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GH-Releasing Peptide-2 Increases Fat Mass in Mice Lacking NPY: Indication for a Crucial Mediating Role of Hypothalamic Agouti-Related Protein

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Ghrelin, an endogenous GH secretagogue, is capable of stimulating adiposity in rodents. Because such adiposity was thought to be mediated by hypothalamic NPY neurons, we investigated by which mechanism a synthetic ghrelin receptor agonist, GHRP-2, would generate a positive energy balance in NPY-deficient [*Npy(−/−)* mice] and wild-type controls. A dose-dependent increase in body weight and food intake was observed during daily sc injections with GHRP-2. Pre- and posttreatment analysis of body composition indicated increased fat mass and bone mass but not lean mass. Respiratory quotient was increased in GHRP-2-treated mice, indicating preservation of fat. Hypothalamic mRNA levels of agouti-related protein (AGRP), an orexigenic melanocortin receptor antagonist, increased after GHRP-2 treatment. Competitive blockade of AGRP action by melanocortin-receptor agonist MT-II prevented GHRP-induced weight gain in *Npy(−/−)* mice. In conclusion, chronic peripheral treatment with a ghrelin receptor agonist induced a positive energy balance leading to fat gain in the absence of NPY. These effects could be mediated in part by AGRP. To date, there are few therapeutics that can produce a positive energy balance. Ghrelin receptor agonists offer a treatment option for syndromes like anorexia nervosa, cancer cachexia, or AIDS wasting. (*Endocrinology* 143: 558–568, 2002)

BOWERS *ET AL.* (1) founded a new area for understanding the regulation of GH release with the discovery of synthetic GH-releasing peptides (GHRPs). More recently, Smith *et al.* (2) discovered nonpeptide mimics of these GHRPs termed GH secretagogues (GHSs). This same group isolated a GHS receptor (GHS-R) cDNA from the pituitary and from the hypothalamus (3), in which it is expressed by hypothalamic NPY-neurons of the arcuate nucleus (4). Expression of GHS-Rs at lower levels has also been shown in heart, lung, pancreas, intestine, and adipose tissue (5). Binding of endogenous and exogenous ligands of the GHS-R to human membranes has been shown in myocardium; adrenal gland; testis; aortic smooth muscle; aortic endothelium; coronary, carotid, lung, ovary, liver, and skeletal muscle; kidney; pituitary and thyroid glands; adipocytes; vena cava; uterus; skin; and lymph nodes (6). Recently, ghrelin (7) and des-Gln-ghrelin (8) were identified as endogenous ligands for the GHS-R. Ghrelin is a 28-residue peptide with an unusual octanoyl side chain at Ser^3 that is primarily produced and secreted by oxyntic glands of the stomach (9). The ability of ghrelin and des-Gln-ghrelin to induce GH secretion from the pituitary has been shown *in vivo* (in rats and humans) and *in vitro* (7, 8, 10–12).

Since the discovery of leptin in 1994 (13), obesity research has been revolutionized by a new understanding of peripheral hormones that interact with neuroendocrine systems to regulate energy balance (14). An equilibrium between anabolic neuropeptides such as NPY/agouti related protein (AGRP) that stimulate energy storage and catabolic neuropeptides such as POMC/cocaine amphetamine-related transcript that decrease energy stores, is currently thought to be at the core of the central regulation of energy balance (14–16), The GHS-R is present in NPY/AGRP neurons and peripheral administration of GHRP-6 increases *c-fos* expression in those same neurons (17–21). Thus, peripheral administration of ghrelin or its mimics should produce a positive energy balance. We demonstrated earlier that peripheral administration of ghrelin induces adiposity in rodents (22). Further, we showed that this induction is mediated by central rather than peripheral GHS-Rs because hyperphagia and sparing of lipid utilization was observed after administration of a intracerebroventricular dose 1000 times smaller than the peripheral dose (22). Such hyperphagia has now been confirmed by others (23–26). Shintani *et al.* (25) recently reported that NPY mRNA expression is increased after chronic intracerebroventricular administration of ghrelin and that administration of an NPY-Y1 antagonist completely reverses ghrelin-induced increase in food intake. One aim of our study was to investigate whether these GHS-R-mediated effects on energy balance are still realized when NPY mRNA expression is eliminated.

Because ghrelin has a short half-life (22), we used the more stable GHRP-2 to study GHS-R-mediated regulation of energy balance, body composition, and hypothalamic gene expression in wild-type and NPY-deficient [*Npy*(−/−) mice] (27, 28). GHRP-2 is a hexapeptide and a GHS that was described by Momany *et al.* (29). Following GHRP-2 treatment for 18 d, both genotypes accrued fat mass and bone mass, but not lean mass. Although adipogenic effects were anticipated for wild-type mice, GHRP-2 also stimulated the accumula-

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Abbreviations: AGRP, Agouti-related protein; CV, calorific value of oxygen; DXA, dual-energy x-ray absorptiometry; EE, energy expenditure; GHRP-2, GH-releasing peptide-2; GHS, GH secretagogue; GHS-R, GHS receptor; G3PDH, glycerol-3-phosphate dehydrogenase; MT-II, melanocortin receptor agonist peptide; RQ, respiratory quotient; RT, reverse transcription; VO_2, volume of oxygen consumed.

tion of adipose tissue mass in *Npy*(−/−) mice. Moreover, this effect was paralleled by an increase in hypothalamic AGRP mRNA expression levels. Therefore, chronic administration of ghrelin receptor agonists increase fat mass independent of NPY, possibly through an up-regulation of the endogenous melanocortin receptor antagonist AGRP.

