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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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

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Ghrelin was initially identified as an endogenous ligand for the GH secretagogue receptor. When administrated exogenously, ghrelin stimulates GH release and food intake. Previous reports in ghrelin-null mice, which do not exhibit impaired growth nor appetite, question the physiologic role of ghrelin in the regulation of the GH/IGF-I axis. In this study, we generated a transgenic mouse that expresses human diphtheria toxin (DT) receptor (DTR) cDNA in ghrelin-secretion cells [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]. Administration of DT to this mouse ablates ghrelin-secretion cells in a controlled manner. After injection of DT into GPDTR-Tg mice, ghrelin-secreting cells were ablated, and plasma levels of ghrelin were markedly decreased [nontransgenic littermates, 70.6 ± 10.2 fmol/ml *vs.* GPDTR-Tg, 5.3 ± 2.3 fmol/ml]. To elucidate the physiological roles of circulating ghrelin on GH secretion and somatic growth, 3-wk-old GPDTR-Tg mice were treated with DT twice a week for 5 wk. The GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in wild-type mice at 5 wk of age. However, those were normalized at 8 wk of age. In contrast, in female mice, there was no difference in GH response to GHRH between GPDTR-Tg mice and controls at 5 or 8 wk of age. The gender-dependent differences in response to GHRH were observed in ghrelin-ablated mice. However, GPDTR-Tg mice did not display any decreases in IGF-I levels or any growth retardation. Our results strongly suggest that circulating ghrelin does not play a crucial role in somatic growth. **(*Endocrinology* 151: 1743–1750, 2010)**

GH secretion is predominantly regulated by two hypothalamic peptides, one factor is GHRH and a second is somatostatin (SST). In 1999, Kojima *et al.* (1) discovered ghrelin as an endogenous ligand for the GH secretagogue receptor (GHS-R or ghrelin receptor) from rats' stomach. Ghrelin, an acylated peptide of 28 amino acids, is synthesized primarily in endocrine cells of the stomach, named X/A-like or ghrelin cells (2). Peripheral administration of ghrelin strongly stimulates GH secretion (1, 3). Because coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Therefore, circulating ghrelin was thought to be the third peptide which

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Abbreviations: BMD, Bone mineral density; CT, computed tomography; DT, diphtheria toxin; DTR, DT receptor; GHS-R, GH secretagogue receptor; GPDTR-Tg, ghrelin-promoter DTR-transgenic; HB-EGF, heparin-binding epidermal growth factor-like growth factor; SST, somatostatin; WT, nontransgenic littermates.

regulates GH secretion. Indeed, patients with a functional mutation in GHS-R, ghrelin receptor, display familial short stature (5). Okimura *et al.* (6), however, demonstrated that circulating ghrelin levels do not correlated with those of GH; also, administration of a GHS antagosist to freely moving rats did not reduce plasma GH levels. Ghrelin knockout mice also exhibit normal growth patterns (7). On the other hand, ghrelin receptor knockout mice exhibit modest, but significant, body weight reductions and decreased serum IGF-I levels (8). Together, these findings question the physiologic significance of ghrelin in the regulation of GH secretion. As always with such model mice, there may be confounding factors, such as developmental adaptation and other compensatory mechanisms. To avoid these factors, it may be necessary to ablate ghrelin after birth or before puberty. Moreover, during the prepubertal and pubertal period, GH-dependent proportional body growth is observed in many mammalian species. The fetal growth is GH-independent, and growth during the early postnatal is only partial dependent upon GH. Therefore, to evaluate whether an absence of circulating ghrelin can influence a somatic growth through GH/IGF-I axis modification, we think that it is appropriate to choose a postweaning model.

In this study, we adopted a diphtheria toxin (DT) receptor (DTR)-mediated conditional and targeted cell ablation strategy to ablate ghrelin secretion cells, X/A-like cell, in a specific and controlled manner (9). We generated a transgenic mouse expressing human DTR cDNA, which encodes human heparin-binding epidermal growth factor-like growth factor (HB-EGF), under the control of the transcriptional regulatory regions of ghrelin. In this mouse, ghrelin-secreting cells express the human DTR and can be ablated after the administration of a small amount of DT. By using this transgenic mouse, we ablated ghrelin-secretion cells after weaning, which allowed us to evaluate the physiologic significance of ghrelin in GH secretion and somatic growth.

Materials and Methods

All animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Procedures were performed in accordance with the principles and guidelines established by that committee.

Plasmid construction and generation of transgenic mice [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]

The pGPDTR plasmid was constructed by replacement of the mouse albumin enhancer/promoter region of pMS7 (9) with a 4.1-kb *Mul*-*Hin*dIII fragment containing the 5′-flanking region of the human ghrelin gene (−4110/−33) derived from the p-4110/-33GHRE plasmid (human ghrelin promoter in pGL3) (Fig. 1A) (10). The 6.4-kb *Not*I-*Xho*I fragment of pGPDTR was microinjected into the pronucleus of fertilized eggs obtained from C57/B6 mice (SLC, Shizuoka, Japan). The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (Japan CLEA, Osaka, Japan) by using standard techniques (11). Founder transgenic mice, identified by PCR analysis, were bred with C57BL/6 mice. Mice were housed in air-conditioned animal quarters, with light between 0800 and 2000 h. Except where noted, animals were fed standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA) and water *ad libitum*.

Semiquantitative PCR

Total RNA was extracted using a Sepasol-RNA kit (Nacalai Tesque, Kyoto, Japan). RT used a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA).

Semiquantitative PCR determined the distribution of the DTR in GPDTR-Tg mice, using the following primers: sense 5′-CCTCCTCTCGGTGCGGG-3′ and antisense 5′-AGTCAC-CAGTGCCGAGAGAACTG-3′. Thirty-five cycles of thermal was performed with 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. Human heart mRNA (purchased from Clontech, Palo Alto, CA) was used as a positive control.

DT injection

DT was purchased from Sigma-Aldrich Japan (Tokyo, Japan). According to the previous report using DTR-mediated cell ablation systems (9), DT was injected im.

Histological procedures

Formalin-fixed, paraffin-embedded tissue sections were immunostained using avidin-biotin peroxidase complex methods (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA) as described (11). Sections were incubated overnight at 4 C with antighrelin-(1–11) antiserum that specifically recognizes acylated ghrelin (final dilution, 1:5000). Tissue sections were also stained with hematoxylin and eosine.

Measurement of plasma ghrelin levels

Measurement of plasma ghrelin levels was performed as reported previously (12). Blood samples drawn from the retro-orbital vein at 1000 h were immediately transferred to chilled siliconized glass tubes containing Na2EDTA (1 mg/ml) and

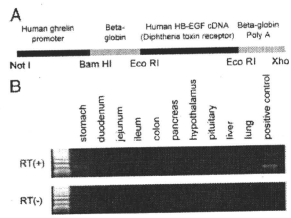

FIG. 1. Generation of GPDTR-Tg mice. A, The GPDTR-Tg construct contained a fusion gene comprised of the 5′flanking lesion of human ghrelin (4085 bp) and the DTR cDNA (human HB-EGF). B, Expression of DTR mRNA in various tissues of GPDTR-Tg mice at 8 wk of age. The human heart mRNA was used as a positive control.

TABLE 1. PCR primers and TaqMan probes

Ghrelin	Sense	5′-GCATGCTCTGGATGGACATG-3′
	Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
	Probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
GH	Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
	Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
	Probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH	Sense	5′-AGGATGCAGCGACACGTAGA-3′
	Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
	Probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
SST	Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
	Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
	Probe	5′-CGAACCCAGCAATGGCACCCC-3′
IGF-1	Sense	5′-ACCCGGACCTACCAAAATGAC-3′
	Antisense	5′-GGTGTGAAGACGACATGATGTGT-3′
	Probe	5′-CACCTGCAATAAAG-3′
GHS-R	Sense	5′-CACCAACCTCTATCCAGCAT-3′
	Antisense	5′-CTGACAAACTGGAAGCGTTTGCA-3′
	Probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′

aprotinin (1000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan). After centrifugation at 4 C to separate out the plasma, hydrochloric acid was added to samples at a final concentration of 0.1 N. Plasma was immediately frozen and stored at −80 C until assayed. Plasma ghrelin concentrations were determined using a ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Real-time PCR analysis

Extraction of total RNA from various tissues and RT was performed as described above. Real-time quantitative PCR used an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the primers and TaqMan probes described in Table 1. The mRNA expression levels of each gene were normalized to that of 18S rRNA. All samples were examined in triplicate in 96-well plates using an ABI Prism 7500 sequence detector according to the manufacturer's protocol.

