of the rabbit β-globin gene. Mutations were created using a QuikChange Site-Directed Mutagenesis kit, according to the manufacturer's instruction. The hSAP-ghrelin plasmid was used as the template for PCR amplification. To replace the AGC codon encoding Ser to a TGG codon encoding Trp, we used two oligonucleotide primers: 5′-GGACATGGCCATGGCAGGCTCCTGGTTCCTGAGCCCAGAGC-3′ and 5′-GCTCTGGGCTCAGGAACCAGGAGCCTGCCATGGCCATGTCC-3′. The mutated construct was verified by sequencing (please see figure 2A). The DNA fragment encoding mutant ghrelin was excised from the plasmid by digestion with *Sal*I and *Hin*dIII (see figure 2B), then purified and microinjected into the pronuclei of fertilized eggs as reported (11). Founder Tg mice were identified by PCR analysis and bred against C57BL/6 mice.

Please refer to Supplemental Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org, for real-time quantitative RT-PCR, semiquantitative PCR, glucose and insulin tolerance tests, measurements of insulin-releasing ability, body weights, body length, body composition, daily food intake, hormonal parameters, and statistical analyses.

Results

Experiment 1, the *in vivo* effects of Trp^3-ghrelin on food intake and GH secretion

To elucidate whether Trp^3-ghrelin has ghrelin-like potency *in vivo*, 8-wk-old male C57BL/6 mice were administrated vehicle, ghrelin, or Trp^3-ghrelin before determining food intake over a period of 2 h. Injection of ghrelin or Trp^3-ghrelin stimulated food intake in a dose-dependent manner (Fig. 1A). The 2-h food intake after injection of 3600 mcg/kg of Trp^3-ghrelin was 0.47 ± 0.04 g, which was 2.2-fold higher than that seen in vehicle-injected mice (0.21 ± 0.02 g/2 h). This level of stimulation was similar to that seen in mice injected with ghrelin at a dose of 360 mcg/kg. Serum GH levels increased after injection of 360 mcg/kg Trp^3-ghrelin to 133.9 ± 46.1 ng/ml, which was 21-fold higher than that seen after vehicle injection (6.4 ± 1.1 ng/ml) and similar to those seen after ghrelin injection at 40 mcg/kg (138.8 ± 26.5 ng/ml, respectively) (Fig. 1B). Injection of ghrelin or Trp^3-ghrelin inhibited glucose-stimulated insuline release in a dose-dependent manner (Fig. 1C). The 10-min insulin response was significantly inhibited by 3600 mcg/kg Trp^3-ghrelin and 360 mcg/kg to the same extent (0.78 ± 0.09 and 0.63 ± 0.06 ng/ml), compared with saline (1.10 ± 0.01 ng/ml). These results indicated that Trp^3-ghrelin stimulates food intake and GH secretion and inhibits glucose-stimulated insulin secretion in a manner similar to ghrelin with a potency approximately 1/10–1/20 (Trp^3-ghrelin needs about 20-fold amount for stimulation of food intake, about 10-fold amount for GH secretion, and about 10-fold amount for

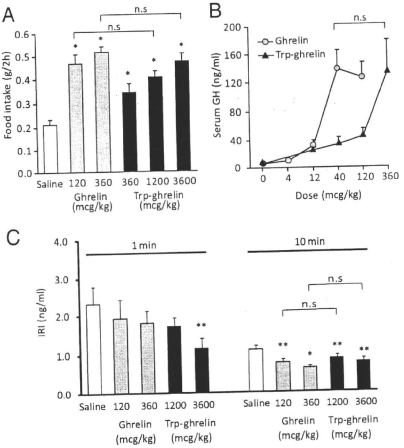

FIG. 1. Effects of the exogenous administration of Trp^3-ghrelin on food intake, GH secretion, and inhibition of glucose stimulated insulin release. A, Two-hour food intake after saline (*open bar*), ghrelin at 120 or 360 mcg/kg (*shaded bars*), or Trp^3-ghrelin at 360, 1200, or 3600 mcg/kg (*closed bars*) ip injection (n = 8–10). B, Serum GH levels were measured 10 min after 4, 12, 40, or 120 mcg/kg ghrelin (*shaded circles*) or 12, 40, 120, or 360 mcg/kg Trp^3-ghrelin (*closed triangles*) iv injection (n = 8–10). C, Serum insulin levels were measured 1 and 10 min after iv injection of 1.0 g/kg glucose, together with saline (*open bars*), ghrlein (120 and 360 mcg) (*shaded bars*), or Trp^3-ghrelin (1200 and 3600 mcg/kg) (*closed bars*) (n = 8). *, $P < 0.01$; **, $P < 0.05$; n.s, not significant compared with saline group; IRI, immunoreactive insulin. Data are presented as the means ± SEM.

inhibition of glucose stimulated insulin release) that of ghrelin.

