The activation of renin-angiotensin system contributes to the development of metabolic syndrome and diabetes as well as hypertension. However, it remains undetermined how renin-angiotensin system is implicated in feeding behavior. Here, we show that angiotensin II type 1 (AT1) receptor signaling regulates the hypothalamic neurocircuit that is involved in the control of food intake. Compared with wild-type Agtr1a+/+ mice, AT1 receptor knock-out (Agtr1a−/−) mice were hyperphagic and obese with increased adiposity on an ad libitum diet, whereas Agtr1a−/− mice were lean with decreased adiposity on a pair-fed diet. In the hypothalamus, mRNA levels of anorexigenic neuropeptide corticotropin-releasing hormone (CRH) were lower in Agtr1a−/− mice than in Agtr1a+/+ mice both on an ad libitum and pair-fed diet. Furthermore, intracerebroventricular administration of CRH suppressed food intake both in Agtr1a+/+ and Agtr1a−/− mice. In addition, the CRH gene promoter was significantly transactivated via the cAMP-responsive element by angiotensin II stimulation. These results thus demonstrate that central AT1 receptor signaling plays a homeostatic role in the regulation of food intake by maintaining gene expression of CRH in hypothalamus and suggest a therapeutic potential of central AT1 receptor blockade in feeding disorders.

Maintaining energy homeostasis through regulated food intake and body weight is fundamental for survival. However, >1 billion people are now classified as obese or overweight, and the prevalence of these conditions is increasing rapidly (1). As a major risk factor for cardiovascular diseases and diabetes, obesity has become a leading public health threat and economic burden worldwide. On the other hand, weight loss is a serious and occasionally life-threatening problem in patients with anorexia nervosa and in cachectic patients suffering from chronic obstructive pulmonary disease and cancer. Although environmental and lifestyle factors contribute to body weight gain or loss, impaired regulation of food intake also underlies the pathogenesis of these eating disorders (2). Recent studies have demonstrated that feeding behavior is under control of the central neural circuit involving the hypothalamus. Hypothalamus contains several nuclei that exert homeostatic control of food intake through orexigenic and anorexigenic signals (2).

The renin-angiotensin system (RAS) plays a crucial role in the physiological control of blood pressure and fluid balance and also participates in the pathological processes in the development of cardiovascular and metabolic diseases. Although the activation of RAS in peripheral organs contributes to the development of obesity and the metabolic syndrome (3–5), it remains undetermined how RAS is implicated in feeding behavior. The effects of angiotensin II (Ang II), a pivotal bioactive molecule of RAS, are mainly mediated through the type 1 (AT1) receptor (6). In rodents, AT1 receptor consists of two subtypes (AT1a and AT1b), but AT1a receptor is predominantly expressed and functionally important in most organs including the heart, blood vessels, kidney, adrenal glands, brain, and adipose tissues (7, 8). Feeding behavior is regulated by multiple orexigenic peptides such as neuropeptide Y (NPY), agouti-related protein (AgRP), orexin, and melanin-concentrating hormone (MCH), and anorexigenic peptides such as corticotropin-releasing hormone (CRH), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) in hypothalamus (2). The AT1a receptor is expressed in hypothalamus, including paraventricular nucleus (PVN), lateral hypothalamic area, perifornical nucleus, and retrochiasmatic area.
AT1 Receptor in PVN Controls Feeding Behavior

(9), but the regulatory role of AT1 receptor signaling in the hypothalamic neuronal circuit remains precisely unknown. In the present study, we demonstrate that AT1a receptor knock-out (Agtr1a−/−) mice were hyperphagic and obese on an ad libitum diet, but not on a pair-fed diet, compared with wild-type Agtr1a+/+ mice and that central AT1 receptor signaling controls food intake by regulating anorexigenic Crh gene expression in hypothalamus.