GH provocative test

GH provocative test was carried out as previously described (12). These experiments were conducted in unanesthelized mice. Human GHRH was purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). Serum samples were collected at 15 and 30 min after sc injection of 180 mcg/kg of GHRH.

Ghrelin-rescue experiments

Osmotic infusion pumps (Alzet Micro-Osmotic pump, Model 1002; Durect Corp., Cupertino, CA) were implanted sc in 3-wk-old male GPDTR-Tg mice. Ghrelin (60 mcg/kg·d; Peptide Institute, Osaka, Japan) or saline was continuously infused through the osmotic infusion pumps. Then mice were started to treat with DT (50 ng/kg twice a week) a day after pump implantation. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out in these mice at the age of 5 wk.

Measurement of serum GH and IGF-I levels

Blood samples were collected from the tail veins of mice. Serum was isolated by centrifugation and stored at −20 C until assayed. Serum GH levels and IGF-I levels were measured using the appropriate EIA kits from SPI-BIO (Bonde, France) and Diagnostic Systems Laboratories, Inc. (Webster, TX), respectively, according to the manufacturers' instructions.

Measurement of body lengths

Mouse body length was measured by manual immobilization and extension of mice to determine nose-to-anus length. All measurements were performed by the same individual in a blind fashion.

Measurement of fat mass and bone mineral density (BMD)

The fat mass (% fat) and BMD of mice were measured by computed tomography (CT) (Laboratory CT; Lacita, Aloka, Japan) under pentobarbital anesthesia.

Statistical analysis

Results are expressed as the means ± SEM. Multiple comparisons between groups were made by Turkey-Krammer test, with α set at $P < 0.05$. The results on body weight and serum GH levels after GHRH injection were analyzed by a two-way ANOVA followed by Tukey's *post hoc* test, with α set at $P < 0.05$. Statistical analyses were carried out with STATVIE 4.0 software (Abacus Concepts, Inc., Berkley, CA).

Results

Generation of transgenic mice in which ghrelin can be ablated in a controlled manner

Transgenic mice

To elucidate physiologic role of ghrelin in GH secretion and somatic growth, we developed transgenic mice in which ghrelin can be ablated in controlled manner. We adopted a DTR-mediated conditional and targeted cell ablation strategy. We created transgenic mice that expressed the gene for the human DTR, human HB-EGF,

under the control of the ghrelin promoter. By injecting transgenes into 184 eggs, we obtained three lines of transgenic mouse (Tg 1-2, Tg 5-1, and Tg 5-8). We continued with the Tg 5-1 transgenic line, because Tg 1-2 animals did not exhibit decreases in plasma ghrelin levels after injection of high-dose DT and Tg 5-8 required high doses of DT (50 mcg/kg) to ablate ghrelin-producing cells (data not shown). In Tg 5-1 transgenic animals, semiquantitative PCR analysis revealed high expression of DTR mRNA in the stomach and weak expression in the duodenum and jejunum. No expression, however, could be detected in the ileum, colon, pancreas, hypothalamus, pituitary, liver, or lung (Fig. 1B). In Tg 5-1 mice, the ghrelin-producing cells of the stomach were ablated by injection with low-dose DT (10 or 50 ng/kg) (Fig. 2, A, B, and D). We therefore designated the Tg 5-1 transgenic line and nontransgenic littermates as GPDTR-Tg mice and wild-type (WT) mice, respectively.

Ablation of ghrelin-producing cell

To determine the dose and timeframe of DT injection, preliminarily studies were performed: GPDTR-Tg mice were injected with saline or DT twice a week at a dose of 10, 30, 50, 100, and 500 ng/kg (on d 0 and 2). Plasma ghrelin levels on d 4 were decreased to approximately 60, 30, 5, 5, and 5% of control mice (Tg mice treated with saline) after 10, 30, 50, 100, and 500 ng/kg of DT injection, respectively. Thus, we judged that 50 ng/kg of DT is the smallest effective dose to reduce plasma ghrelin. The final results using 10 and 50 ng/kg of DT were described below. Next, GPDTR-Tg mice were injected with 50 ng/kg of DT with four schedules: once a week (on d 0), twice a week (on d 0 and 2), three times a week (on d 0, 2, and 4), or daily (from d 0 to 6), and plasma ghrelin levels were measured on d 7. The once-a-week injection of DT was insufficient, but the twice-a-week injection of DT had enough effect on reduction in plasma ghrelin concentration.

To ablate ghrelin-producing cells, 8-wk-old male WT and GPDTR-Tg mice were injected im with 10 or 50 ng/kg DT daily on d 0 and 2 and analyzed on d 4. WT mice treated with saline or DT and GPDTR-Tg mice treated with saline were used as control mice.

To evaluate the effects of DT injection on ghrelin-producing cell, we analyzed stomach by immunohistochemical analysis with antighrelin antisera (Fig. 2A) and real-time PCR (Fig. 2, B and C). DT injection reduced in a dose-dependent manner both the number of ghrelin-positive cells and the expression of ghrelin mRNA in the stomach of GPDTR-Tg mice (Fig. 2, A and B). DT injection did not produce in any abnormalities in WT mice, because

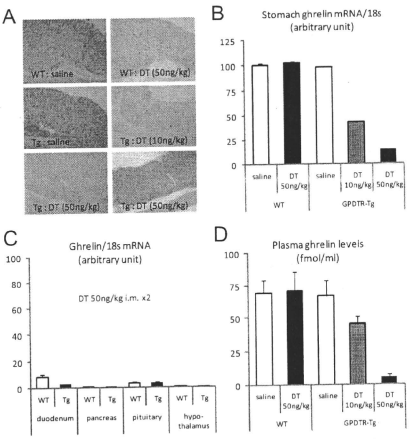

FIG. 2. Ablation of ghrelin-secretion cells. Eight-week-old male GPDTR-Tg mice (Tg) and nontransgenic littermates (WT) were injected with saline or 10 or 50 ng/kg of DT (im) on d 0 and 2, then analyzed on d 4. A, Histological analysis of stomach sections. Immunohistochemical analysis of ghrelin peptide expression and hematoxylin and eosin staining. Original magnification, ×100. B, Ghrelin mRNA levels in the stomach. C, Ghrelin mRNA levels in the duodenum, pancreas, pituitary, and hypothalamus of GPDTR-Tg and WT mice injected with 50 ng/kg of DT. D, Plasma ghrelin levels in GPDTR-Tg and WT mice. For B–D, data represent the means ± SEM (n = 8).

these mice do not possess the DTR, making them insensitive to DT. In transgenic animals, DT injection also reduced ghrelin mRNA expression in the duodenum, but not the pancreas, pituitary, or hypothalamus (Fig. 2C). Plasma ghrelin levels in GPDTR-Tg mice treated with 10 and 50 ng/kg of DT were decreased to approximately 60 and 5–7% of control mice, respectively (Fig. 2D). These results suggested that this transgenic mouse model is a useful tool for evaluating the physiologic role of circulating ghrelin.

Histological analysis with hematoxylin and eosin staining revealed that no inflammatory cell infiltration was seen in the stomach (Fig. 2A), small intestine, colon, pancreas, pituitary, and hypothalamus of the GPDTR-Tg mice with 50 ng/kg of DT injection. Other historical abnormalities were also not observed in these tissues (data not shown).

