Leptin Resistance and Enhancement of Feeding Facilitation by Melanin-Concentrating Hormone in Mice Lacking Bombesin Receptor Subtype-3

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Mice lacking either bombesin receptor subtype (BRS)-3 or gastrin-releasing peptide receptor (GRP-R) exhibit feeding abnormalities. However, it is unclear how these receptors are associated with feeding regulation. In BRS-3-deficient mice, we found hyperphagia, subsequent hyperleptinemia, and brain leptin resistance that occurred after the onset of obesity. To explore the cause of this phenomenon, we examined changes in feeding responses to appetite-related neuropeptides in BRS-3–deficient, GRP-R–deficient, and wild-type littermate mice. Among orexigenic neuropeptides, the hyperphagic response to melanin-concentrating hormone (MCH) was significantly enhanced in BRS-3–deficient mice but not in GRP-R–deficient mice. In addition, the levels of MCH-R and prepro-MCH mRNAs in the hypothalamus of BRS-3–deficient mice were significantly more elevated than those of wild-type littersmates. There was no significant difference in feeding between BRS-3–deficient and wild-type littermate mice after treatment with bombesin (BN), although the hypophagic response to low-dose BN was significantly suppressed in the GRP-R–deficient mice. These results suggest that upregulation of MCH-R and MCH triggers hyperphagia in BRS-3–deficient mice. From these results, we assume that the BRS-3 gene deletion upsets the mechanism by which leptin decreases the expression of MCH-R and that this effect may be mediated through neural networks independent of BN-related peptides such as GRP-R.

Owing to technical progress in molecular biology, dozens of substances that regulate appetite have been identified (1). Of these substances, bombesin (BN) has been known as a feeding suppressant since 1979 (2). Two BN-related peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB), are detected in the mammalian brain (3). Both GRP and NMB regulate feeding, because treatment with these peptides, as well as BN, decreased the amount of metabolite consumed (4–6). Meanwhile, five types of BN receptors have been cloned, namely the GRP receptor (GRP-R) (7,8), NMB receptor (NMB-R) (9), BN receptor subtype-3 (BRS-3) (10), BB4 (11), and BRS-3.5 (12). Endogenous ligands for BRS-3 and BRS-3.5 have not yet been determined. Among these receptors, GRP-R, NMB-R, and BRS-3 are found in the mammalian brain, and mice lacking each receptor have been generated (6,13–15). It is reported that the mice lacking BRS-3 become obese as a result of metabolic defects and hyperphagia (14), whereas the mice lacking GRP-R are overweight and show alterations in feeding patterns after 45 weeks of age (16). From these reports, it is assumed that both GRP-R and BRS-3 are located in brain regions involved in appetite suppression. However, because the localization of GRP-R mRNA is different from that of BRS-3 mRNA in the mammalian brain (17–19), these receptors are thought to affect feeding behavior by different means. In BRS-3–deficient mice, it was shown that hyperphagia occurred despite high plasma insulin and leptin levels at 23 weeks postnatal (14), suggesting that the peripheral hormonal information about appetite could not be transmitted properly to the brain. Therefore, we can expect that BRS-3–deficient mice will have insulin and leptin resistance.

In the mammalian hypothalamus, the arcuate nucleus is thought to be the center for sensing hormonal information from blood, because leptin and insulin receptors are abundant there (20–23). In this nucleus, BRS-3 mRNA is expressed in mice and rats (18,19). Other than BRS-3, several orexigenic neuropeptides, such as neuropeptide Y (NPY) and agouti-related protein (AgRP), are found in this nucleus (24). Anorexigenic neuropeptides such as α-melanocyte–stimulating hormone (MSH) and cocaine- and amphetamine-regulated transcript (CART) also coexist within this nucleus (24). It is reported that NPY and AgRP are also produced by the same cells (25), and proopiomelanocortin (POMC), the precursor of α-MSH, is localized within the
same nerves containing CART in the lateral portion of the arcuate nucleus (26). Both NPY/AgRP and POMC/CART neurons in this nucleus are putative targets of leptin because leptin concentration in blood altered the concentrations of both NPY and POMC (22,27). In support of this, NPY/AgRP neurons express leptin receptors (28–30), and activities of POMC/CART neurons are increased by leptin (31).