EXPERIMENTAL PROCEDURES

Animals—Generation of Agtr1a−/− mice on the C57BL/6J Jcl background has been described previously (10). Male mice were used in this study. We maintained mice in individual cages under a temperature- and humidity-controlled condition with a 12 h light-dark cycle. Mice were allowed free access to water and standard chow (343.1 kcal/100 g; crude protein 25.1%; crude fat 4.8%; crude fiber 4.2%; crude ash 6.7%; nitrogen-free extract 50.0%; Clea Japan, Tokyo, Japan) on an ad libitum-feeding. For pair-feeding experiments, Agtr1a−/− mice were restricted to the amount of food consumed by ad libitum-fed Agtr1a+/+ mice, as described previously (11). Pair feeding was started on mice at 5 weeks of age, and continued for a period of 11 weeks. All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

Cold Exposure Test—Mice were kept at 4 °C for 60 min and then kept at room temperature for 30 min. Rectal body temperatures were measured with a thermometer (BWT-100; Bio Research Center, Nagoya, Japan) at 0, 60, 75, and 90 min during the experiment.

Blood and Urine Analysis—Blood glucose levels were determined by using ACCU-CHEK Blood Glucose Meter (Roche Diagnostics, Basel, Switzerland). For a glucose tolerance test, a glucose load was injected i.p. (1 g/kg body weight) after a 16 h fast. Blood glucose concentrations were measured at 0 (before), 30, 60, 90, and 120 min after the injection. For insulin tolerance test, insulin (Humulin R; Eli Lilly, Indianapolis, IN) was injected i.p. (1 unit/kg body weight) after a 1-h fast. Blood glucose concentrations were measured at 0 (before), 15, 30, 45, and 60 min after the injection. Serum leptin concentrations were measured at 0 (before), 15, 30, 60, 90, and 120 min after the injection. Serum leptin concentrations were assayed by using mouse leptin quantikine ELISA kit (R&D Systems) according to the manufacturer’s protocol. Urine concentrations of catecholamines were measured by HPLC in the laboratory of SRL, Inc. (Tokyo, Japan).

Histological Analysis—The liver and epididymal fat were excised, immediately fixed in 10% neutralized formalin, and embedded in paraffin. Sections at 5 μm were stained with hematoxylin and eosin.

In Situ Hybridization—The mice were decapitated, and the brains were rapidly removed and frozen. Frozen sections at 12 μm were used for in situ hybridization, as described previously (12). Antisense probes were 3′-end labeled with 35S by using oligonucleotides complementary to mRNAs of Npy, Agpp, Hcrt, Pmcn, Crh, Pomc, and Cartpt. A semi-quantitative image analysis was performed with the MCID Image Analysis System (Imaging Research, St. Catharines, Ontario, Canada).

Intracerebroventricular Administration of CRH—The mice were anesthetized with inhalation of diethyl ether. Vehicle or 0.2 μg of CRH (Sigma-Aldrich, St. Louis, MO) in a total volume of 1 μl was administered intracerebroventricularly (i.c.v.) to the anesthetized mice by using a Hamilton syringe with a catherter PH03 (Hamilton Company, Reno, NV) into the right lateral ventricle using the coordinates: 0.5 mm caudal to bregma, 1 mm lateral to sagital suture, and 2 mm in depth.

Luciferase Assays—HEK293 cells expressing the AT1 receptor were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and were starved under a serum-free condition for 48 h before stimulation with 10−7 M of Ang II (Sigma-Aldrich) (13). The Crh luciferase reporter plasmids, with or without the expression vector for dominant-negative form of cAMP-responsive element (CRE)-binding protein (DN-CREB), was transfected by the FuGENE 6 Transfection Regent (Roche Diagnostics), according to the manufacturer’s instructions. pRL-SV40 (Promega, Madison, WI) was co-transfected as an internal control. Luciferase activities were measured 24 h after Ang II stimulation by using the Dual-Luciferase reporter assay system (Promega). Experiments were repeated at least three times in triplicate, and representative data are shown. The Crh luciferase reporter plasmids and the expression vector for dominant-negative form of CRE-binding protein (DN-CREB) (14) were a generous gift from Dr. Y. Iwasaki (Kochi University).