The effects of a reduction in circulating ghrelin after weaning on the GH/IGF-I axis and somatic growth

To study the effects of postweaning reductions in circulating ghrelin on the GH/IGF-I axis and somatic growth, 3-wk-old WT and GPDTR-Tg mice were treated with DT

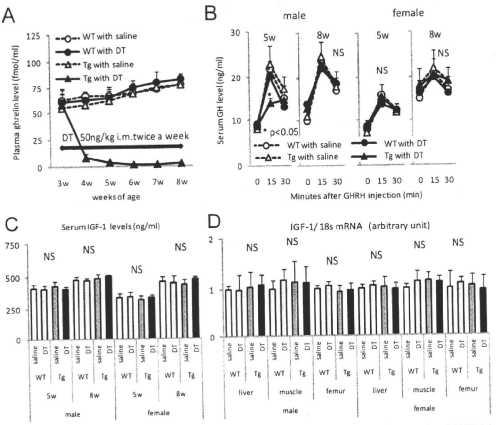

FIG. 3. The effects of a postweaning reduction in circulating ghrelin on the GH/IGF-I axis. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A, Plasma ghrelin levels before and after DT injection. B, GH response to GHRH administration (180 μg/kg sc) in GPDTR-Tg and WT mice at 5 and 8 wk of age. C, Serum IGF-I levels of GPDTR-Tg and WT mice at 5 and 8 wk of age. D, IGF-I mRNA levels in liver, skeletal muscle, and femur in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

(50 ng/kg) or saline twice a week for 5 wk (from 3 to 8 wk old). After DT injection, plasma ghrelin levels of GPDTR-Tg mice decreased rapidly. In GPDTR-Tg mice, ghrelin levels were undetectable by 5 wk of age, remaining so thereafter (Fig. 3A). The data obtained from GPDTR-Tg mice were compared with those from three groups of control mice (WT with saline, WT with DT, and GPDTR-Tg with saline).

To elucidate whether a postweaning reduction in circulating ghrelin can influence GH secretion, we measured basal serum GH levels and performed GH provocative test with GHRH. There were no differences in basal serum GH levels between GPDTR-Tg mice treated with DT and control mice in either males or females at 5 or 8 wk of age. GH provocative test with GHRH showed some intriguing results (Fig. 3B). The GH responses to GHRH in male GPDTR-Tg mice treated DT were significantly lower than those in three controls at 5 wk of age. However, those responses were normalized at 8 wk of age. On the other hand, there were no differences in GH response to GHRH among four groups (WT with saline or DT, and Tg with saline or DT) in females at 5 or 8 wk of age.

To elucidate whether temporarily attenuation of GH responses to GHRH can affect IGF-I regulation, we investigated serum IGF-I levels and IGF-I mRNA expressions in the liver, skeletal muscle, and distal femur. There were no differences in serum IGF-I levels among any animal groups in either males or females at 5 or 8 wk of age (Fig. 3C). There were also no differences in IGF-I mRNA expressions in the liver, skeletal muscle, or distal femur among any animal groups at 5 wk of age (Fig. 3D). We then investigated the effects of decreases in circulating ghrelin on the expression of mRNA encoding GHRH and SST within the hypothalamus and encoding GH and GHS-R in the pituitary. There were no differences in mRNA expression levels of these mediators among any animal groups in male and female at 5 wk of age (Fig. 4).

As expected from the results of the IGF-I studies, no evidence of growth retardation could be found in either male or female GPDTR-Tg mice treated with DT during the observation period. There were no difference in body weight or length in comparison with three groups of control mice at any point (Fig. 5, A and B, for male; and Fig. 5, D and E, for female animals). CT analysis of body composition demonstrated that there were no differences in percent fat or BMD among any animal groups at 5 and 8 wk of age (Fig. 5C for male, and Fig. 5F for female animals).

There were no differences in weekly food intake from 3 to 8 wk of age [WT *vs.* GPDTR-Tg (treated with DT); male, 18.4 ± 0.5 *vs.* 18.9 ± 0.7; female, 18.4 ± 1.0 *vs.* 18.5 ± 0.6 (g/wk)]. These results suggested that although GH responses to GHRH were temporarily reduced under conditions of decrease in circulating ghrelin, somatic growth was not impaired.

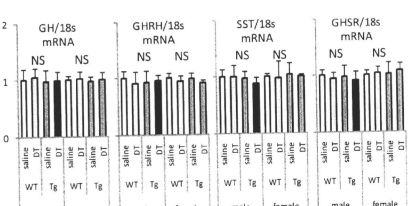

FIG. 4. The effects of a postweaning reduction in circulating ghrelin on the expression of mRNA encoding GHRH, SST,GH and GHS-R. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). Pituitary mRNA levels of GH and GHS-R and hypothalamic mRNA levels of GHRH and SST in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

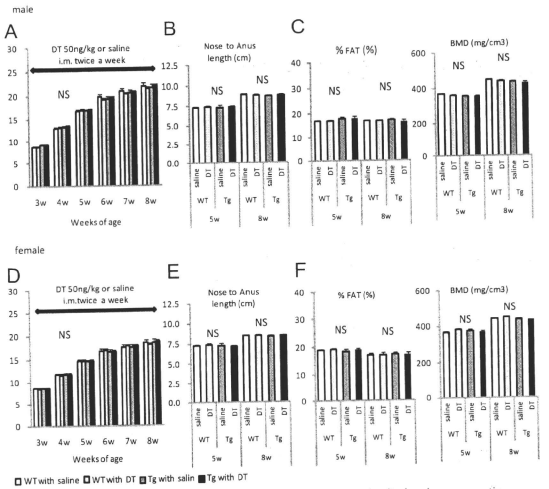

FIG. 5. The effects of a postweaning reduction in circulating ghrelin levels on somatic growth. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A and D, Changes in body weight in male (A) and female mice (D). B and E, Nose to anus length in male (B) and in female mice (E) at 5 and 8 wk of age. C and F, Body composition (% Fat) and BMD as analyzed by CT in male (C) and in female mice (F) at 5 and 8 wk of age. Data represent the means ± SEM (n = 12).

GH response to GHRH in the ghrelin-rescued GPDTR-Tg mice

To elucidate whether GH responsiveness to GHRH can be ameliorated by ghrelin replacement in the ghrelin-ablated mice, GH provocative test were carried out in the DT-treated GPDTR-Tg mice whose circulating ghrelin were rescued by continuously administration of ghrelin with osmotic pump. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out at the age of 5 wk.

GH responsiveness to GHRH was ameliorated by ghrelin replacement. Serum GH levels at 0, 15, and 30 min after GHRH administration in the ghrelin-rescued mice were 7.8 ± 1.6, 26.2 ± 4.2, and 12.3 ± 0.8 ng/ml, respectively, whereas those in mice without ghrelin replacement were 6.8 ± 1.5, 10.9 ± 2.6, and 11.3 ± 1.6 ng/ml, respectively. These results suggested that attenuated response to GHRH seen in ghrelin-ablated mice without ghrelin replacement was due to acute ghrelin deficiency.

Discussion

In this study, we generated transgenic mice expressing the DTR driven by the transcriptional regulatory machinery of ghrelin. Injection of DT into this mouse can ablate ghrelin-secreting cells. Approximately 70–80% of circulating ghrelin originates from the stomach (13). Ghrelin-producing cells are also found throughout the small intestine, with the duodenum producing approximately one-tenth that of the stomach (14). Semiquantitative PCR revealed that DTR was only expressed in stomach and not in pituitary, hypothalamus, and pancreas and the intensity of the band of DTR in stomach was very low. Three possibilities might be considered to explain this result. The first is the low efficiency of gene transfection. Three lines of GPDTR-Tg mice that we generated in this study were inserted with low copy numbers of transgene (DTR cDNA). Thus, the expression levels of DTR mRNA could be very low even in stomach. The second is the efficiency of gene expression. In this study, we designed a fusion gene comprising the 4085-bp fragment contained a partial sequence of the 5′-flanking region of the human ghrelin gene and human DTR. The efficiency of gene expression driven by this fragment might be lower than those driven by the original ghrelin promoter region. The last, except gastrointestinal tract, transcription of ghrelin gene might be driven by a different size of fragment of the 5′-franking region. Immunohistochemical and PCR analyses demonstrated that ghrelin-secreting cells in the stomach and duodenum were ablated after DT injection into GPDTR-Tg mice, resulting in marked reduction of plasma ghrelin levels. In contrast, ghrelin-producing cells of the pituitary and hypothalamus were unaffected. Thus, this transgenic mouse is a useful model to explore the role of circulating ghrelin, because plasma ghrelin levels can be abrogated in a controlled manner without altering pituitary and hypothalamic ghrelin mRNA expression levels.

