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 littermates. 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. *Diabetes* 53:570–576, 2004

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AgRP, agouti-related protein; BN, bombesin; BRS, bombesin receptor subtype; CART, cocaine- and amphetamine-regulated transcript; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP-R, gastrin-releasing peptide receptor; MCH, melanin-concentrating hormone; MSH, melanocyte-stimulating hormone; NMB, neuromedin B; NPY, neuropeptide Y; ORX, orexin; POMC, proopiomelanocortin.

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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

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same neurons 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 BRS-3 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 BRS-3–deficient mice, we investigated the feeding responses of BRS-3–deficient, GRP-R–deficient, and wild-type mice to the various orexigenic or anorexigenic agents to mark the first step in situating BRS-3 in the functional downstream of leptin and also to reveal the difference in functions mediated by GRP-R or BRS-3.

RESEARCH DESIGN AND METHODS

Animals. Male BRS-3–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 *BRS3* or *GRPR* allele, respectively (14,15). We genotyped mice by PCR as described previously (14,15) and used F8–10 generations for BRS-3, or F9 and 10 generations for GRP-R of 129/SV×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 immunoassay 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 fixed in a stereotaxic instrument (model 900; David Kopf Instruments, Tujunga, CA) with a neonatal rat/mouse adapter (#51625) in which an incisor bar was set at 4.0 mm below the interaural line. The surface of the skull was kept horizontal. Then, a 24-gauge guide cannula with stainless steel tubing 7.0 mm below the pedestal (C316G/Spc; Plastics One, Roanoke, VA) was set in a mounting holder (MH-325; Plastics One). The skull was drilled, and stainless steel screws were attached to the skull. The guide cannula was positioned at 2.5 mm ventral, 0.2 mm posterior, and 1.0 mm right of the bregma and cemented. Handling of operated animals was performed for 15 min every day to accustom mice to the manipulation. Four days after the implantation of the guide cannula, we changed the pellet food to powder-type food to measure more precisely the amount of food intake. During the injection, a 31-gauge internal cannula with stainless steel tubes extending 1.0 mm below the guide cannula (C316I/Spc; Plastics One) was inserted, and the injection was executed under free-moving conditions for 1 min using a multisyringe infusion pump (KDS 230; kdScientific, New Hope, PA). After the experiments, 1% fast green solution was infused into the lateral ventricle under deep pentobarbital anesthesia, and we confirmed that the dye was spread through the ventricular system in each animal.

Effect of feeding facilitation. To survey the effect of orexigenic agents on facilitation of food intake, we injected the orexigenic agents into animals during the daytime when free-fed mice show less feeding activity. NPY (#4158), MCH (#4369), AgRP (#4366), and orexin-A (ORX, #4346) were purchased from the Peptide Institute (Osaka, Japan). Seven days after the implantation of the guide cannula, animals received an injection of NPY (5 μg), MCH (10 μg), AgRP (5 μg), or ORX (2.5 μg) dissolved in 5 μl of saline at 1300, and the amount of food consumed at 0.5, 1, 2, and 3 h after the injection was measured. Five microliters of sterilized saline was used as a control.

Effects of feeding suppression. To survey the inhibition of food intake by anorexigenic agents, we injected anorexigenic agents into mice habituated to consume food only in the daytime. Mouse leptin (#AFP362C) was donated by the National Hormone & Peptide Program, the National Institute of Diabetes and Digestive and Kidney Diseases. α-MSH (#4057), BN (#4086), and CART (#4350) were purchased from the Peptide Institute. After a 7-day recovery period, food access was restricted from 1300 to 1900 each day. The amount of food consumed was recorded daily at 1330, 1400, 1500, 1600, and 1900. Food consumption gradually increased, and it took 7–9 days to adapt the mice to this restricted feeding schedule (data not shown). After this adaptation period, leptin (3 μg), α-MSH (3 or 0.3 μg), BN (1 or 0.05 μg), or CART (0.5 μg) was injected at 1300, and food consumed was recorded at 0.5, 1, 2, 3, and 6 h after the injection.