One of the regions that receives projections from the arcuate nucleus is the lateral hypothalamic area (32), where expression of BR3-mRNA is detected in mice (18). In the lateral hypothalamus, a large number of cell bodies contain melanin-concentrating hormone (MCH) and orexin (ORX)/hypocretin, which participate in increasing food intake and decreasing energy expenditure (33–35). Because fibers immunoreactive for NPY, AgRP, and α-MSH are distributed in the region where MCH- or ORX/hypocretin-immunoreactive cells are found (36), it is possible that these latter cells receive hormonal information detected in the arcuate nucleus.

Starting with leptin resistance found in BR3–deficient mice, we investigated the feeding responses of BR3–deficient, GRP-R–deficient, and wild-type mice to the various orexigenic or anorexigenic agents to mark the first step in situating BR3 in the functional downstream of leptin and also to reveal the difference in functions mediated by GRP-R or BR3-

**RESEARCH DESIGN AND METHODS**

**Animals.** Male BR3–deficient mice and their wild-type littermates and male GRP-R–deficient mice and their wild-type littermates were generated by breeding male C57BL/6J mice with female mice heterozygous for the disrupted BR3 or GRPR allele, respectively (14,15). We genotyped mice by PCR as described previously (14,15) and used F8–10 generations for BR3- or F9 and 10 generations for GRP-R of 120/5V×C57BL/6J hybrid mice. Mice were housed under controlled temperature (26°C) and photoperiod (14 h light:10 h dark, lights on from 0700 to 2100). Mice received pellet-type food (CE-2; Japan Clea, Tokyo, Japan) and tap water ad libitum unless specified otherwise. The animal experiments were performed in accordance with the guidelines of the Tokyo Medical and Dental University (Tokyo, Japan), and the experimental design was approved by the animal investigation committee of the Tokyo Medical and Dental University.

**Measurement of food intake and body weight from postnatal weeks 13 to 20.** The amount of food consumed by mice for a period of 72 h each week (Friday to Sunday) was measured using pellet-type food, and the mean amount of food consumed per day was calculated. Body weights of animals were examined at 1300 on Thursday each week.

**Enzyme immunoassay of leptin.** Blood was withdrawn from the mouse tail and collected using a heparinized capillary (Drummond Scientific, Broomall, PA). Plasma leptin concentrations were measured using a mouse leptin enzyme-linked immunosassay kit (Cat. no. 200726; Morinaga Institute of Biological Science, Yokohama, Japan). The least detectable concentration of leptin was 0.2 ng/ml, and variations between assays were <10%.

**Cannulae implantation and substance administration.** Twenty-five 30-week-old mice were deeply anesthetized by diethylether and intubated for 10–15 min using a multipurpose thermometer (BAT-10; Physitemp Instruments, Clifton, NJ). For intracerebroventricular infusion, a guide cannula was fixed and mice were handled as in the feeding experiments. After a 7-day recovery period, the body temperature of each animal was measured at 1500, and BM (1 µg) dissolved in 5 µl of saline was injected. Body temperature was monitored at 15, 30, 45, 60, and 120 min after the injection.

**Data analysis.** All data are expressed as mean ± SD. The number of animals used is indicated in parentheses in each figure. Two-way ANOVA with
we compared plasma leptin concentration (Fig. 2) in BRS-3–deficient mice at 13 weeks postnatal (A, left; *P < 0.01). The first significant increase in daily food intake in BRS-3–deficient mice appeared at 16 weeks postnatal (A, right; *P < 0.05), and increases in food intake were observed at 17, 18, and 20 weeks postnatal. There was no significant difference in body weight or daily food intake between GRP-R–deficient and wild-type littermate mice from 13 to 20 weeks postnatal (B).

Repeated measurement followed by Fisher projected least significant difference at each time point was used for comparison of body weight, food intake from 13 to 20 weeks postnatal (Fig. 1), cumulative food intake (Figs. 2A, 3, and 4), and rectal temperature (Fig. 5). Two-way factorial ANOVA (genotype × region) followed by Fisher projected least significant difference was used for ppMCH and MCH-R mRNAs in BRS-3–deficient and wild-type littermate mice (Fig. 6D and E). A two-tailed unpaired t test was used at each time point when we compared plasma leptin concentration (Fig. 2A and B), body weight (Fig. 2A, 4B, and 2B), and ppMCH and MCH-R mRNAs in GRP-R–deficient and wild-type littermate mice (Fig. 6D and E). Semi-log regression analysis was used to calculate correlation coefficients (Fig. 2C). To compare the difference in the correlation coefficients, we used the conventional method for comparison of two correlation coefficients. All statistical analyses, except the comparison of two correlation coefficients, were performed using StatView 5.0 (SAS Institute, Cary, NC). Comparison of two correlation coefficients was calculated using Microsoft Excel 2001. *P < 0.05 was considered significant.