Statistics—The results are expressed as the mean ± S.E. Differences in measured values were evaluated with an analysis of variance using Fisher’s t test and an unpaired Student’s t test. Values of p < 0.05 were considered statistically significant.

RESULTS

Agtr1a−/− Mice Are Obese and Hyperphagic under ad Libitum Feeding—We first compared the body weight between male Agtr1a+/+ and Agtr1a−/− mice on the C57BL/6 background. When maintained ad libitum on standard laboratory chow, Agtr1a−/− mice were significantly heavier than Agtr1a+/+ mice after 3 weeks of age and weighed 11.5 ± 2.9% more than Agtr1a+/+ mice at 24 weeks of age (Fig. 1, A–C). Although the tibial length was indistinguishable, liver and epididymal fat were significantly heavier in Agtr1a−/− mice (Fig. 1C). Because an excess of body weight results from an imbalance between food intake and energy expenditure, we compared the food intake between Agtr1a+/+ and Agtr1a−/− mice. The daily food intake of Agtr1a−/− mice was significantly more throughout the course of observation, than that of Agtr1a+/+ mice (Fig. 1D).

On the other hand, i.p. glucose tolerance test and insulin tolerance test revealed that the glucose disposal and the hypoglycemic effect of insulin were pronounced in Agtr1a−/− mice compared with Agtr1a+/+ mice (Fig. 2). These results suggest that Agtr1a−/− mice have better glucose tolerance and insulin sensitivity despite increased food intake, body weight, and adiposity. Although the core body temperatures were comparable under basal conditions (Agtr1a+/+, 38.5 ± 0.1 °C; Agtr1a−/−, 38.4 ± 0.1 °C; n = 8 per group; p = 0.234), Agtr1a−/− mice showed enhanced thermogenic response after cold exposure, compared with Agtr1a+/+ mice (Fig. 3), suggesting that energy expenditure was increased in Agtr1a−/− mice, as reported previously (15). In addition, clinical and experimental studies have...
demonstrated that pharmacological inhibition of RAS improves insulin sensitivity in part through increased glucose uptake in skeletal muscle (3). We suppose that the beneficial effect of Agtr1a deficiency on glucose metabolism overcomes the detrimental effect of increased body weight and adiposity in Agtr1a−/− mice.

Agtr1a−/− Mice Weigh Less Than Agtr1a+/+ Mice under Pair feeding—Next, we examined the relevance of hyperphagia to increased body weight gain and adiposity in Agtr1a−/− mice.

For this purpose, we restricted 5-week-old Agtr1a−/− mice to the amount of food consumed by ad libitum-fed Agtr1a+/+ littermate mice and continued pair-feeding for 11 weeks. On a pair-fed diet, the body weight gain of Agtr1a−/− mice was less than that of Agtr1a+/+ mice, and Agtr1a−/− mice weighed 11.9 ± 2.3% less than Agtr1a+/+ mice at 16 weeks of age (Fig. 4, A and B). In parallel with body weight, the weights of liver and epididymal fat in pair-fed Agtr1a−/− mice were comparable with or less than those of Agtr1a+/+ mice (liver: p = 0.806; epididymal fat: p < 0.01, Fig. 4B). Histological analysis revealed that hepatic steatosis and adipocyte hypertrophy were promi-
potentiality that a down-regulation of anorexigenic
expression is stimulated by leptin and that a CRH antago-

nist attenuates the leptin-induced reduction in food intake and
reward circuits to increase food intake without altering the
corticosterone levels through undefined compensatory mecha-
nisms. Importantly, administration of CRH by i.c.v. injection
reduced food intake during2ht ot h e indistinguishable level
between Agtr1a+/− and Agtr1a+/+ mice (Fig. 6). These results
strongly suggest that down-regulation of anorexigenic Crh is
the underlying mechanism of hyperphagia in Agtr1a−/−.