The physiologic roles of ghrelin in the regulation of GH secretion remain unclear, because previous reports using rodents deficient or reduced in ghrelin signals have given conflicting results (7, 8, 15, 16). Sun *et al.* (7) reported that ghrelin-deficient mice did not exhibit any growth retardation or decreases in serum IGF-I levels. Wortley *et al.*

(15) also were unable to observe any significant differences between ghrelin-deficient mice and WT mice in body weight or basal serum GH levels, when fed a standard diet. Moreover, Zigman *et al.* (16) demonstrated there was no significant difference in serum IGF-I levels between ghrelin receptor knockout and WT mice. Sun *et al.* (8), however, showed that ghrelin receptor knockout mice exhibited only a small reduction in body weight and serum IGF-I levels. In addition, Pantel *et al.* (5) showed that two unrelated families with short statue have a missense mutation of GHS-R. This mutation impairs the constitutive activity of the GHS-R. They also reported a young patient with growth delay who has a recessive partial isolated GH deficiency due to GHS-R mutations (17). These results indicate importance of ghrelin/GHS-R signals in GH secretion and somatic growth.

The purpose of this study is to evaluate whether an absence of circulating ghrelin can influence GH secretion and somatic growth via GH/IGF-I axis in mammals. First, we investigated basal serum GH levels and the GH response to GHRH. Although basal serum GH levels in the ghrelin-abrogated mice did not differ from those seen in WT mice, the GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in WT mice at 5 wk of age. As coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Indeed, GH responsiveness to GHRH was ameliorated by ghrelin replacement in the ghrelin-ablated mice. However, the attenuated response to GHRH in the ghrelin-ablated mice had persisted only for a short term. The GH responses to GHRH in male GPDTR-Tg mice were recovered and were not different from those in WT mice at 8 wk of age. It is possible that an adaptation to reduced circulating ghrelin occurred within a short term. Indeed, Popovic *et al.* (18) reported that 10 patients who underwent total-gastrectomy at least 2 yr ago, a state of acquired chronic hypoghrelinemia, exhibited normal GH response to GHRH compared with normal subjects. Meanwhile, in female mice, there were no differences in either basal serum GH levels or GH response to GHRH between WT and GPDTR-Tg mice at 5 or 8 wk of age. The secretory pattern of GH in rodents is sexually differentiated. In male rats, GH is secreted in episodic pattern with low levels between pulses, whereas in females, the pulses are lower and plasma GH levels between pluses are higher than males (19). The secretory pattern of GH differs between male and female by 30 d of age (20). Gonadal steroids are thought to produce the sexual differences in GH secretion. We assumed that the sexual differences in GH response to GHRH in ghrelin-ablated mice may depend on gonadal steroids.

As GH secretion is pulsatile in nature, a single measurement of GH concentration in blood would not adequately reflect endogenous GH secretion. To estimate the amplitude and frequency of GH pulses, short-interval blood sampling under a conscious state is required. Such studies are difficult to perform in mice. Instead, we investigated serum IGF-I levels, skeletal muscle IGF-I mRNA expression, and anthropometric parameters that reflect pulsatile GH release under similar nutritional conditions (21). Serum IGF-I levels and IGF-I mRNA expression in skeletal muscle did not decrease in the ghrelin-abrogated mice in comparison with WT mice. These results suggest that circulating ghrelin does not play a dominant role in the GH/IGF-I axis. Due to significant differences between species in the regulation of GH secretion (21), we have to give careful considerations to apply the results of animal experiments concerning GH secretion directly to humans; insulin-induced hypoglycemia is a potent stimulus of GH secretion in humans, whereas rats respond to the stress of hypoglycemia by decreasing GH secretion (22, 23). L-arginine is a potent GH secretagogue in humans, but does not (or less overtly) stimulate GH secretion in rats (21, 24).

Somatic growth is affected not only by GH and IGF-I but also by thyroid hormones, sex steroids, and glucocorticoids. It also depends on genetic background and nutrition. Adequate nutrition is one of the most important factors affecting somatic growth. In present study, there were no differences in food intake between the ghrelin-abrogated mice and WT mice. Body weight, length, and body composition also were not influenced by plasma ghrelin levels. These results suggest that circulating ghrelin does not play a dominant role in somatic growth.

We cannot exclude the possibility that hypothalamic ghrelin may regulate GH secretion, as hypothalamic ghrelin-secreting cells were preserved in this animal model. Shuto *et al.* (25) demonstrated that transgenic rats expressing antisense GHS-R mRNA within the arcuate nucleus of the hypothalamus displayed growth retardation, suggesting that ghrelin/GHS-R systems in the hypothalamus function in the regulation of GH. Further studies will be needed to elucidate the role of hypothalamic ghrelin in GH secretion.

In summary, we have succeeded in generating transgenic mice in which circulating ghrelin can be abrogated in a controlled manner after birth. Our results suggest that circulating ghrelin does not play a crucial role in somatic growth.

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Orexin decreases mRNA expressions of NMDA and AMPA receptor subunits in rat primary neuron cultures

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ABSTRACT

Orexin is one of the orexigenic neuropeptides in the hypothalamus. Orexin neurons in the lateral hypothalamus (LH) project into the cerebral cortex and hippocampus in which the receptors are distributed in high concentrations. Therefore, to elucidate the actions of orexin in the cerebral cortex, we examined its effects on the mRNA expressions of N-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A, NR2B) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits (GluR1, GluR2) following 6-day application of orexin-A or orexin-B to rat primary cortical neuron cultures. The mRNAs of NR1 and NR2A subunits were significantly decreased by orexin-A and orexin-B at concentrations over 0.1 μM and 0.01 μM, respectively. The mRNA expression of NR2B subunit was also significantly decreased by orexin-A and orexin-B only at the concentration of 1 μM. Moreover, orexin-A and orexin-B at concentrations over 0.01 μM significantly decreased the mRNA expressions of AMPA receptor subunits, GluR1 and GluR2. The present study demonstrated that orexins significantly suppressed RNA expressions of NMDA and AMPA receptor subunits in cortical neuron cultures, suggesting that orexin may regulate the higher functions of the cerebral cortex as well as be involved in energy regulation in the hypothalamus.

1. Introduction

The hypothalamus is a center of energy regulation containing orexigenic and anorexigenic neuropeptides, and its dysregulation is thought to induce obesity [36]. Obesity is a risk factor for metabolic diseases, such as type 2 diabetes mellitus, hypertension and hyperlipidemia [26]. Among these neuropeptides, orexins (orexin-A, orexin-B) containing neurons were observed to be present in the lateral hypothalamus (LH) with projections into the cerebral cortex, hippocampus and amygdala [13,30], which are the essential regions for controlling cognition [21], anxiety and depression [24]. Orexins in LH were reported to be up-regulated in obese rodent models [28]. The actions of orexins are mediated via two receptors, orexin-1 (OX1R), and orexin-2 (OX2R), that are coupled with G-proteins [34]. While

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orexin-A has equal affinity for OX1R and OX2R, orexin-B has an appreciably greater (approximately 10-fold) affinity for OX2R [34]. In situ hybridization studies have shown that high levels of orexin-1 receptor and orexin-2 receptor mRNA occur in the cerebral cortex [15,21]. Endogenous orexins have diverse physiological functions related to food intake [34], arousal [13], sleep-waking cycle [31,41], sleep disorder [22], nociception [7], and learning and memory [1,2,16,20,40], anxiety and depression [24].

In the cerebral cortex, the ionotropic glutamatergic nervous system, including *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) glutamate receptors, plays a crucial role in the regulation of learning and memory within cortico-hypothaoamic networks [4,5,8]. The NMDA receptors are composed of NR1, NR2A-D, and NR3 subunits, with the NR1 subunit being a necessary component of functional NMDA receptor channels [10,29,33]. The AMPA receptors composed of GluR1-4 subunits are distributed in the cerebral cortex, and also play an important role in neural plasticity related to learning and memory with interaction NMDA receptor signaling [6,35]. It was reported that depletion of NR2A subunit of NMDA receptor in mice induced memory dysfunction [23], and that depletion of NR2B subunit of NMDA receptor in mice impairment of spatial learning [12]. Intracerebroventricular administrations of AMPA receptor antagonists CNQX and NBQX, were reported to induce impairment of memory [6,35]. Orexin was demonstrated to increase glutamatergic activity in several brain areas innervated by orexin neuronal fibers, including the LH/PFA [14] Glutamate and orexin are colocalized within fibers in the tuberomamillary nucleus [14] and hypothalamus [14], and the postsynaptic action of orexin to stimulate pyramidal cells in the prefrontal cortex was facilitated by glutamate in a synergistic fashion [14]. In addition, glutamate release was increased in response to systemic administration of orexin-A in both the locus coeruleus and amygdala [14].