Fluorescence real-time RT-PCR for prepro-MCH and MCH receptor mRNAs. A portion of the cerebral cortex, hypothalamus, and medulla oblongata in each mouse was dissected out by the methods described below. Using a brain slicer (MBS-01C; Muromachi Kikai, Tokyo, Japan), a coronal slice of 2-mm thickness covering the anterior hypothalamic area to the mamillary body was obtained. The hypothalamic tissue was obtained from this slice with incisions made horizontally at the level of the medial lemniscus and laterally at the level of the cerebral peduncle. For obtaining the cerebral cortex, incisions were made between the basolateral amygdala and piriform cortex and in the corpus callosum, using the left portion of the same slice. The medulla oblongata was dissected under a dissection microscope and included the nucleus tractus solitarius anteriorly and the area postrema posteriorly. These tissues were immediately transferred to TRIzol (Invitrogen, Carlsbad, CA) and homogenized in 1.5-ml microtubes. Total RNAs were extracted by following a protocol indicated by the manufacturer. Single-stranded cDNA was synthesized from 1 μg of total RNA with a Superscript II Reverse Transcription Kit (Invitrogen). To quantify prepro-MCH (ppMCH) and MCH receptor (MCH-R) mRNAs, we used the ABI Prism 7900HT system (Applied Biosystems, Foster City, CA). To detect ppMCH (GenBank Accession #AK020723) and MCH-R (GenBank Accession #AY049011) mRNAs, PCR primer sets for ppMCH (forward 353–377: 5′-CCACTGAGTCTGGCTGTA AAACCT-3′ and reverse 449–473: 5′-TTCTTCATCCCCAATTTCTCTCTT-3′) and MCH-R (forward 3096–3117: 5′-CATCATGCCTTCAGTGTTTGGT-3′ and reverse 3198–3220: 5′-TGATGAAGATGTCAGGGACGTT-3′) were purchased from Proligo Japan (Kyoto, Japan). Carboxyfluorescein (FAM)- and arboxytetramethylrhodamine (TAMRA)-labeled TaqMan probes for ppMCH (396–421: 5′-CCGTAGCCTTCCCAGCTGAGAATGG-3′) and MCH-R (3164–3188: 5′-CCGTGGTGAAGAAATCCAAGCTGCA-3′) were purchased from Applied Biosystems. In each well of the plate, 10 μl of 2× TaqMan Universal PCR Master mix (4304437; Applied Biosystems), 11 pmol of primers, 1.5 μl of cDNA samples, and either 5.4 pmol of ppMCH probe or 4.3 pmol of MCH-R probe were applied to give a total volume of 20 μl/well. PCR settings were as follows: 95°C for 5 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The amount of each mRNA was standardized by the graded dilution of highly concentrated cDNA samples from the hypothalamus. As an endogenous reference for total mRNA level, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was determined from the respective standard curve using a TaqMan Rodent GAPDH control kit (AP.BIO 4308313). Dividing each mRNA level by the GAPDH level resulted in a normalized mRNA value.

Rectal temperature. Body temperature of GRP-R–deficient or BRS-3–deficient mice and their wild-type littermates at 25–30 weeks postnatal was monitored under ambient temperature (26°C) using a rectal probe connected to 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 BN (1 μg) dissolved in 5 μl of saline was injected. Body temperature was monitored at 15, 30, 45, 60, 90, 120, and 180 min after the injection.

Data analysis. All data are expressed as mean ± SE. The number of animals used is indicated in parentheses in each figure. Two-way ANOVA with

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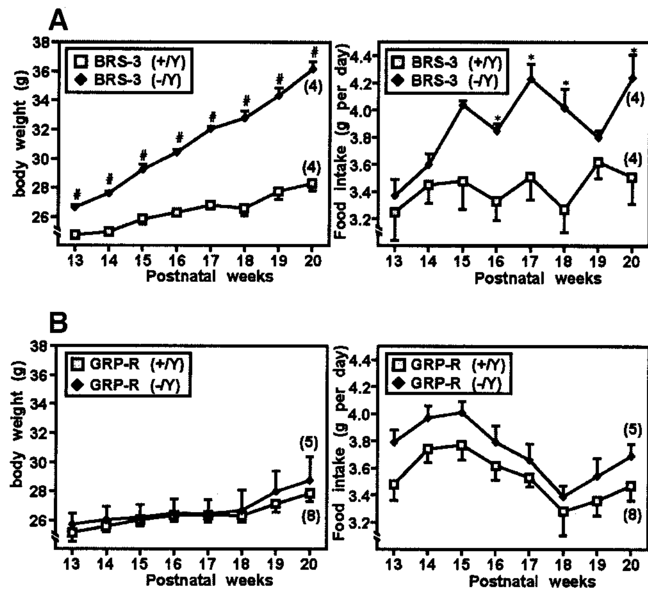