Experiment 2, generation of Tg mice overexpressing Trp^3-ghrelin

Two Tg mouse lines, Tg6-2 and Tg6-5, were obtained. Hepatic transgene expression in Tg6-2 and Tg6-5 mice was 3.02 ± 1.15 and 0.07 ± 0.01 in arbitrary units, respectively, after normalization to preproghrelin mRNA expression levels seen in the stomachs of non-Tg littermates (non-Tg mice) (1.00 ± 0.18). No expression of preproghrelin mRNA was seen in the livers of non-Tg mice (Fig. 2C).

Two RIA methods [RIA recognizing the N-terminal region of ghrelin (N-RIA) and RIA recognizing the C-terminal region of ghrelin (C-RIA)] were performed to measure plasma ghrelin, des-acyl ghrelin, and Trp^3-ghrelin concentrations. In a previous study, N-RIA has been demonstrated to recognize only the acylated N-terminal region of ghrelin, whereas C-RIA recognizes the C-terminal region of ghrelin, making it possible to detect both acylated ghrelin and des-acyl ghrelin (12). We determined whether Trp^3-ghrelin could be detected by one or both of these RIA systems. When synthetic Trp^3-ghrelin was added to plasma samples from wild-type mice, Trp^3-ghrelin could only be detected by C-RIA, not N-RIA (data not shown). At 8 wk of age, plasma ghrelin concentrations measured by N-RIA did not differ among genotypes. Total plasma ghrelin concentrations, including ghrelin, des-acyl ghrelin, and Trp^3-ghrelin, measured by C-RIA were significantly elevated in Tg mice (Fig. 2, D and E). To determine precise plasma Trp^3-ghrelin concentration, we also performed HPLC on Tg6-2 samples (Fig. 2F). Plasma ghrelin (40.5 ± 10.2 *vs.* 36.6 ± 4.4 fmol/ml) and des-acyl ghrelin (167.5 ± 51.8 *vs.* 235.7 ± 44.8 fmol/ml) concentrations did not differ among genotypes.

Plasma Trp^3-ghrelin concentrations in Tg6-2 was 3437.8 ± 571.6 (2546.4–5101.7) fmol/ml, which was approximately 85-fold (3437.8/40.5 = 84.9-fold) higher than plasma ghrelin (acylated ghrelin) concentrations seen in non-Tg mice. Because Trp^3-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo* (experiment 1), plasma Trp^3-ghrelin concentrations in Tg6-2 were calculated to have an activity approximately 6-fold greater than that of ghrelin (acylated ghrelin) seen in non-Tg mice (84.9-fold × 1/10–1/20 = 4.2–8.5-fold). Total ghrelin concentrations measured by C-RIA in the Tg mice were roughly constant throughout the day.

We then analyzed the phenotype of the Tg6-2 line. Tg mice overexpressing Trp^3-ghrelin (Tg6-2 line) were abbreviated as Trp^3-ghrelin-Tg mice.

The analysis of the phenotypes of Trp^3-ghrelin-Tg mice

During postnatal development, there were no significant differences in somatic growth between Trp^3-ghrelin-Tg and non-Tg mice (Supplemental Fig. 1, A and B). Consistent with these results, no changes in serum GH and IGF-I concentrations were observed in Trp^3-ghrelin-Tg mice (Supplemental Fig. 1, C and D). The average food intake of Trp^3-ghrelin-Tg mice did not differ from that of non-Tg mice (Supplemental Fig. 1E). Trp^3-ghrelin-Tg mice consumed the largest food portions during the dark phase (75.4 ± 2.7%), similar to the behavior seen in non-Tg mice (75.9 ± 1.6%). There were no differences between 10-wk-old Trp^3-ghrelin-Tg and non-Tg mice in pituitary and hypothalamic mRNA levels of factors involved in GH secretion and food intake (Supplemental Fig. 2, A and B). In addition, glucose metabolism in Trp^3-ghrelin-Tg mice did not differ from that seen in non-Tg mice in early life (Supplemental Fig. 1, F and G).