Recent studies have shown that central administration of orexin-A and orexin-B has effects on learning and memory but the literature concerning the role of the orexin system in cognition remains controversial. Aou et al. have reported that i.c.v. administered orexin-A produced memory impairment in water maze performance in a 2-day training protocol [2]. On the contrary, Akbari et al. reported that the intra-CA1 injection of the selective OX1R antagonist impaired acquisition, consolidation and retrival of spatial memory in Morris water maze task [1]. They also reported that the intra-dentate gyrus administration of OX1R antagonist impaired acquisition and consolidation of Morris water maze task, but had no effect on retrieval in spatial memory [1].

We postulated that the ionotropic glutamatergic nervous system in the cerebral cortex may be modulated by orexins. To test this, we used the primary cortical neuronal culture, which is well understood and an established method for examining the physiological effect on adult brain plasticity of a specific substance on the ionotropic glutamatergic nervous system [38]. In the present study, we examined the mRNA expressions of NMDA and AMPA receptor subunits after chronic application of orexin-A and orexin-B by using rat cultured primary cortical neurons.

2. Materials and methods

2.1. *Rat cultured primary cortical neurons*

Timed pregnant Sprague–Dawley rats were obtained from Japan SLC, Inc. (Japan) on gestational day 18. The animals were anesthetized with pentobarbital sodium (50 mg/kg, ip; Abbott, Abbott Park, Ill, USA) and sacrificed by cervical dislocation. The fetuses were delivered and decapitated. For each experiment, fetuses were extracted from four maternal rats. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at Kyoto University, Chiba University and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to optimize the comfort and to minimize the use of animals. The cultured primary cortical neurons were prepared according to previous reports [9,18,19] with minor changes. The cerebral cortex was dissected and cut into small pieces in Hanks' balanced salt solution (HBSS: Ca^{2+} and Mg^{2+} free) (Invitrogen, Carlsbad, CA, USA). The tissue was then dispersed with 0.025% trypsin-EDTA solutions (Invitrogen, Carlsbad, CA USA). DNase (final concentration; 0.2 μg/ml) (Sigma, St. Louis, MO, USA), soybean trypsin inhibitor (final concentration; 2 μg/ml) (Sigma, St. Louis, MO, USA), $MgSO_4$ (final concentration; 0.24 mM) were added. The cell suspension was centrifuged at 800 × *g* for 5 min, and the supernatant was aspirated. The cells were suspended in Neurobasal Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% of B27 containing antioxidants (Invitrogen, Carlsbad, CA, USA), L-glutamine (final concentration; 0.5 mM), antibiotic–antimycotic solution (final concentration; 1%) (Nacalai tesque, Kyoto, Japan), then DNase (final concentration; 0.2 μg/ml) (Sigma, St. Louis, MO, USA), soybean trypsin inhibitor (final concentration; 2 μg/ml) (Sigma, St. Louis, MO, USA) and $MgSO_4$ (final concentration; 0.24 mM). The cell suspension was centrifuged at 800 × *g* for 5 min, the supernatant was aspirated, and the remainder was washed with Neurobasal Medium. This procedure was repeated 3 times. Live cells were counted using a hemocytometer, and the cell suspension was then diluted with Neurobasal Medium at 2×10^6 cells/ml. Cells were seeded onto poly-D-lysine-coated 6-well plates (BD Bioscience, Discovery Lab ware, Bedford, MA, USA) at 2×10^6 cells/ml well. All cultures were maintained in Neurobasal medium at 37 °C in 95% humidified air and 5% CO_2. On the 3rd day of culture, cytosine β-D-arabino-furanoside hydrochloride (final concentration; 10 μM) (Sigma, St. Louis, MO, USA), a selective inhibitor of DNA synthesis, was added for 72 h to the culture to prevent further proliferation of non-neuronal cells.

Orexin-A and orexin-B (0.01 μM, 0.1 μM and 1 μM) were applied to the culture wells on the 6th day of culture, and the cultured cells were incubated for 6 days. The culture medium which contained orexin-A, orexin-B or vehicle was changed every 3 days. On the 12th day of culture, the total cellular RNA was extracted from two cultured wells as one RNA sample using an RNeasy Mini Kit (Quiagen Sciences, MA, USA). The samples were stored at −20 °C until assay. All experiments consisted of 3–6 repetitive runs. For each run, two to six mRNA samples were obtained. Orexin-A and orexin-B were from

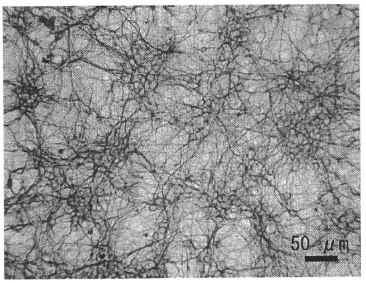

Fig. 1 – Rat cultured cortical neurons stained with anti-MAP2 antibody.

Peptide Institute Inc. (Osaka, Japan). Neuronal degeneration was assessed every 3 days after neuropeptide application by efflux of lactate dehydrogenase using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Co., Madison, WI, USA) [19].

2.2. *Immunohistochemical method*

For verification of cultured cortical neurons, expression of microtubule-associated protein-2 (MAP-2) predominantly in the dendrites of neurons was observed (Fig. 1). Cortical neuron cultures were prepared on D-poly-L-lysine-coating slide glasses (Matsunami, Osaka, Japan) according to the above noticed method. Cultured neurons at day 6th were fixed with 4% paraformaldehyde in 15 mM phosphate buffered saline (PBS) (pH7.3) for 30 min at room temperature (RT). To enhance penetration of the cell membrane, sections were treated with 0.25% Triton-X in PBS for 10 min at RT. To reduce non-specific background staining, sections were treated with 3% goat serum and 5% bovine serum albumin (BSA) in PBS for 30 min at RT. For MAP-2 protein staining, sections were incubated with anti-MAP-2 rabbit antibody (1:200; Sigma, St. Louis, USA) in PBS containing 4% goat serum, 0.3% BSA and 0.3% Triton-X over night at 4 °C. Antibody was detected using the Vectastain ABC Elite kit (PK-6101, Vector Laboratories, CA, USA). DAB substrate kit (SK-4100, Vector Laboratories) was used for visualization.

2.3. *Real-time RT-PCR*

Quantitative real-time RT-PCR was performed in the ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR green dye. Quantitative PCR was prepared in duplicate with 50 μl of reaction mixture in MicroAmp optical 96-well reaction plates. Each reaction well contained 12.5 μl of RNA sample, 25 μl of SYBR Green PCR Master Mix, 0.5 μl of RT Mix (Quiagen Sciences, MA, USA), 7 μl RNAase free water, and 25 pmol each of forward and reverse primers. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: [sense 5′-TGCACCACCAACTGCTTAGC-3′, antisense 5′-GGATGCAGGGATGATGTTCTG-3′], for OX1R, [sense 5′-GCATATCCACCTGGCCTGAA-3′, antisense 5′-CCACCATGCCAACGAGATCC-3′] [42], for OX2R, [sense 5′-CTACGCTCTTCTGCTATTGA-3′, antisense 5′-ACTGGCATGCTGATACATAC-3′] [41], for NR1 subunit, [sense 5′-AAGCCCAACGCCATACAGAT-3′, antisense 5′-AGGCGGGTGGCTAACTAGGA-3′] [44], for NR2A, [sense 5′-GCTACACACCCTGCACCAATT-3′, antisense 5′-CACCTGGTAACCTTCCTCAGTGA-3′] [6], for NR2B subunit, [sense 5′-CCCAACATGCTCTCTCCCTTA A-3′, antisense 5′-CAGCTAGTCGGCTCTCTTGGTT-3′] [3], for GluR1 subunit, [sense 5′-TTCCTGTTGACACATCCAATCAAT-3′, antisense 5′-ATGGTCGATAATGCTAATGAGAGCTT-3], and for GluR2 subunit, [sense 5′-CCTAGCTTCCCAACAGATGGC-3′, antisense 5′-GAGGTATGCGAACTTGTCCCA-3′]. Real-time RT-PCR was conducted according to our previous report [25]. All gene-specific mRNA expression values were normalized against the internal housekeeping gene GAPDH. The results are presented as the mean ± S.E.M. of 8–31 RNA samples per group.