FIG. 1. Body weight and daily food intake in BRS-3–deficient (*A*) and GRP-R–deficient mice (*B*) from 13 to 20 weeks postnatal. The BRS-3–deficient mice were significantly heavier than wild-type littermates at 13 weeks postnatal (*A*, *left*; #$P < 0.01$). The first significant increase in daily food 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 GPR-R–deficient and wild-type littermate mice from 13 to 20 weeks postnatal (*B*).

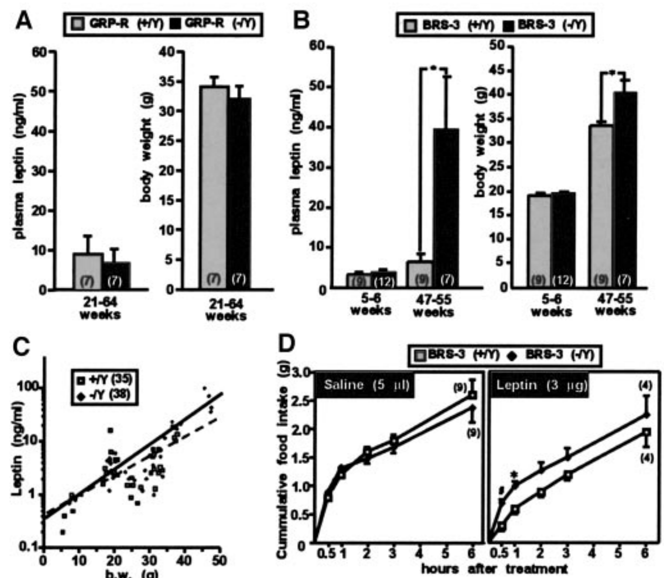

FIG. 2. Plasma leptin level and leptin resistance in BRS-3–deficient mice. No significant difference was observed between GRP-R–deficient and wild-type littermate mice in plasma leptin concentration or body weight at 21–64 weeks postnatal (*A*). Although there was no difference in either plasma leptin 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 ($r^2 = 0.674$) and wild-type littermate mice ($r^2 = 0.451$) (*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: $\log(Y) = 0.0415 \times -0.4352$ for the solid line and $\log(Y) = 0.0328 \times -0.3628$ 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*).

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. 2*D*, 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. 6*D* and *E*). A two-tailed unpaired *t* test was used at each time point when we compared plasma leptin concentration (Fig. 2*A* and *B*), body weight (Fig. 2*A* and *B* and 6*B*), and ppMCH and MCH-R mRNAs in GRP-R–deficient and wild-type littermate mice (Fig. 6*D* and *E*). Semi-log regression analysis was used to calculate correlation coefficients (Fig. 2*C*). 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. 1*A*, right). They gained more weight than their wild-type littermates at 13 weeks postnatal, and the difference became more prominent with age (Fig. 1*A*, 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. 1*B*).

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. 2*A*). 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. 2*B*). 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. 2*B*). A significant correlation between body weight and plasma leptin level was found in both BRS-3–deficient ($r^2 = 0.674$) and wild-type littermate mice ($r^2 = 0.451$, $P < 0.001$; Fig. 2*C*). No significant difference between these two correlation coefficients was detected ($P = 0.15$). Figure 2*D* demonstrates the leptin resistance of BRS-3–deficient mice at 25–30 weeks of age. Under restricted feeding conditions, there was no significant difference in cumulative food intake between BRS-3–deficient and wild-type mice with saline treatment (Fig. 2*D*, left). After leptin treatment, the feeding suppression in BRS-3–deficient mice was significantly inhibited at 0.5 and 1 h after treatment ($P < 0.01$ and 0.05, respectively) compared with that in wild-type littermates (Fig. 2*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