AT1 Receptor Signaling Regulates Crh Gene Transcription via
cAMP-responsive Element—Next, to examine how AT1 receptor
signaling regulates Crh gene transcription, we transfected a
luciferase reporter containing the −907 bp promoter of the
human Crh gene (−907 Crh-Luc) into HEK293 cells expressing
AT1 receptor. After stimulation with 10−7 m of Ang II, a strong
transactivation of the Crh gene promoter was observed (Fig.
7A). Deletion of the Crh promoter between −907 and −220
markedly reduced the fold activation by Ang II stimulation,
although deletion between −907 and −330 had a marginal
effect (Fig. 7A). Deletion of the cAMP-responsive element
(CRE) located between −330 and −220 of the Crh promoter
(14, 25) significantly reduced the fold activation by Ang II stim-
ulation (Fig. 7B), and co-transfection of an expression vector for
DN-CREB diminished Ang II-induced fold activation of −907
Crh-Luc in a dose-dependent manner (Fig. 7C). These results
suggest that the AT1 receptor signaling regulates Crh gene tran-
scription via CRE.

DISCUSSION

Our present study demonstrated a hitherto undefined role of
the AT1 receptor signaling in the regulation of hypothalamic
neurocircuit that is involved in the control of food intake. Ang II
is produced by cleavage of angiotensinogen by renin and ACE.
Mice deficient for angiotensinogen (Agt) or renin (Ren1c) are
similarly resistant to diet-induced weight gain (26, 27). On a
high-fat diet, no differences in food intake were observed
between these mutant mice and wild-type mice, although the
food intake of Agt−/− mice was significantly more than that of
Agt+/− mice on a standard chow diet (26). On the other hand,
mice deficient for ACE (Ace) are lean, exhibiting unchanged
food intake and increased energy expenditure (28). In someth

irrespective of the leptin levels, was the cause of hyperphagia in
Agtr1a−/− mice.

i.c.v. Injection of CRH Reduces Food Intake in Agtr1a−/− Mice—
CRH is a major regulator of the hypothalamo-pituitary-adrenocortical axis and also functions as an endogenous inhibitor of
food intake (2). Indeed, i.c.v. injection of CRH reduced sponta-
neous or deprivation-induced feeding (22), but Crh−/− mice
did not exhibit an increase in food intake under physiological
conditions (23). In general, appetite in animals and humans is
closely related to the glucocorticoid levels. Although Crh−/−
mice showed a markedly low corticosterone levels (23), the
serum corticosterone concentrations were not significantly dif-
different between Agtr1a+/− and Agtr1a+/+ mice on an ad libi-
tum diet (supplemental Fig. S1). Interestingly, it has been
reported that the food intake of Crh−/− mice was significantly
higher than that of Crh+/+ mice, when the corticosterone levels
were adjusted to the equal levels through adrenalectomy and
corticosterone replacement (24). We speculate that down-regu-
lation of Crh in Agtr1a−/− mice activates downstream satiety
and reward circuits to increase food intake without altering the
corticosterone levels through undefined compensatory mecha-
nisms. Importantly, administration of CRH by i.c.v. injection
reduced food intake during 2 h to the indistinguishable level
between Agtr1a−/− and Agtr1a+/+ mice (Fig. 6). These results