RESULTS
Food intake and body weight from 13 to 20 weeks postnatal. In BRS-3–deficient mice, the first significant increase in food intake appeared at 16 weeks postnatal (*P < 0.05), and augmentation was also observed at 17, 18, and 20 weeks postnatal (Fig. 1A, right). They gained more weight than their wild-type littermates at 13 weeks postnatal, and the difference became more prominent with age (Fig. 1A, left). Concerning GRP-R–deficient mice, body weight and daily food intake did not differ from their wild-type littermates from 13 to 20 weeks postnatal (Fig. 1B).

Plasma leptin level and leptin resistance in BRS-3–deficient mice. No significant difference was observed between GRP-R–deficient and wild-type littermate mice in plasma leptin concentration or body weight at 21–64 weeks postnatal (Fig. 2A). At 5–6 weeks postnatal, there was no difference in plasma leptin level or body weight between BRS-3–deficient and wild-type littermate mice (Fig. 2B). However, the plasma leptin level of heavier BRS-3–deficient mice compared with wild-type littermates was significantly higher at 47–55 weeks postnatal (*P < 0.05; Fig. 2B). A significant correlation between body weight and plasma leptin level was found in both BRS-3–deficient (r² = 0.674) and wild-type littermate mice (r² = 0.451) (C; logarithmic scale of the ordinate). Solid and broken lines indicate the regression lines of BRS-3–deficient mice and wild-type littermates, respectively. The regression lines are given by the following equations: log(Y) = 0.0415* − 0.4352 for the solid line and log(Y) = 0.0328* − 0.3628 for the broken line. Under the condition in which mice were habituated to have access to food exclusively in daytime, there was no significant difference in feeding response after saline treatment (D, left) at 25–30 weeks of age. Feeding suppression after leptin treatment in BRS-3–deficient mice was significantly inhibited at 0.5 and 1 h after treatment (*P < 0.01 and *P < 0.05, respectively) compared with that in wild-type littermates (D, right).

BRS-3–deficient mice were observed at 17, 18, and 20 weeks postnatal. Although there was no difference in either plasma leptin level or body weight between BRS-3–deficient and wild-type littermate mice at 5–6 weeks postnatal (B), the plasma leptin level of heavier BRS-3–deficient mice (B, right; *P < 0.05) was significantly higher than that of wild-type littermate mice (B, left; *P < 0.05) at 47–55 weeks postnatal. A positive correlation between body weight (abscissa) and plasma leptin level (ordinate) was found in both BRS-3–deficient (r² = 0.674) and wild-type littermate mice (r² = 0.451) (C; logarithmic scale of the ordinate). Solid and broken lines indicate the regression lines of BRS-3–deficient mice and wild-type littermates, respectively. The regression lines are given by the following equations: log(Y) = 0.0415* − 0.4352 for the solid line and log(Y) = 0.0328* − 0.3628 for the broken line. Under the condition in which mice were habituated to have access to food exclusively in daytime, there was no significant difference in feeding response after saline treatment (D, left) at 25–30 weeks of age. Feeding suppression after leptin treatment in BRS-3–deficient mice was significantly inhibited at 0.5 and 1 h after treatment (*P < 0.01 and *P < 0.05, respectively) compared with that in wild-type littermates (D, right).

Effects of feeding-promoting peptides. For screening neuropeptides that participate in expression of leptin resistance, several orexigenic neuropeptides were injected one by one during the daytime in free-fed mice (Fig. 3). Significant enhancement of feeding facilitation after MCH treatment was observed in BRS-3–deficient mice at 0.5 and 2 h after the treatment (*P < 0.05 vs. wild-type littermates). There was no significant difference between BRS-3–deficient and wild-type littermates in cumulative food intake.
after NPY, ORX, and AgRP treatments (Fig. 3A). No significant difference between GRP-R–deficient and wild-type littermate mice was found after treatment with any orexigenic agents tested (Fig. 3B). After ORX and MCH treatment, behavioral symptoms other than feeding were also observed. Locomotor activities were enhanced after ORX treatment and suppressed after MCH treatment in all groups.