Materials and Methods

Preparation of peptides

GHRP-2 was prepared, HPLC purified, and lyophilized at Lilly Research Laboratories. The amino acid sequence was D-Ala.D-β-Nal. Ala.Trp.Phe.Lys.NH_2, and the molecular weight was 767.9 g. For sc injections, GHRP-2 was dissolved in PBS to achieve concentrations of 2.5 μg/100 μl and 10.0 μg/100 μl. Melanocortin receptor agonist peptide (MT-II) also was manufactured at Lilly Research Laboratories using the Merrifield process.

Animals

Eighty-four male mice [42 *Npy(−/−)*] mice and 42 wild-type mice (genetic background: 129 SvCPJ) were studied. Both genotypes were divided into three treatment groups (n = 14) of equivalent mean body weight. Technical settings limited the number of animals per study group for RT-PCR (Biomek), DXA analysis, and measurement of respiratory quotient (RQ). Experimental subgroups were chosen randomly. Mice were housed individually in a temperature-controlled (22 C) environment with 12-h light and 12-h dark (1800 to 0600 h). All mice had *ad libitum* access to pelleted mouse food (5008 PMI, Nutrition International, Richmond, IN) and tap water. Animals were between 14 and 18 wk of age. Wild-type mice (129SV strain) were purchased from Taconic Farms, Inc., Germantown, NY; *Npy(−/−)* mice (40), originally provided to us by Dr. Richard Palmiter, were raised at Taconic Farms, Inc. Mice were injected sc once daily between 0500 h and 1800 h with 0.1 ml containing 0 μg, 2.5 μg (3.26 nmol), or 10 μg (13.0 nmol) GHRP-2 over 18 d. Doses were injected 2 h after initiating indirect calorimetric analysis and 1 h before decapitation at 1500 h on d 18.

Food intake and body weight were measured daily at 0800 h. After 18 d, animals were killed by decapitation. Trunk blood was collected and hypothalami were rapidly removed and frozen on dry ice. Blood was kept on wet ice until plasma was separated and stored at −80 C until assayed. Plasma IGF-I, insulin, and corticosterone were measured by RIA (Linco Research, St. Charles, MO). Blood glucose was measured by glucose oxidase method using i-STAT data station (i-STAT Corp., East Windsor, NJ) for clinical chemistry analysis.

The study protocol used in these experiments was approved by the Animal Care Committee of Eli Lilly & Co., Lilly Research Laboratories (Indianapolis, IN). All animal experiments were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Indirect calorimetry

Twenty-four-hour energy expenditure (EE) and RQ were measured by indirect calorimetry using an open circuit calorimetry system (Oxymax, Columbus Instruments International Corp., Columbus, OH). The instrument was calibrated before each experiment using standard gas mixtures containing known concentrations of CO_2, N_2, and O_2. After injection, animals were placed in calorimeter chambers containing food and water in rooms maintained under identical conditions as those described above. Gas sampled from each of 10 chambers was first dried by a condenser. The volume of oxygen consumed (VO_2) and carbon dioxide produced in an hour was measured using a paramagnetic oxygen sensor and a spectrophotometric CO_2 sensor for 24 h. RQ was calculated as the ratio of carbon dioxide produced to VO_2. EE was calculated as the product of calorific value of oxygen and VO_2 per kilogram of body weight, where the calorific value of oxygen = 3.815 + 1.232*RQ. Total calories expended were calculated to determine daily fuel utilization. To calculate proportion of protein, fat, and carbohydrate that is used during that 24-h period, the proposal of Flatt *et al.* and Rose *et al.* (30, 31) was used, and it was assumed that protein utilization was equivalent to protein intake for adult animals. Using formulae and constants derived by Elia and Livesey (32), we calculated the percent of daily fuel utilization derived from carbohydrate and fat. Daily caloric intake was calculated as (mass of daily food intake in g)*(physiological fuel value of the diet in kcal/g).