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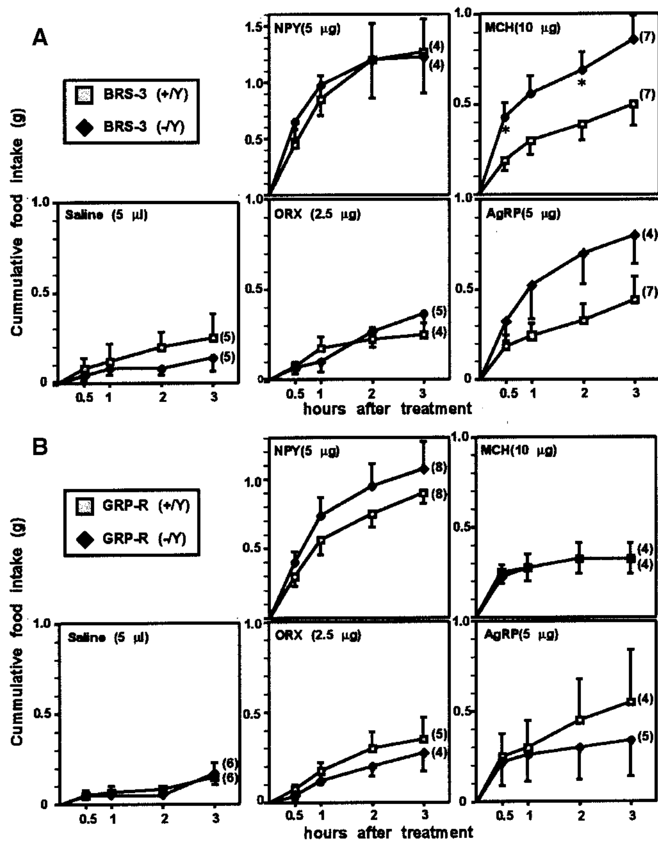

FIG. 3. Effects of orexigenic peptides on feeding. In these experiments, mice were kept in a free-fed condition. Mice received an injection of saline or orexigenic neuropeptides during the daytime when they show less feeding activity. Effects of feeding facilitation in BRS-3–deficient and GRP-R–deficient mice are demonstrated in *A* and *B*, respectively. There was no significant difference between BRS-3–deficient and wild-type littermate mice after saline treatment (*A*). A significant enhancement in 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; *A*). There was no significant difference between BRS-3–deficient and wild-type littermate mice in cumulative food intake after NPY, ORX, and AgRP (*A*) treatments. No significant difference was detected between GRP-R–deficient and wild-type littermate mice after saline, NPY, MCH, ORX, and AgRP (*B*) treatments.