We conducted a precise evaluation of glucose metabolism using more aged mice. Thus we continued rearing

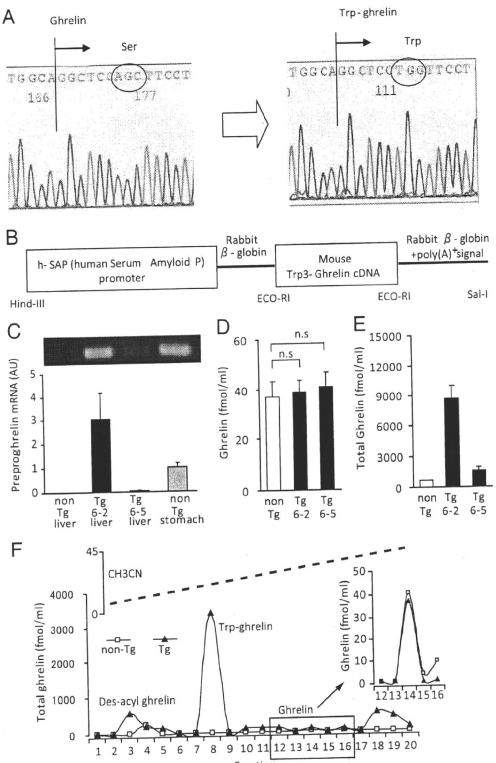

FIG. 2. Generation of Trp^3-ghrelin overexpressing Tg mice. A, A mutant construct in which the AGC codon encoding Ser, the third amino acid of ghrelin that is modified by n-octanoic acid, was replaced by a TGG codon encoding Trp. B, The construct encoding Trp^3-ghrelin used to generate Tg mice was a fusion gene of the hSAP promoter combined with the mutated cDNA of mouse ghrelin. C, The expression levels of preproghrelin mRNA or mutated preproghrelin mRNA. D, Plasma concentrations of ghrelin (acylated form) were measured by N-RIA (n = 8–10). E, Plasma concentrations of total ghrelin, which included ghrelin, des-acyl ghrelin, and Trp^3-ghrelin, were measured by C-RIA (n = 8–10). F, Representative results of HPLC analysis (non-Tg, *open square*; Tg6-2, *closed triangle*). n.s, Not significant. Data are presented as the means ± SEM (C–E).

these mice to 1 yr of age. Some intriguing results on glucose metabolism were obtained from 1-yr-old Trp^3-ghrelin-Tg mice. Although there were no differences between Trp^3-ghrelin-Tg and non-Tg mice in anthropometric parameters, including body weight, total body fat percentage, and lean body mass, Trp^3-ghrelin-Tg mice exhibited impaired glucose tolerance and reduced insulin sensitivity (Fig. 3, A–F); blood glucose levels after glucose injection were significantly higher than those in non-Tg mice. The acute phase of insulin secretion typically seen in response to glucose tended to be suppressed in Trp^3-ghrelin-Tg mice (P = 0.11) (Fig. 3, C and D). In addition, the hypoglycemic response after the injection with insulin was blunted in Trp^3-ghrelin-Tg mice (Fig. 3E). There were no differences, however, in pancreatic insulin mRNA levels between 1-yr-old Trp^3-ghrelin-Tg and non-Tg mice (Fig. 3F). Because glucose tolerance and insulin sensitivity are influenced by GH, we examined whether GH secretion was augmented in 1-yr-old Trp^3-ghrelin-Tg mice. Serum GH and IGF-I levels were unchanged in Trp^3-ghrelin-Tg mice in comparison with those seen in non-Tg mice at 1 yr of age (Fig. 4, A and B). There was no difference between 1-yr-old Trp^3-ghrelin-Tg and non-Tg mice in ghrelin or GOAT mRNA within the stomach or in plasma acylated ghrelin concentrations, which reflects the intrinsic secretion of ghrelin (Fig. 4, C and D). Because ghrelin can also affect the lipid metabolism, we measured serum nonesterified fatty acid, total cholesterol, and triglyceride levels. However, there was no significant difference in them (non-Tg *vs.* Tg: nonesterified fatty acid, 0.74 ± 0.03 *vs.* 0.82 ± 0.04 mEq/liter, P = 0.12; total cholesterol, 122.5 ± 9.8 *vs.* 143.4 ± 7.4 mg/dl, P = 0.10; triglyceride, 162.6 ± 12.8 *vs.* 159.5 ± 10.8 mg/dl, P = 0.85).

Discussion

It is challenging to generate ghrelin gain-of-activity models, because ghrelin requires posttranscriptional modification, an octanoylation of Ser^3. GOAT is responsible for this octanoylation of ghrelin, which confers its biological activity (10, 13). In this study, we succeeded in generating Tg mice overexpressing Trp^3-ghrelin, a ghrelin analog that does not require posttranscriptional modification with GOAT for activity. Because expression of the mutated-ghrelin transgene was driven by the hSAP promoter, Trp^3-ghrelin was continuously secreted from the liver after birth. Plasma concentrations of Trp^3-ghrelin of Tg mice were calculated to have an equivalent activity