2.4. *Data analysis*

Statistical analysis of the data was carried out by analysis of variance (ANOVA) followed by Dunnett's multiple range test. Statistical significance was defined as $p < 0.05$.

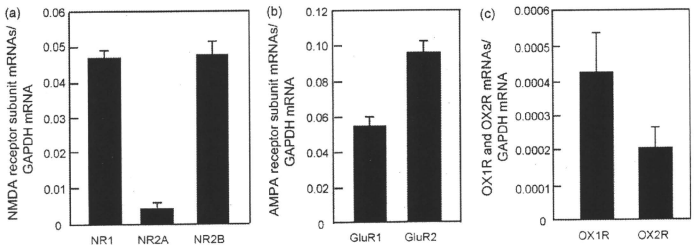

Fig. 2 – mRNA expressions of *N*-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A, NR2B) (a), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits (GluR1, GluR2) (b), and orexin receptors (OX1R, OX2R) (c) in rat cultured cortical neurons. Values are mean ± S.E.M. from 8 to 31 RNA samples.

3. Results

mRNAs of NMDA receptor subunits (NR1, NR2A, NR2B) and AMPA receptor subunits (GluR1, GluR2) were detected in the rat cultured primary cortical neurons (Fig. 2a and b), which agreed with previous reports [17,45]. The mRNA expressions of OX1R and OX2R were also detected in the cultured neurons (Fig. 2c).

Orexin-A at concentrations of 0.1 and 1 μM significantly decreased the mRNA expressions of NR1 ($F(6, 102) = 10.76$, $p < 0.01$; Dunnett multiple range test; 0.1 μM, $p = 0.0107$; 1 μM, $p < 0.01$) and NR2A ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3a and b). Orexin-B at the concentrations 0.01, 0.1 and 1 μM significantly decreased the mRNA expressions of NR1 ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.01 μM, $p = 0.0194$; 0.1 μM, $p < 0.0194$; 1 μM, $p < 0.01$) and NR2A ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.01 μM, $p < 0.01$, 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3a and b). The mRNA expression of NR2B was significantly decreased by both orexin-A and orexin-B at the concentration of 1 μM ($F(6,102) = 8.22$, $p < 0.01$; Dunnett multiple range test; orexin-A, 1 μM, $p < 0.01$; orexin-B, 1 μM, $p < 0.01$) (Fig. 3c). With regard to the mRNA expressions of AMPA receptor subunits, the mRNA expressions of both GluR1 and GluR2 were significantly decreased by both orexin-A and orexin-B at 0.01, 0.1 and 1 μM (GluR1: $F(6,108) = 18.18$, $p < 0.01$; Dunnett multiple range test; orexin-A, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$; orexin-B, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3d), (GluR2: $F(6,114) = 12.92$, $p < 0.01$; Dunnett multiple range test; orexin-A, 0.01 μM, $p = 0.0215$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$; orexin-B, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3e).

While the inhibitory effect of orexin-B on the mRNA expressions of these receptor subunits was about 2-fold as potent as that of orexin-A at the same concentration, there

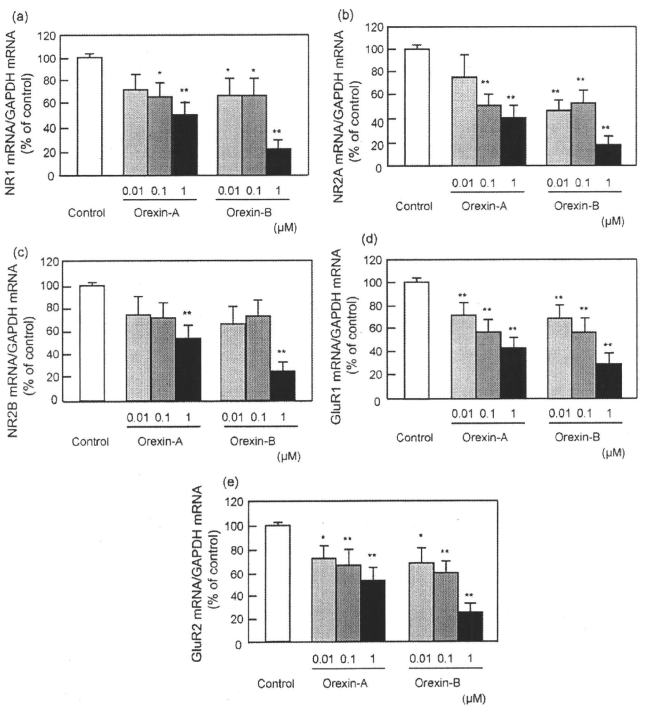

Fig. 3 – Changes of mRNA expressions of NR1 (a), NR2A (b), NR2B (c), GluR1 (d) and GluR2 (e) subunits induced by incubation for 6 days with orexin-A and orexin-B (0.01, 0.1, 1 μM). Data are presented as percent of control. The values are mean ± S.E.M. from 11 RNA samples. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

was no statistical difference between orexin-A and orexin-B at every concentration.

In the present experiments, neuronal degeneration was not observed at 3 days or at 6 days after the application of neuropeptides (data not shown).

4. Discussion

Recent studies have shown that central administration of orexin-A and orexin-B has effects on learning and memory. In the cerebral cortex, glutamatergic system is important in cognition. Recently it was reported that the feeding response to orexin-A in the perifornical region of the lateral hypothalamus is mediated by the glutamatergic systems, especially NMDA receptors [14]. Several lines of increasing evidence indicate that orexin modulates plasticity and NMDA receptor currents in the hippocampus [2,37] and potentiates glutamatergic transmission in prefrontal cortex [27]. In this study, orexins were applied to rat cultured primary cortical neurons for 6 days to evaluate their chronic effect because cortical neurons of obese animals are supposed to be chronically stimulated by the high concentrations of orexins up-regulated in the brain of obese animals. The present study demonstrated that orexin-A and orexin-B significantly down-regulated the mRNA expressions of NMDA and AMPA receptor subunits in rat cultured primary cortical neurons on 6-day application. These findings indicate that orexin might modulate the excitatory transmission in the cerebral cortex by altering the subunit ratio of NMDA and AMPA receptors.

Several lines of evidence indicate that the ability of the NR2A subunit to modulate NMDA receptor currents may be involved in the modulation of thresholds for long-term potentiation (LTP) and learning behavior [23,43]. Moreover, the NR2B transgenic mice, in which the NMDA receptor function is enhanced by the NR2B subunit transgene in neurons of the forebrain, were reported to show enhancement of cognition [39]. These findings show that NR2A and NR2B subunits play a pivotal role in learning and memory processing. In this regard, orexin has been reported to increase plasticity and NMDA receptor currents in the hippocampus and potentiates glutamatergic transmission in the prefrontal cortex in electrophysiological studies [2,27,37], suggesting that orexin may enhance cognitive abilities. Although recent behavioral studies have shown that central administration of orexin-A and orexin-B have effects on learning and memory, the literature concerning the role of the orexin system in cognition remains controversial. Aou et al. have reported that intracerebroventricular administration of orexin-A produced memory impairment in water maze performance in a 2-day training protocol [2], which may be supported by the present findings demonstrating the down-regulation of NMDA receptor subunits, NR1, NR2A and NR2B by orexin. In contrast, Akbari et al. reported that blockade of OX1R in the CA1 region or dentate gyrus of the hippocampus impaired memory processing in the Morris Water Maze task [1]. The discrepancy may be attributed to the different action in each region or different experimental methods.