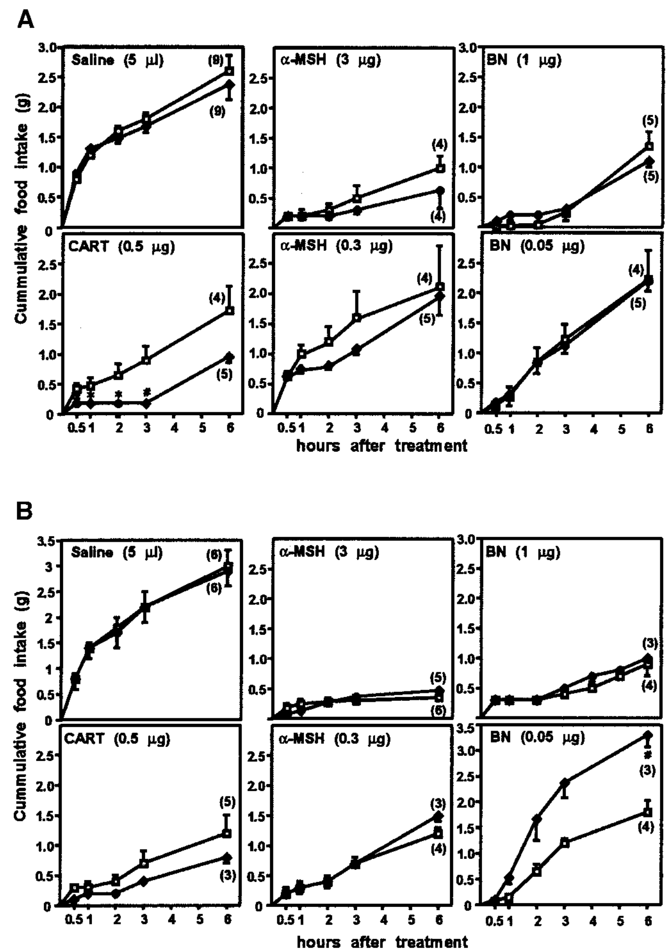

FIG. 4. Effect of anorexigenic neuropeptides on feeding. Mice were habituated to consume food during the daytime. Saline or anorexigenic neuropeptides were injected in these mice just before they were given access to food in the daytime. The effects of feeding inhibition by these agents in BRS-3–deficient and GRP-R–deficient mice are demonstrated in *A* and *B*, respectively. There was no significant difference in food intake between BRS-3–deficient mice and wild-type littermates after saline treatment (*A*). The inhibitory effect of food consumption by CART in BRS-3–deficient mice was significantly enhanced compared with that in wild-type littermates at 0.5, 1, 2, and 3 h (*A*, 0.5–2 h, *$P < 0.05$; 3 h, #$P < 0.01$). There was no significant difference between BRS-3–deficient and wild-type littermates in feeding inhibition after α-MSH or BN (*A*) treatments. *A*: □, BRS-3 (+/Y); ♦, BRS-3 (−/Y). In GRP-R–deficient mice, low-dose (0.05 μg) BN suppressed the cumulative food intake at 3 h after treatment, but its effect was significantly blunted at 6 h, compared with that in wild-type littermates (*B*; *$P < 0.05$). High-dose (1 μg) BN was similarly potent in both mice (*B*). There was no significant difference between GRP-R–deficient and wild-type littermate mice after treatments with either CART or α-MSH (*B*). *B*: □, GRP-R (+/Y); ♦, GRP-R (−/Y).

after NPY, ORX, and AgRP treatments (Fig. 3*A*). No significant difference between GRP-R–deficient and wild-type littermate mice was found after treatment with any orexigenic agents tested (Fig. 3*B*). 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. 4*A*). 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. 4*B*). 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. 6*D*). Low expressions of ppMCH mRNAs in the cerebral cortex and medulla

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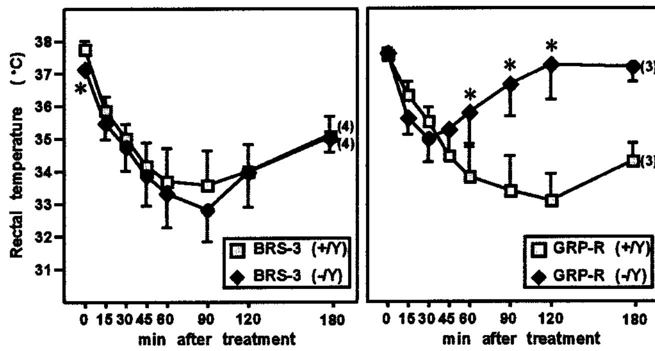

FIG. 5. Hypothermic effect of BN in the BRS-3– and GRP-R–deficient mice. We measured the rectal temperature of BRS-3– and GRP-R–deficient mice after BN treatment. The temperature of BRS-3–deficient mice before treatment was significantly lower than that of wild-type littermates (*left*, *$P < 0.05$). 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*, *$P < 0.05$).