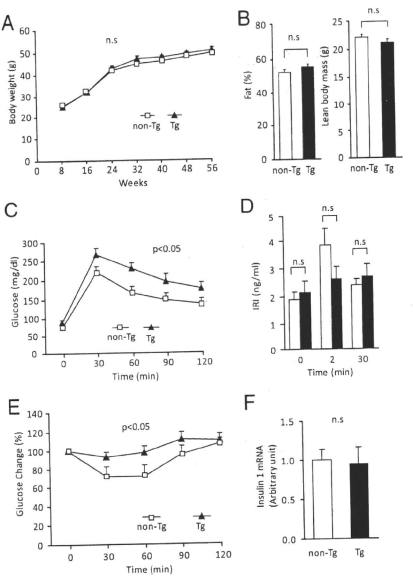

FIG. 3. Analysis of Trp^3-ghrelin Tg mice. A, Changes of body weight in Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 20–25). B, Body fat percentage and lean body mass, as determined by computer tomography, in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14–16). C, Glucose tolerance test (0.75 g/kg) was performed in 52-wk-old Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 14–16; *, $P < 0.05$ in comparison with non-Tg littermates). D, Serum insulin levels at baseline, 2 min, and 30 min after ip glucose injection of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14–16, $P = 0.11$ in comparison with non-Tg littermates). E, Insulin tolerance test after treatment with 1.5 U/kg regular insulin in 52-wk-old Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 14–16; *, $P < 0.05$ in comparison with non-Tg littermates). F, Insulin 1 mRNA levels in the pancreases of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14). IRI, Immunoreactive insulin; n.s, not significant. Data are presented as the means ± SEM.

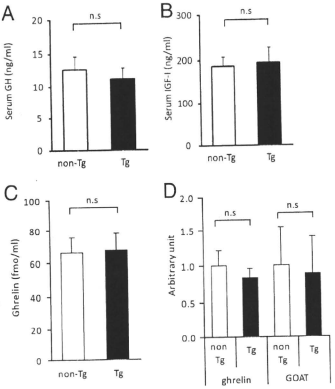

FIG. 4. We examined the levels of GH/IGF-I axis factors, plasma ghrelin levels, and ghrelin and GOAT mRNA levels in the stomachs of 52-wk-old Trp^3-ghrelin-Tg mice. A and B, We measured serum GH (A) and IGF-I (B) levels in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 10). C, Plasma ghrelin levels in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 10). D, The mRNA levels of ghrelin and GOAT in the stomachs of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14). n.s, Not significant. Data are presented as the means ± SEM.

as 4.2- to 8.5-fold higher levels of acylated ghrelin in non-Tg mice. We think that this unique mouse model is a useful tool to evaluate the long-term pathophysiological and/or pharmacological effects of ghrelin or ghrelin analogs and provides insight into the physiological roles of ghrelin/GHS-R systems.

Bewick *et al.* (8) developed ghrelin-overexpressing mice using the endogenous ghrelin promoter. Although this mouse model was suitable to investigate the physiological role of ghrelin, it is not suitable to explore the pathophysiological or pharmacological effects of ghrelin, because the plasma ghrelin levels achieved in these mice were only 1.5-fold greater than that seen in non-Tg mice at the highest. Reed *et al.* (5) also developed ghrelin-overexpressing mice using the neuron-specific enolase promoter, reaching circulating ghrelin levels approximately 5-fold higher than those seen in non-Tg mice. Because these mice primarily produced ghrelin in the brain, it remains unclear whether the phenotype of these mice resulted from elevations in peripheral ghrelin and/or central ghrelin. Kirchner *et al.* (13) generated Tg mice simultaneously expressing human ghrelin and GOAT in the liver under the control of the human apolipoprotein E promoter. When fed a standard diet, these mice lack the circulating fatty-acid-modified forms of ghrelin, demonstrating high circulating concentrations of des-acyl ghrelin only. These mice exhibited elevated concentrations of fatty-acid-modified forms of ghrelin only when given a diet rich in medium-chain triglycerides. It may be difficult to characterize the phenotype of the mice precisely, especially the metabolic phenotype, under such a diet.

Trp^3-ghrelin-Tg mice exhibited normal growth patterns and feeding behaviors. These results are consistent with previous results; ghrelin loss-of-function mice, ghrelin-deficient mice, or ghrelin-receptor-null mice all have normal growth rates, food intake, and body compositions (14–17). One-year-old Trp^3-ghrelin-Tg mice demon-

strated impaired glucose tolerance and reduced insulin sensitivity, although there were no differences in body weight or composition between Trp[3]-ghrelin-Tg and non-Tg mice. When ghrelin-receptor-null mice were maintained on long-term standard chow, they had lower blood glucose levels with low-to-normal insulin levels in comparison with wild-type mice, although they exhibited similar body weights and composition (14). Ghrelin-receptor-null mice appeared to have enhanced insulin sensitivity in comparison with wild-type mice. In addition, Gauna *et al.* (18) demonstrated that administration of ghrelin to wild-type mice reduced insulin sensitivity. It was also reported that ghrelin inhibited glucose-stimulated insulin release (19–21).