The subunit of the AMPA receptor, GluR2 was reported to be involved in regulation of synaptic plasticity and sustaining LTP [11,43]. On the other hand, the blockage of AMPA receptor by the antagonists, CNQX and NBQX in rats were demonstrated to induce cognitive impairment [6,36]. Therefore, the AMPA receptor is also demonstrated to be potentially involved in learning and memory processes. It was recently demonstrated that orexin neurons robustly express neuronal activity-regulated pentraxin (Narp), secreted neuronal pentraxin, which has been implicated in regulating clustering of AMPA receptors [32]. The present findings with rat neuron cultures, showing that orexins exhibited down-regulation of mRNA expressions of the AMPA receptor subunits GluR1 and GluR2, suggest the possibility of impairment of AMPA receptor functions.

Orexin, one of the orexigenic neuropeptides in the hypothalamus, projects neurons from the lateral hypothalamus to the cerebral cortex and hippocampus. These observations including our present results indicate that orexin may have several regulatory actions on NMDA receptor and AMPA receptor activities. Further detailed experiments are needed to elucidate the pathophysiological significance of orexins in the hypothalamo-cortical system.

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Orexins increase mRNA expressions of neurotrophin-3 in rat primary cortical neuron cultures

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ABSTRACT

Orexins and melanin-concentrating hormone (MCH) as orexigenic neuropeptides are present in the lateral hypothalamus, and their receptors are distributed in the cerebral cortex and hippocampus. In the present study, the regulatory effects of orexin-A, orexin-B and MCH on neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) expressions were examined in primary cortical neuron cultures using quantitative real-time PCR. Both orexin-A and orexin-B on 6-day exposure significantly increased the NT-3 mRNA at concentrations of 0.01, 0.1 and 1 μM. Orexin-A and B at 1 μM led to an increase of twofold or more over the control. However, no such NT-s mRNA increase occurred with exposure to MCH at the same concentrations as orexins. The mRNA expression of BDNF was significantly increased only by orexin-B at 1 μM. These findings suggest that orexins, but not MCH, may be an inducer of NT-3 in the cerebral cortex.

Orexins (orexin-A, orexin-B) and melanin-concentrating hormone (MCH) are orexigenic neuropeptides predominantly present in the lateral hypothalamus [23]. The orexin receptors (OX1R, OX2R) and MCH receptors (MCHR1) are distributed at high concentrations in the cerebral cortex and hippocampus [12]. These areas are postulated to play an important role in regulating the higher functions of the central nervous system, such as learning and memory, based on neuronal plasticity. In this regard, orexin has been reported to play a critical role in neuronal plasticity relevant to addiction in the ventral tegmental area and in long-term potentiation of synaptic transmission in the hippocampus [4,24]. MCH was also found to increase hippocampal synaptic transmission via increased synaptic efficacy [29]. Recently, we demonstrated that in rat primary cortical neuron cultures orexins and MCH decreased the expression of subunits of the NMDA (*N*-methyl-d-aspartate) receptor and the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor [31]. These findings suggest that orexin and MCH may regulate the higher functions, such as cognition and emotion, of the central nervous system as well as energy regulation. Moreover, neurotrophic factors also play an important role in regulating neuronal plasticity in the brain. The neurotrophin family includes brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5) [15]. They exert their biological functions via each of the specific tyrosine kinase receptors (Trk) [2,5]. In the mature nervous system, neurotrophic factors, especially BDNF and NT-3, are demonstrated to be widely distributed in the brain, where they regulate the activity-dependent synaptic plasticity which is involved in the learning and memory regulation [22,27].

In this study, to explore the functions of orexin and MCH in the cerebral cortex, we examined the effects of chronic application of orexin and MCH on NT-3 and BDNF mRNA expressions using primary cortical neuron cultures.

For preparing rat cultured primary cortical neurons timed pregnant Sprague–Dawley rats were obtained from Japan SLC, Inc. (Japan) on gestational day 18. The animals were anesthetized with pentobarbital sodium (50 mg/kg, ip; Abbott, Abbott Park, IL, USA) and sacrificed by cervical dislocation. The fetuses were delivered and decapitated. In each experiment, fetuses were extracted from four maternal rats. All experiments were performed in accordance with the guideline established by the Institutional Animal Investigation Committee at Kyoto University (Med Kyo 06514), Chiba

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University (2006362). Every effort was made to optimize the comfort and to minimize the use of animals. According to our previous report [31], the cultured primary cortical neurons were prepared. Briefly, the cerebral cortex was dissected and cut into small pieces in Hanks' balanced salt solution (HBSS: Ca^{2+} and Mg^{2+} free) (Invitrogen, Carlsbad, CA, USA). The tissue was then dispersed with 0.025% trypsin-EDTA solutions (Invitrogen, Carlsbad, CA USA). The cell suspension was centrifuged at 800 × g for 5 min. The cells were suspended in Neurobasal Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% of B27 containing antioxidants (Invitrogen, Carlsbad, CA, USA), L-glutamine (final concentration 0.5 mM) and antibiotic–antimycotic solution (final concentration 1%) (Nacalai tesque, Kyoto, Japan). The cell suspension was centrifuged at 800 × g for 5 min. Live cells were counted using a hemocytometer, and the cell suspension was then diluted with Neurobasal Medium at 2×10^6 cells/ml. Cells were seeded onto poly-D-lysine-coated 6-well plates (BD Bioscience, Discovery Labware, Bedford, MA, USA) in 2×10^6 cells/ml well. All cultures were maintained in Neurobasal Medium at 37 °C in 95% humidified air and 5% CO_2. At the 3rd day of culture, cytosine β-D-arabino-furanoside hydrochloride (final concentration 10 μM) (Sigma, St. Louis, MO, USA), a selective inhibitor of DNA synthesis, was added for 72 h in culture to prevent further proliferation of non-neuronal cells.

Orexin-A, orexin-B and MCH (0.01, 0.1 and 1 μM) were applied to cultured wells on the 6th day of culture, and the cultured cells were incubated for 6 days. Cultured medium which contained neuropeptides was changed every 3 days. On the 12th day of culture, the total cellular RNA was extracted from two cultured wells as one RNA sample using an RNeasy Mini Kit (Quiagen Sciences, MA, USA). The samples were stored at −20 °C until assay. All experiments consisted of three to six repetitive runs. For each experiment, two to six mRNA were obtained. Orexin-A, orexin-B and MCH were purchased from Peptide Institute Inc. (Osaka, Japan). Neuronal degeneration was assessed every 3 days after neuropeptide application using the efflux of lactate dehydrogenase by CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Co., Madison, WI, USA) [31].

Quantitative real-time RT-PCR was conducted according to our previous report [31]. Quantitative real-time RT-PCR was performed with the ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR green dye. Quantitative PCR was conducted in duplicate with 50 μl of reaction mixture in MicroAmp optical 96-well reaction plates. Each reaction well contained 12.5 μl of RNA sample, 25 μl of SYBR Green PCR Master Mix, 0.5 μl of RT Mix (Quiagen Sciences, MA, USA), 7 μl RNAase free water, and 25 pmol each of forward and reverse primers. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: [sense 5′-TGCACCACCAACTGCTTAGC-3′, antisense 5′-GGATGCAGGGATGATGTTCTG-3′], for OX1R, [sense 5′-GCATATCCACCTGGCCTGAA-3′, antisense 5′-CCACCATGCCAACGAGATCC-3′] [28], for OX2R, [sense 5′-CTACGCTCTTCTGCTATTGA-3′, antisense 5′-ACTGGCATGCTGATACATAC-3′] [28], for MCHR1, [sense 5′-TCA GCT TGG GCT ATG CTA ACA G-3′, antisense 5′-CAA CAC CAA GCG TTT TCG AA-3′ [8], for BDNF, [sense 5′-GGTCACAGTCCTGGAGAAAG-3′, antisense 5′-GCTTATCCTTATGAACCGCC-3] [30], and for NT-3, [sense 5′-TGCAGAGCATAAGAGTCACC-3′, antisense 5′-AAGTCAGTGCTCGGACGTAG-3′] [30]. All gene-specific mRNA expression values were normalized against the internal housekeeping gene GAPDH.

The results are presented as the mean ± S.E.M. of 8–31 RNA samples per group. Statistical analysis of the data was carried out by analysis of variance (ANOVA) followed by Dunnett's multiple range test. Statistical significance was defined as $p < 0.05$.