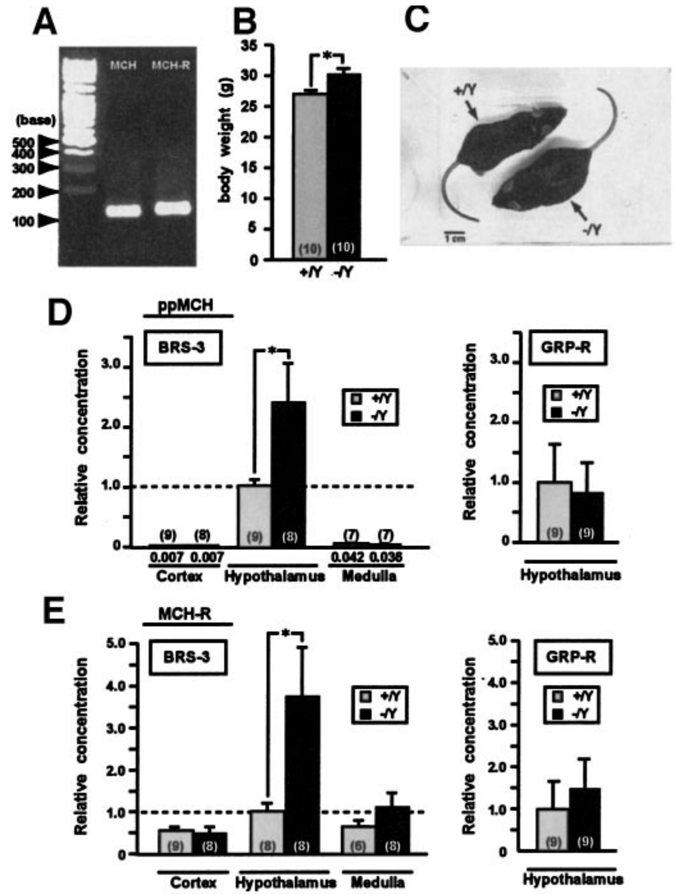

FIG. 6. Expression level of ppMCH and MCH-R mRNAs in the brain. We quantified the expression level of ppMCH and MCH-R mRNAs in several brain regions using primer sets that could detect ppMCH and MCH-R mRNAs in RT-PCR (*A*). We confirmed the absence of any PCR product when the reverse transcription step was omitted. We removed brain tissue samples from BRS-3–deficient mice and their littermates at 16 weeks postnatal, when the body weight (*B*; *$P < 0.05$) and size (*C*) were already different between these mice. The expression level of ppMCH mRNA in the hypothalamus of BRS-3–deficient mice was significantly higher compared with that of wild-type littermates (*D*; *$P < 0.05$). The ppMCH mRNA in the cerebral cortex and medulla oblongata were expressed at very low levels compared with the hypothalamus in both BRS-3–deficient and wild-type littermate mice (*D*). Similarly, the expression level of MCH-R mRNA in the hypothalamus of BRS-3–deficient mice was significantly higher than that of wild-type littermates (*E*; *$P < 0.05$), although the expression levels of MCH-R mRNAs in the cerebral cortex and medulla oblongata were not significantly different between BRS-3–deficient and wild-type littermate mice (*E*). In GRP-R–deficient mice aged 24–44 weeks, both ppMCH and MCH-R mRNA were similarly detected in the hypothalamus, as in the wild-type mice.

oblongata compared with the hypothalamus were similar in both mice (Fig. 6*D*). In addition, the expression level of MCH-R mRNA in the hypothalamus of BRS-3–deficient mice was significantly higher than that of wild-type littermates ($P < 0.05$; Fig. 6*E*), although the levels in the cerebral cortex and medulla oblongata were comparable. In contrast, GRP-R–deficient and wild-type mice showed similar mRNA levels of ppMCH and MCH-R in the hypothalamus (Fig. 6*D* and *E*).

Hypothermic effect of BN in BRS-3–deficient or GRP-R–deficient mice. To assess whether the low effectiveness of BN in GPR-R–deficient mice but not in BRS-3–deficient mice is a general phenomenon, we measured the body temperature in both mice after 1 μg of BN treatment (Fig. 5). The body temperature in BRS-3–deficient mice before treatment was significantly lower than that of wild-type littermates ($P < 0.05$). However, in both BRS-3–deficient and wild-type littermate mice, the body temperature decreased with BN treatment and remained low for up to 180 min. In contrast to BRS-3–deficient mice, body temperature before treatment was comparable between GRP-R–deficient and wild-type littermate mice, the decrease in body temperature was mild, and a rapid recovery of body temperature starting at 60 min was detected in the GRP-R–deficient mice.

DISCUSSION

Hyperphagia, along with hypometabolism, is the main cause of obesity in BRS-3–deficient mice (14). This appeared after 16 weeks postnatal (Fig. 1*A*), and hyperleptinemia as well as leptin resistance were observed subsequently (Fig. 2*B* and *D*). Because plasma leptin levels increased in parallel with body weight in both BRS-3–deficient and wild-type mice (Fig. 2*C*), 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, hyperphasia and leptin resistance are the

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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 A^y/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 hypothermic 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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