In conclusion, we succeeded in generating Tg mice overexpressing a ghrelin analog. The mice presented in this study will serve as a useful tool for evaluating the long-term effects of ghrelin or ghrelin analogs. In addition, the method provided in this study may be useful in the generation of gain-of-function models for hormones that require posttranscriptional modification.

Acknowledgment

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This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan; and by research grants from the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. **Ariyasu H, Takaya K, Iwakura H, Hosoda H, Akamizu T, Arai Y, Kangawa K, Nakao K** 2005 Transgenic mice overexpressing desacyl ghrelin show small phenotype. Endocrinology 146:355–364
3. **Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M** 2005 Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18–24
4. **Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K** 2005 Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. J Biol Chem 280:15247–15256
5. **Reed JA, Benoit SC, Pfluger PT, Tschöp MH, D'Alessio DA, Seeley RJ** 2008 Mice with chronically increased circulating ghrelin develop age-related glucose intolerance. Am J Physiol Endocrinol Metab 294:E752–E760
6. **Wei W, Qi X, Reed J, Ceci J, Wang HQ, Wang G, Englander EW, Greeley Jr GH** 2006 Effect of chronic hyperghrelinemia on ingestive action of ghrelin. Am J Physiol Regul Integr Comp Physiol 290: R803–R808
7. **Zhang W, Chai B, Li JY, Wang H, Mulholland MW** 2008 Effect of des-acyl ghrelin on adiposity and glucose metabolism. Endocrinology 149:4710–4716
8. **Bewick GA, Kent A, Campbell D, Patterson M, Ghatei MA, Bloom SR, Gardiner JV** 2009 Mice with hyperghrelinemia are hyperphagic and glucose intolerant and have reduced leptin sensitivity. Diabetes 58:840–846
9. **Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL** 2008 Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132:387–396
10. **Matsumoto M, Kitajima Y, Iwanami T, Hayashi Y, Tanaka S, Minamitake Y, Hosoda H, Kojima M, Matsuo H, Kangawa K** 2001 Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. Biochem Biophys Res Commun 284:655–659
11. **Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga H, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K** 1999 Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 48:1822–1829
12. **Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2002 Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Endocrinology 143:3341–3350
13. **Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschöp MH** 2009 GOAT links dietary lipids with the endocrine control of energy balance. Nat Med 15:741–745
14. **Zigman JM, Nakano Y, Coppari R, Balthasar N, Marcus JN, Lee CE, Jones JE, Deysher AE, Waxman AR, White RD, Williams TD, Lachey JL, Seeley RJ, Lowell BB, Elmquist JK** 2005 Mice lacking ghrelin receptors resist the development of diet-induced obesity. J Clin Invest 115:3564–3572
15. **Sun Y, Ahmed S, Smith RG** 2003 Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23:7973–7981
16. **Sun Y, Wang P, Zheng H, Smith RG** 2004 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 101:4679–4684
17. **Wortley KE, Anderson KD, Garcia K, Murray JD, Malinova L, Liu R, Moncrieffe M, Thabet K, Cox HJ, Yancopoulos GD, Wiegand SJ, Sleeman MW** 2004 Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc Natl Acad Sci USA 101:8227–8232
18. **Gauna C, Meyler FM, Janssen JA, Delhanty PJ, Abribat T, van Koetsveld P, Hofland LJ, Broglio F, Ghigo E, van der Lely AJ** 2004 Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. J Clin Endocrinol Metab 89: 5035–5042
19. **Reimer MK, Pacini G, Ahrén B** 2003 Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. Endocrinology 144:916–921
20. **Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T** 2009 A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. Am J Physiol Endocrinol Metab 297:E802–E811
21. **Dezaki K, Sone H, Koizumi M, Nakata M, Kakei M, Nagai H, Hosoda H, Kangawa K, Yada T** 2006 Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. Diabetes 55:3486–3493

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-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0141 Received February 3, 2010. Accepted May 18, 2010.
First Published Online June 16, 2010

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

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 × 15 × 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).