**Effects of feeding-suppressing peptides.** Figure 4 demonstrates the effect of feeding inhibition when treating the mice habituated to daytime feeding with anorexigenic agents. The inhibitory effect on feeding as a result of CART in BRS-3–deficient mice was more significantly enhanced than in their wild-type littermates at 0.5, 1, 2, and 3 h (0.5–2 h, *P* < 0.05; 3 h, *P* < 0.01). There was no significant difference between BRS-3–deficient mice and wild-type littermates in feeding inhibition after treatments with either α-MSH or BN (Fig. 4A). Conversely, the suppressive effect of 1 μg of BN was significant in GRP-R–deficient mice, although a low dose (0.05 μg) of BN was not effective (*P* < 0.05). There was no significant difference between GRP-R–deficient and wild-type littermate mice in cumulative food intake after treatment with other anorexigenic agents (Fig. 4B). After α-MSH and BN treatments, all mice showed grooming behavior. In addition, after CART treatment, movement-associated tremors were found in all animals and continued for 2 h.

**Expression levels of ppMCH and MCH-R mRNAs in the brain.** To examine the neural basis of overfeeding after MCH treatment in BRS-3–deficient mice, we quantified the expression levels of ppMCH and MCH-R mRNAs in the various brain regions (Fig. 6). The expression level of ppMCH mRNA in the hypothalamus of BRS-3–deficient mice was significantly elevated compared with that of wild-type littermates (*P* < 0.05; Fig. 6D). Low expressions of ppMCH mRNAs in the cerebral cortex and medulla...
b::mice, the temperature before treatment was signi-
ificantly lower than that of wild-type littermates (left, *P < 0.05). However, in both BRS-3–deficient and wild-type littermate mice, the temperature decreased with BN treatment and remained low for up to 180 min (left). In GRP-R–deficient mice, the temperature before treatment was not different from that of wild-type mice, the hypothermic effect of BN was mild, and a rapid recovery of temperature starting at 60 min was detected (right, *P < 0.05).

**Discussion**

Hyperphagia, along with hypometabolism, is the main cause of obesity in BRS-3–deficient mice (14). This appeared after 16 weeks postnatal (Fig. 1A), and hyperleptinemia as well as leptin resistance were observed subsequently (Fig. 2B and D). Because plasma leptin levels increased in parallel with body weight in both BRS-3–deficient and wild-type mice (Fig. 2C), we recognize that hyperleptinemia is due to the gain in body weight developed in BRS-3–deficient mice and is not caused by abnormalities of the secretory mechanism of leptin from adipose tissues. Both leptin resistance and hyperleptinemia appeared not only in the mice showing diet-induced obesity (37) but also in mice lacking obesity-related genes (38). Considering this point, it can be concluded that leptin resistance may be caused by a defect in intracellular signaling downstream of the leptin receptor and/or direct dysfunctions of neuropeptides and their receptors regulated by leptin. For example, leptin action is known to be involved in the functions of various neuropeptide receptors, such as NPY Y1 and Y2 receptors (39,40) and melanocortin 4 receptor (41), in addition to alternations of neuropeptide expressions, such as NPY, AgRP, POMC, and CART (32). In BRS-3–deficient mice, only MCH facilitated the feeding more effectively than in wild-type mice, and feeding response was similar to wild-type littermates after treatments with other orexigenic neuropeptides such as NPY and ORX and anorexigenic neuropeptides such as α-MSH and BN. Thus, it is suggested that in BRS-3–deficient mice, hyperphagia and leptin resistance are the...
outcome of a deterioration in the MCH signaling pathway and are not attributed to the intracellular leptin signaling that may also affect other pathways. After AgRP treatment, we found a tendency toward enhancement of feeding facilitation. Because AgRP is known to participate mainly in the long-term facilitation of feeding (42), it might be difficult to isolate the effect of AgRP in a short-term experiment.