Hypothalamic Crh Expression Is Decreased in ad Libitum-
and Pair-fed Agtr1a−/− Mice—Feeding behavior is regulated
by multiple orexigenic and anorexigenic peptides in hypothal-
amus (2). To elucidate the mechanism underlining hyperphagia in
Agtr1a−/− mice, we performed semi-quantitative in situ
hybridization analysis of appetite-related peptide genes in the
hypothalamus (2). Despite hyperphagia, the expression levels of
orexigenic Npy and Agrp in arcuate nucleus and Hcrt in lateral
hypothameric area were lower in ad libitum-fed Agtr1a−/−
mice than in Agtr1a+/+ mice (Fig. 5A). In contrast, the expres-
sion levels of Npy and Agrp were higher in pair-fed Agtr1a−/−
mice (Fig. 5B). It has been reported that Npy and Agrp mRNA
expression are suppressed in the state of high leptin levels and
enhanced in the state of low leptin levels (17–19). In Agtr1a−/−
mice, the Npy and Agrp expressions were in inverse proportion
to the levels of leptin concentration and adiposity. The expres-
sion levels of anorexigenic Pomc and Cartpt were decreased in
Agtr1a−/− mice, which were statistically indistinguishable from
those in Agtr1a+/+ mice. Notably, the expression levels of
anorexigenic Crh in PVN were significantly decreased in both
ad libitum- or pair-fed Agtr1a−/− mice, compared with
Agtr1a+/+ mice (Fig. 5, A and B). It has been reported that the
Crh expression is stimulated by leptin and that a CRH antago-
nist attenuates the leptin-induced reduction in food intake and
body weight (20, 21). Therefore, these results raised the possi-
bility that a down-regulation of anorexigenic Crh, occurring
nent in ad libitum-fed Agtr1a−/− mice, but not in pair-fed
Agtr1a−/− mice (Fig. 4C). In general, weight gain and fat con-
tent are associated with increased levels of leptin (16). The
serum leptin concentrations of Agtr1a−/− (n = 8) and Agtr1a−/− (n = 8) mice on an ad libitum diet at 16 weeks of
age. *, p < 0.05. RT, room temperature.

FIGURE 3. Agtr1a−/− mice show enhanced thermogenic response after
cold exposure. Changes in body temperature after cold exposure in
Agtr1a+/− (n = 8) and Agtr1a−/− (n = 8) mice on an ad libitum diet at 16 weeks of
age. *, p < 0.05. RT, room temperature.
as the renal abnormalities observed in Agt<sup>−/−</sup> mice are quantitatively similar to those of mutant mice homozygous for both AT<sub>1a</sub> and AT<sub>1b</sub> (29, 30), it is possible that the effect of Ang II on AT<sub>1b</sub> receptor might contribute to feeding behavior in Agtr1a<sup>−/−</sup> mice. However, the compensatory effect of Ang II receptor subtypes was minimal, because the mRNA levels of the AT<sub>1b</sub> (Agtr1b) and AT<sub>2</sub> (Agtr2) receptors in the hypothalamus did not differ significantly between Agtr1a<sup>+/+</sup> and Agtr1a<sup>−/−</sup> mice. 

TABLE 1

|                | Urine volume (µl/day) | Urinary norepinephrine (ng/day) | Urinary epinephrine (ng/day) | Urinary dopamine (ng/day) | Leptin (ng/ml) |
|----------------|----------------------|--------------------------------|-----------------------------|---------------------------|----------------|
| Agtr1a<sup>++</sup> |          |                                |                             |                           |                |
| Agtr1a<sup>−/−</sup> |          |                                |                             |                           |                |

<sup>a</sup>p < 0.01.  
<sup>b</sup>p < 0.05.
mice (supplemental Fig. S2). Kouyama and colleagues reported that Agtr1a−/− mice showed attenuation of high-fat diet-induced weight gain and adiposity, which was accompanied by unchanged food intake and increased energy expenditure (15). However, they also described that Agtr1a−/− mice ate more on a calorie basis than Agtr1a+/+ mice on a standard chow diet (15). In our hands, Agtr1a−/− mice were initially hyperphagic, compared with Agtr1a+/+ mice, when maintained ad libitum on a high-fat diet (supplemental Fig. S3A). However, the difference in daily food intake between Agtr1a−/− and Agtr1a+/+ mice became insignificant after 6 weeks of high-fat diet (supplemental Fig. S3B). The reasons for this phenomenon are currently unclear, but unpalatable taste of high-fat chow and altered metabolic status due to high-fat diet might potentially contribute to it. As a consequence, Agtr1a−/− weighed 9.8 ± 3.7% more than Agtr1a+/+ mice after 6 weeks of high-fat diet (supplemental Fig. S3B), but the difference in body weight between Agtr1a−/− and Agtr1a+/+ mice was not statistically significant (p = 0.053). We assume that to gain or to