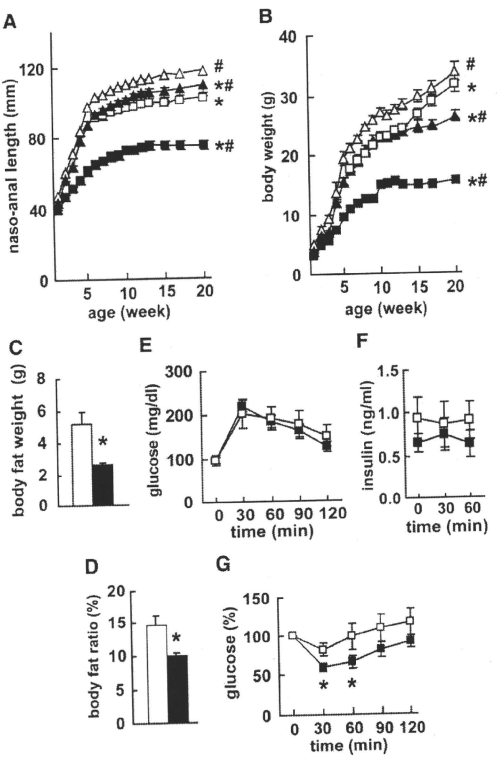

FIG. 1. Growth, body fat accumulation, and glucose and insulin tolerance. A and B, Growth curves of male wild-type (Non-Tg/*Nppc*$^{+/+}$, *open squares*, n = 6), CNP-Tg/*Nppc*$^{+/+}$ (*open triangles*, n = 11), Non-Tg/*Nppc*$^{-/-}$ (*closed squares*, n = 5), and CNP-Tg/*Nppc*$^{-/-}$ (*closed triangles*, 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).

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

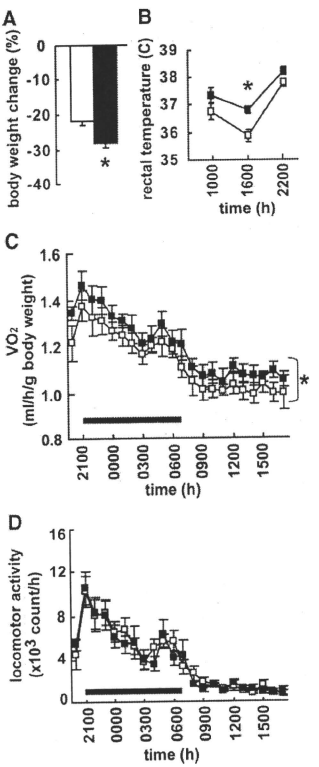

FIG. 2. Parameters of energy expenditure. A, Body weight changes in CNP-Tg/*Nppc*$^{+/+}$ (*open column*, n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed column*, n = 6) during 48-h starvation are shown in percentages to body weight before the starvation. *, $P < 0.05$ *vs.* CNP-Tg/*Nppc*$^{+/+}$ mice 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.

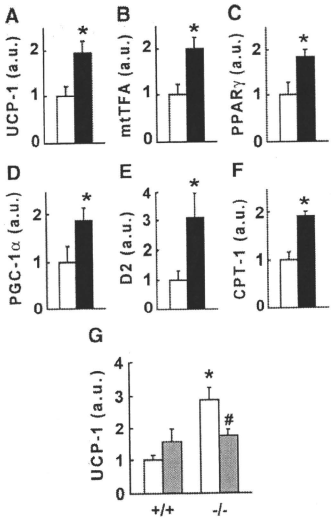

FIG. 3. mRNA expression levels of genes participating in thermogenesis and energy expenditure in brown adipose tissue. Levels of UCP-1 (A), mtTFA (B), PPAR-γ (C), PGC-1α (D), D2 (E), and muscle-type CPT-1 (F) mRNA in CNP-Tg/*Nppc*$^{+/+}$ (*open columns*, n = 6) and CNP-Tg/*Nppc*$^{-/-}$ mice (*closed 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

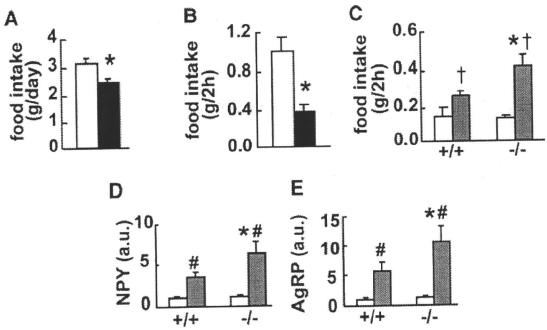

FIG. 4. Food intake and hypothalamic mRNA expression. A, Food intake of male mice at 17 wk of age upon *ad libitum* feeding is shown in absolute values. B, Food intake of male mice at 17 wk of age during the first 2 h at refeeding after 48-h starvation is shown in absolute values. In A and B, *open* 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

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.

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.

Acknowledgments

We thank Dr. Takashi Akamizu and Dr. Hiroyuki Ariyasu for technical advice and discussions, Hirokazu Tsujimoto and Yoshie Fukuchi for technical assistance, and Ayumi Ishida, Shiho Takada, and Aki Egami for secretarial assistance. We also thank the staffs of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University for assistance in animal experiments.