Quantitative RT-PCR analysis showed the presence of mRNAs of orexin receptors (OXR1, OXR2) and MCH receptor (MCHR1) in rat primary cortical neuron cultures used in this study (Fig. 1). Moreover, the basal expressions of NT-3 and BDNF mRNAs were also detected in the cultured cortical neurons (Fig. 2).

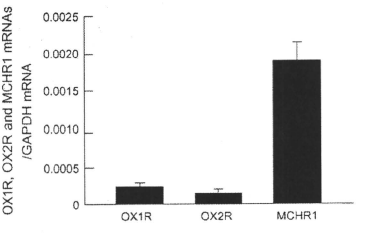

Fig. 1. mRNA expressions of orexin receptors (OX1R, OX2R) and MCHR1 in rat cultured cortical neurons. The values are mean ± S.E.M. from 9 to 10 RNA samples.

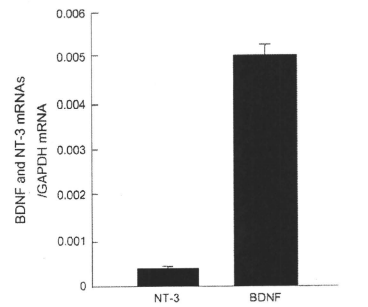

Fig. 2. mRNA expressions of BDNF and NT-3 in rat cultured cortical neurons. The values are mean ± S.E.M. from 8 to 31 RNA samples.

The mRNA expression of NT-3 following 6-day exposure to orexin-A at 0.01, 0.1 and 1 μM markedly increased to 1.57, 1.84 and 2.31 of control levels, respectively (Fig. 3). In a similar manner, orexin-B at 0.01, 0.1 and 1 μM also led to significant increases to 1.55, 1.63 and 2.49-fold of the control levels, respectively (Fig. 3).

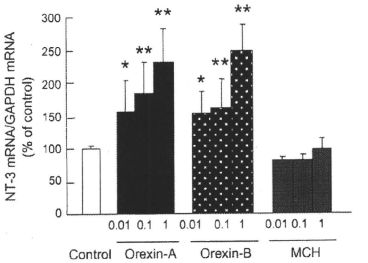

Fig. 3. Changes of mRNA expression of NT-3 induced by incubation for 6 days with orexin-A, orexin-B and MCH (0.01, 0.1, and 1 μM). Data are presented as a percentage of the control. The values are mean ± S.E.M. from 11 RNA samples. *$p < 0.05$, **$p < 0.01$ vs. control group.

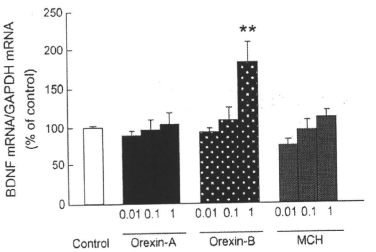

Fig. 4. Changes of mRNA expression of BDNF induced by incubation for 6 days with orexin-A, orexin-B and MCH (0.01, 0.1, and 1 μM). Data are presented as a percentage of the control. The values are mean ± S.E.M. from 11 RNA samples. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

On the other hand, no change in the mRNA expression of NT-3 occurred on exposure to MCH (Fig. 3). The effects of these neuropeptides on BDNF mRNA expression were examined with the same RNA samples. Orexin-B at 1 μM significantly increased BDNF mRNA to 1.82-fold the control level, but its lower concentrations did not affect the mRNA expression of BDNF (Fig. 4). Neither orexin-A nor MCH changed the BDNF mRNA expression (Fig. 4).

In the present study, neuronal degeneration was not detected at 3 or 6 days after the application of neuropeptides (data not shown).

In the present study, the mRNA expression of NT-3 in primary cortical neuron cultures was markedly increased at 6 days after application of orexin-A and B, but not MCH. Moreover, only a high concentration of orexin-B significantly increased the expression of BDNF mRNA, while orexin-A and MCH did not. These findings suggest that orexins may be potent inducers of NT-3 in the cerebral cortex. Orexin and MCH are restrictedly present in the lateral hypothalamus and their neurons project to the cerebral cortex and hippocampus which contain their receptors. The radioimmunoreactive contents of orexins and MCH in the lateral hypothalamus have been shown to significantly increase in obese rodent models [19].

In the adult brain, especially in the cerebral cortex and hippocampus, BDNF and NT-3 act as neurotransmitter and neuromodulator in the central nervous system, and they act on TrkB and TrkC [5,25], respectively. Both BDNF and NT-3 have been implicated in the genesis of new synapses, which may be important for structural aspects of neuronal plasticity [18]. Neurotrophin expression in neurons is mainly regulated by neuronal depolarization [10,13], which may be an important mechanism in neuronal plasticity and may influence neuronal susceptibility to excitotoxicity. Chronic depolarization induced by K^+ (25 mM) in primary cultures of rat cerebellar neurons sustained a persistent increase of BDNF expression which is accompanied by a drastic decrease in NT-3 expression [7,10]. In contrast to the up-regulation of BDNF mRNA, the level of NT-3 mRNA does not change either after injection of kainic acid [1] or after kindled seizures [9]. Moreover, Rocamora et al. [21] have shown with an experimental model of limbic seizures that the dramatic increase of NGF and BDNF expression is accompanied by a fivefold decrease of NT-3 mRNA in dentate gyrus granule cells [21]. The reciprocal regulation of BDNF and NT-3 has also been observed in the dentate gyrus granule cells following cerebral ischemia [16,26]. Reduction of NT-3 mRNA in the hippocampal dentate gyrus was also demonstrated after long-term potentiation [6] and status epileptitus [3,20].

Several lines of evidence demonstrate the involvement of the glutamate nervous system in BDNF expression. Activation of the NMDA receptor, an ionotrophic glutamate receptor, increases BDNF gene expression in cortical neuron cultures [11]. Continuous culture exposure to non-toxic concentrations of NMDA resulted in a prolonged increase in BDNF mRNA expression in primary cultures of rat cerebellar granule neurons. In addition, AMPA also induced a concentration-dependent increase in BDNF mRNA and protein expression [17]. Moreover, an AMPA receptor potentiator (LY392098) was reported to increase BDNF mRNA levels, while it did not change in either NT-3 or NT-4 mRNA. Activation of both L-type Ca^{2+} channels and mitogen-activated protein (MAP) kinases contribute to AMPA receptor-mediated increases in BDNF mRNA [14]. AMPA antagonist CNQX, but not MK-801, suppresses kinase-induced increases in BDNF mRNA in hippocampal neuron cultures [32], while the activation of GABAergic transmission reduces the mRNA levels of BDNF [33]. These findings indicate that the neural depolarization induced by the activation of glutamate receptors increases BDNF expression.

These above-mentioned observations clearly demonstrate that NT-3 expression is suppressed by neuronal depolarization and, moreover, reciprocally regulated in contrast to BDNF expression in the brain. However, in the present experiment using primary cultured cortical neurons, orexin-A and B significantly up-regulated NT-3 mRNA expression, and orexin-B, to a lesser but significant extent, up-regulated the expression of BDNF mRNA. Although the mechanisms leading to reduced NT-3 mRNA expression induced by orexins has not yet been elucidated, orexins may be potent inducers of NT-3 in the cerebral cortex.

As above mentioned NT-3 as well as BDNF plays an important role in neurotransmission and neuronal plasticity in the brain, and its synthesis and release are regulated by neuronal depolarization. The present study demonstrated that orexins, but not MCH, increased the expression of NT-3 mRNA. It seems likely orexin might act on some neurons which was different from neurons containing MCH receptors. Orexin is well known as orexigenic neuropeptide in the hypothalamus and is regulated by hunger status. Orexin released in response to the peripheral metabolic signals may increase NT-3 mRNA in the cerebral cortex, resulting in the modulation of neuronal transmission in the cerebral cortex.

The findings in the present study indicate that these neuropeptides involved in energy regulation may regulate the expressions of NT-3 and BDNF in the cerebral cortex, indicating that these neuropeptides can regulate the activity of the cerebral cortex via changes in neuronal plasticity. These findings offer information for understanding the functional significance of NT-3 in obese animals and a new insight into the bidirectional interaction between energy regulation and higher functions of the limbic system, such as learning/memory and emotion.

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