We demonstrated that MCH-R and ppMCH mRNAs were upregulated in the hypothalamus of BRS-3–deficient mice but not in GRP-R–deficient mice. These results suggest that MCH-R and MCH overexpression in mice lacking BRS-3 induce hyperphagia. In other words, BRS-3 may balance the appetite via inhibition of MCH-R and/or MCH expression in normal mice, and a dysfunction of BRS-3 causes the dysregulation of MCH and/or MCH-R.

Distributions of MCH-R and MCH mRNAs are well documented in the rat and mouse brain. Whereas MCH is localized in the lateral hypothalamic area and zona incerta (33), MCH-R mRNA is found extensively in the cerebral cortex, hypothalamus, and limbic system (43,44). The lateral hypothalamic area contains both MCH (33) and BRS-3 mRNAs (18). In addition, it has been reported that MCH-R mRNA is expressed in appetite-related brain regions such as the paraventricular nucleus, arcuate nucleus, the dorsomedial nucleus and lateral hypothalamic area of the hypothalamus, and the medial and central nuclei of the amygdala (43,44). A previous report indicated that BRS-3 is localized in these nuclei in the hypothalamus (18). From these coincidences, we speculated that BRS-3 modulates the activity of neurons containing MCH or expressing MCH-R.

Kokkotou et al. (45) indicated that MCH-R mRNA expressed extensively in fasting and ob/ob mice, and the expression decreased after leptin treatment. They also reported that MCH secretion is not related to this negative regulation, because MCH-R mRNA expression is unaffected by gene deletion of MCH. These facts indicate that MCH-R is directly regulated by leptin. In BRS-3–deficient mice, the expression of MCH-R mRNA was enhanced despite hyperleptinemia, suggesting an important role for BRS-3 in the connection of leptin action to MCH-R expression.

It is interesting that the feeding suppression after CART treatment was enhanced in BRS-3–deficient mice. We can explain this phenomenon as a compensatory activation of the neural system, with the receptive site for CART in opposition to the activation of the MCH and MCH-R system. This hypothesis can be assumed after a previous report that hyperleptinemia in A2/a mice upregulates CART expression in the arcuate nucleus (46). However, CART is reported to colocalize not only with POMC in the arcuate nucleus (26) but also with MCH in the lateral hypothalamus (47), i.e., in both anorexigenic and orexigenic pathways in the hypothalamus. We observed movement-associated tremors in all mice that were treated with CART, and CART-containing fibers were found in the motor-related nuclei such as substantia nigra (48). Taking this complexity into consideration, we could not conclude that the compensatory activation of a receptor for CART results in the hypersensitivity to CART in BRS-3–deficient mice.

After 0.05 μg of BN treatment, a partial inhibitory effect on feeding was suppressed in the mice lacking GRP-R. Previously, it was shown that the glucose intake suppression after peripheral administration of BN does not occur in GRP-R–deficient mice (13). The present study analyzed the effect of central administration of BN, suggesting the importance of brain GRP-R in BN-induced feeding suppression. High-dose (1 μg) BN masks the low responsiveness of GRP-R–deficient mice. This may reflect the effect of BN action via NMB-R. However, BN-induced suppression of feeding occurred equally in both BRS-3–deficient and wild-type littermate mice even at a low dose, confirming that BN is not a potent ligand of BRS-3. The hypothalamic effect of BN was different between GRP-R–deficient and BRS-3–deficient mice in terms of the minimum body temperature reached and time point of recovery. On the basis of these results, it is likely that there is a difference between BRS-3 and GRP-R, not only in the nature of receptors but also in the neural networks of appetite or thermal control in which they are embedded. The absence of feeding facilitation enhancement after MCH treatment in GRP-R–deficient mice and lower body temperature at ambient room temperature in BRS-3–deficient mice support the possibility that the neural network involving BRS-3 is completely separated from the one in which BN-related peptides are inserted.

In conclusion, we found that hyperleptinemia, leptin resistance, and feeding facilitation were induced by MCH treatment in BRS-3–deficient mice. These results suggest that BRS-3 gene deletion upsets the mechanism by which leptin decreases MCH-R expression because MCH-R was overexpressed in BRS-3–deficient mice. In addition, in the network controlling feeding and body temperature, BRS-3 is possibly independent of the neural pathway, including BN-related peptides and GRP-R.

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