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This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Health and Labour Sciences Research Grants, and Research Grants for Cardiovascular Diseases (19C-7 and 20C-3) from the Ministry of Health, Labour, and Welfare, the Japan Smoking Foundation, the Cell Science Research Foundation, the Suzuken Memorial Foundation, the Takeda Science Foundation, the Takeda Medical Research Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Japan Foundation for Applied Enzymology, and the Tanabe Medical Frontier Conference.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Nakao K, Yasoda A, Ebihara K, Hosoda K, Mukoyama M** 2009 Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases. J Mol Med 87:1029–1039
2. **Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K, Imura H** 1992 Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130:229–239
3. **Kojima M, Minamino N, Kangawa K, Matsuo H** 1990 Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). FEBS Lett 276:209–213
4. **Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H** 1992 Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β: possible existence of "vascular natriuretic peptide system." J Clin Invest 90:1145–1149
5. **Komatsu Y, Nakao K, Itoh H, Suga S, Ogawa Y, Imura H** 1992 Vascular natriuretic peptide. Lancet 340:622
6. **Komatsu Y, Itoh H, Suga S, Ogawa Y, Hama N, Kishimoto I, Nakagawa O, Igaki T, Doi K, Yoshimasa Y, Nakao K** 1996 Regulation of endothelial production of C-type natriuretic peptide in coculture with vascular smooth muscle cells: role of the vascular natriuretic peptide system in vascular growth inhibition. Circ Res 78:606–614
7. **Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, Nakamura K, Nakao K, Kurihara T, Komatsu Y, Itoh H, Tanaka K, Saito Y, Katsuki M, Nakao K** 2001 Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc Natl Acad Sci USA 98:4016–4021
8. **Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL** 2004 Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci USA 101:17300–17305
9. **Komatsu Y, Nakao K, Suga S, Ogawa Y, Mukoyama M, Arai H, Shirakami G, Hosoda K, Nakagawa O, Hama N, Kishimoto I, Imura H** 1991 C-type natriuretic peptide (CNP) in rats and humans. Endocrinology 129:1104–1106
10. **Tamura N, Garbers DL** 2003 Regulation of the guanylyl cyclase-B receptor by alternative splicing. J Biol Chem 278:48880–48889
11. **Kishimoto I, Tokudome T, Horio T, Soeki T, Chusho H, Nakao K, Kangawa K** 2008 C-type natriuretic peptide is a Schwann cell-derived factor for development and function of sensory neurones. J Neuroendocrinol 20:1213–1223
12. **Berthoud HR, Morrison C** 2008 The brain, appetite, and obesity. Annu Rev Psychol 59:55–92