FIGURE 5. Hypothalamic Crh expression is decreased in ad libitum- and pair-fed Agtr1a−/− mice. A, quantitative in situ hybridization analysis of hypothalamic orexigenic and anorexigenic peptides in ad libitum-fed Agtr1a−/− and Agtr1a+/+ mice at 16 weeks of age. The number of sections for each analysis is indicated in the bars. *, p < 0.05. Scale bars, 1 mm. B, quantitative in situ hybridization analysis of hypothalamic orexigenic and anorexigenic peptides in pair-fed Agtr1a+/+ and Agtr1a−/− mice at 16 weeks of age. The number of sections for each analysis is indicated in the bars. *, p < 0.05. Scale bars, 1 mm.

FIGURE 6. i.c.v. injection of CRH reduces food intake in Agtr1a−/− mice. Food intake over 2 h after i.c.v. administration of CRH or vehicle in Agtr1a+/+ and Agtr1a−/− mice at 8 weeks of age. *, p < 0.05 versus vehicle; **, p < 0.01 versus vehicle; ###, p < 0.01 versus Agtr1a+/+. NS, not significant.
lose weight may be determined upon a delicate balance between the counteracting effects of the central versus peripheral function of AT1 receptor signaling in Agtr1a⁻/⁻ mice. Our Agtr1a⁻/⁻ mice seem to be more susceptible to body weight gain than those used by Kouyama et al. (15), on a standard or high-fat chow, which might be caused by the differences in the extent of energy expenditure, potentially resulting from different housing conditions. Further studies will be needed to elucidate the differential regulation of feeding behavior by RAS components.

It has been reported that neuroendocrine dysfunction including CRH hyperactivity has been associated with anorexia nervosa (31), and AT1 receptor expression in the PVN is increased in several stress models, contributing to enhanced CRH production and release (32). Although AT1 receptor blockers are widely used to treat hypertension, the effects of AT1 receptor blockers on feeding behavior have not been well documented in clinical practice. Subcutaneous administration of an AT1 receptor blocker olmesartan in wild-type mice lowered blood pressure to the level comparable with that of Agtr1a⁻/⁻ mice (supplemental Fig. S4A), but the body weight and daily food intake did not differ significantly between olmesartan- and vehicle-treated mice (supplemental Fig. S4B). In addition, the expression levels of hypothalamic appetite-related peptides genes were unchanged (supplemental Fig. S4C). On the other hand, i.c.v. administration of olmesartan significantly increased food intake in wild-type mice (supplemental Fig. S5). These data indicate that peripheral administration of olmesartan is insufficient to inhibit the AT1 receptor signaling in the PVN and thus has little effect on hypothalamic neurocircuits and food intake. However, we propose that the hypothalamic AT1 receptor signaling will be a therapeutic target against neurotic and stress-induced eating disorders, if we develop an AT1 receptor blocker with high ability to penetrate the PVN nuclei and to inhibit the hypothalamic AT1 receptor signaling effectively.

We conclude that the AT1 receptor signaling is involved in feeding behavior by regulating Crh gene expression in the PVN. Elucidation of the mechanism by which the AT1 receptor signaling regulates feeding behavior via neuropeptides circuits will provide a clue to the management of cardiovascular, metabolic, and eating disorders.

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