13. **Herman JP, Langub Jr MC, Watson Jr RE** 1993 Localization of C-type natriuretic peptide mRNA in rat hypothalamus. Endocrinology 133:1903–1906
14. **Langub Jr MC, Dolgas CM, Watson Jr RE, Herman JP** 1995 The C-type natriuretic peptide receptor is the predominant natriuretic peptide receptor mRNA expressed in rat hypothalamus. J Neuroendocrinol 7:305–309
15. **Kim-Saijo M, Akamizu T, Ikuta K, Iida Y, Ohmori K, Matsubara K, Matsuda Y, Suzuki M, Matsuda F, Nakao K** 2003 Generation of a transgenic animal model of hyperthyroid Graves' disease. Eur J Immunol 33:2531–2538
16. **Iwakura H, Akamizu T, Ariyasu H, Irako T, Hosoda K, Nakao K, Kangawa K** 2007 Effects of ghrelin administration on decreased growth hormone status in obese animals. Am J Physiol Endocrinol Metab 293:E819–E825
17. **Koide H, Shibata T, Yamada N, Asaki T, Nagao T, Yoshida T, Noguchi Y, Tanaka T, Saito Y, Tatsuno I** 2007 Tumor suppressor candidate 5 (TUSC5) is expressed in brown adipocytes. Biochem Biophys Res Commun 360:139–145
18. **Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N, Aizawa-Abe M, Yoshimasa Y, Nakao K** 1999 Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. Diabetes 48:2028–2033
19. **Ohinata K, Inui A, Asakawa A, Yoshikawa M** 2001 Novel actions of proadrenomedullin N-terminal 20 peptide (PAMP). Peptides 22:1809–1816
20. **Negishi T, Kawasaki K, Suzaki S, Maeda H, Ishii Y, Kyuwa S, Kuroda Y, Yoshikawa Y** 2004 Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ Health Perspect 112:1159–1164
21. **Lowell BB, Spiegelman BM** 2000 Towards a molecular understanding of adaptive thermogenesis. Nature 404:652–660
22. **Mascaró C, Acosta E, Ortiz JA, Marrero PF, Hegardt FG, Haro D** 1998 Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor. J Biol Chem 273:8560–8563
23. **Weis BC, Cowan AT, Brown N, Foster DW, McGarry JD** 1994 Use of a selective inhibitor of liver carnitine palmitoyltransferase I (CPT I) allows quantification of its contribution to total CPT I activity in rat heart. Evidence that the dominant cardiac CPT I isoform is identical to the skeletal muscle enzyme. J Biol Chem 269:26443–26448
24. **Langenickel TH, Buttgereit J, Pagel-Langenickel I, Lindner M, Monti J, Beuerlein K, Al-Saadi N, Plehm R, Popova E, Tank J, Dietz R, Willenbrock R, Bader M** 2006 Cardiac hypertrophy in transgenic rats expressing a dominant-negative mutant of the natriuretic peptide receptor B. Proc Natl Acad Sci USA 103:4735–2740
25. **Madden CJ, Morrison SF** 2009 Neurons in the paraventricular nucleus of the hypothalamus inhibit sympathetic outflow to brown adipose tissue. Am J Physiol Regul Integr Comp Physiol 296:R831–R843
26. **Nakamura K, Morrison SF** 2008 A thermosensory pathway that controls body temperature. Nat Neurosci 11:62–71
27. **Langub Jr MC, Watson Jr RE, Herman JP** 1995 Distribution of natriuretic peptide precursor mRNAs in the rat brain. J Comp Neurol 356:183–199
28. **Vesely DL, San Miguel GI, Hassan I, Gower Jr WR, Schocken DD** 2001 Atrial natriuretic hormone, vessel dilator, long-acting natriuretic hormone, and kaliuretic hormone decrease the circulating concentrations of total and free T_4 and free T_3 with reciprocal increase in TSH. J Clin Endocrinol Metab 86:5438–5442
29. **Müller D, Hida B, Guidone G, Speth RC, Michurina TV, Enikolopov G, Middendorff R** 2009 Expression of guanylyl cyclase (GC)-A and GC-B during brain development: evidence for a role of GC-B in perinatal neurogenesis. Endocrinology 150:5520–5529
30. **Simpson PJ, Miller I, Moon C, Hanlon AL, Liebl DJ, Ronnett GV** 2002 Atrial natriuretic peptide type C induces a cell-cycle switch from proliferation to differentiation in brain-derived neurotrophic factor- or nerve growth factor-primed olfactory receptor neurons. J Neurosci 22:5536–5551
31. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
32. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
33. **Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M** 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 123:1120–1128
34. **Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD** 1998 Role of the Y5 neuropeptide Y receptor in feeding and obesity. Nat Med 4:718–721
35. **Larhammar D, Salaneck E** 2004 Molecular evolution of NPY receptor subtypes. Neuropeptides 38:141–151
36. **Montani JP, Antic V, Yang Z, Dulloo A** 2002 Pathways from obesity to hypertension: from the perspective of a vicious triangle. Int J Obes 26 (Suppl 2):S28–S38
37. **Arch JR** 2008 The discovery of drugs for obesity, the metabolic effects of leptin and variable receptor pharmacology: perspectives from β3-adrenoceptor agonists. Naunyn-Schmied Arch Pharmacol 378:225–240
38. **Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga H, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K** 1999 Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 48:1822–1829
39. **Masuzaki H, Ogawa Y, Aizawa-Abe M, Hosoda K, Suga J, Ebihara K, Satoh N, Iwai H, Inoue G, Nishimura H, Yoshimasa Y, Nakao K** 1999 Glucose metabolism and insulin sensitivity in transgenic mice overexpressing leptin with lethal yellow agouti mutation: usefulness of leptin for the treatment of obesity-associated diabetes. Diabetes 48:1615–1622
40. **Tanaka T, Hidaka S, Masuzaki H, Yasue S, Minokoshi Y, Ebihara K, Chusho H, Ogawa Y, Toyoda T, Sato K, Miyanaga F, Fujimoto M, Tomita T, Kusakabe T, Kobayashi N, Tanioka H, Hayashi T, Hosoda K, Yoshimatsu H, Sakata T, Nakao K** 2005 Skeletal muscle AMP-activated protein kinase phosphorylation parallels metabolic phenotype in leptin transgenic mice under dietary modification. Diabetes 54:2365–2374
41. **Friedman JM, Halaas JL** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763–770
42. **Cusin I, Zakrzewska KE, Boss O, Muzzin P, Giacobino JP, Ricquier D, Jeanrenaud B, Rohner-Jeanrenaud F** 1998 Chronic central leptin infusion enhances insulin-stimulated glucose metabolism and favors the expression of uncoupling proteins. Diabetes 47:1014–1019
43. **Satoh N, Ogawa Y, Katsuura G, Numata Y, Tsuji T, Hayase M, Ebihara K, Masuzaki H, Hosoda K, Yoshimasa Y, Nakao K** 1999 Sympathetic activation of leptin via the ventromedial hypothalamus: leptin-induced increase in catecholamine secretion. Diabetes 48:1787–1793