In vivo analysis of body composition by dual-energy x-ray absorptiometry

Body composition was measured on d −2 and d 18 of the treatment period by dual-energy x-ray absorptiometry (DXA) using a Norland p-DXA (Norland, WI) (distributed by South East Medical Equipment Co., Mandeville, LA) (33). The system provides a noninvasive method for quantification of whole-body composition and is based on the differential attenuation of high- and low-energy x-rays by the tissues in the scan area. Soft tissues attenuate the energy beam less than bone; of the soft tissue mass, fat tissue attenuates the beam less than lean tissue. Fat mass consists primarily of adipose tissue, but lean mass includes organs, tendons, cartilage, blood, and body water in addition to skeletal muscle. In the present study, fat mass, lean mass, and bone mineral content (bone mass) were measured and are reported. Mice were anesthetized with inhalation of isoflurane and placed on the instrument platform in ventral position. Measurements were performed at a speed of 7 mm/sec and a resolution of 0.5 × 0.5 mm. Bone surface area was monitored and set at constant values by adjusting the histogram averaging width. Quality controls using phantom ID2232 and Calibration Standard 82315 (Norland) were performed regularly before starting measurements.

RT-PCR

Quantification of NPY-, AGRP- and glycerol-3-phosphate dehydrogenase (G3PDH) mRNA levels was performed by RT-PCR. Hypothalamic RNA was extracted and purified using TRIzol (Life Technologies, Inc., Gaithersburg, MD). First-strand cDNA was prepared from 0.1, 0.5, and 1.0 μg of DNase-I-treated total RNA using an Oligo dT primer and SuperScript II reverse transcriptase (Life Technologies, Inc.). To control for genomic DNA contamination, identical reactions were prepared without reverse transcription (RT). RT reactions were generated in duplicate for each animal at each concentration and products were pooled and diluted to 200 μl with dH_2O. PCR primers were generated and conditions were optimized for amplification of NPY, AGRP, and G3PDH from pooled cDNA from each group of animals. Optimization determined primer concentration, annealing temperature, template concentration, and cycle number. Final conditions for semiquantitative PCR employed 50-μl reactions (10 pM each primer), 1 μl cDNA synthesized from 0.5 μg of RNA (hypothalamus), 0.2 mM dNTPs, 2.5 mM $Mg(OAc)_2$, and Advantage polymerase mix (CLONTECH Laboratories, Inc., Carlsbad, CA). All PCR were prepared in duplicate for each animal sample using a BioMek 2000 liquid pipetting robot (Robotics, Beckman Coulter, Inc., Fullerton, CA). Reactions were incubated at 94 C for 2 min (to denature TaqStart antibody in the polymerase mix) and then cycled at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min. Aliquots were taken from each sample at 19, 22, and 25 cycles for G3PDH at 27, 30, and 33 cycles for NPY and at 28, 31, and 34 cycles for AGRP. Optimization experiments demonstrated that under these conditions G3PDH, NPY, and AGRP were in exponential phase of amplification. Quantification of PCR products was based on Southern blots of the reactions using internal ^{32}P-labeled oligonucleotide probes to G3PDH, NPY, and AGRP. Image analysis was performed using a Personal FX phosphor-imager and quantified with QuantityOne software (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistics

All values are given as mean ± SEM. One-way ANOVA followed by Tukey test for multiple comparisons was used and performed using SigmaStat 2.03 (SPSS, Inc., Chicago, IL).

Results

GHRP-2 increases body weight in wild-type and Npy(−/−) mice

Peripheral daily administration of GHRP-2 caused a significant increase in body weight of both wild-type and *Npy(−/−)* mice (Fig. 1). Body weight was increased in a dose-dependent manner from 30.7 ± 1.0 g to 33.2 ± 1.0 g (2.5 μg GHRP-2) and from 31.3 ± 1.6 g to 34.8 ± 1.6 g (10 μg GHRP-2) in wild-type mice, (n = 42, $P < 0.001$, significant from d 2). Interestingly, induction of weight gain by GHRP-2 showed a similar but dose-independent increase after daily administration of 2.5 μg (from 29.8 ± 0.9 g to 33.2 ± 1.1 g) or 10 μg (from 29.7 ± 1.0 g to 33.1 ± 0.9 g) in *Npy(−/−)* mice (n = 42, $P < 0.001$, significant from d 5). Maximal weight gain achieved by the 10-μg dose was observed with the 2.5-μg dose suggesting that the *Npy(−/−)* mice were more sensitive to stimulation of weight gain by GHS. Control animals were injected with PBS for the same period of time and showed no significant changes in body weight (wild-type mice: from 31.4 ± 1.4 g to 32.0 ± 1.2 g [$P = 0.3$], *Npy[−/−]* mice: from 31.3 ± 1.4 g to 32.2 ± 1.4 g, [$P = 0.8$]) (Fig. 1).

Effect of GHRP-2 on food intake

GHRP-2 tended to increase daily food intake in a dose-dependent manner in both genotypes. However, this change was not statistically significant when measured after 24 h ($P > 0.089$, n = 84) (Fig. 2). Total food consumption after daily injections of 10 μg GHRP-2 was 89.9 ± 4.8 g (vehicle group: 74.5 ± 3.4 g) in wild-type mice, and 81.9 ± 4.2 g, (vehicle group: 74.9 ± 5.2 g) in *Npy(−/−)* mice. Although total food intake after 18 d did not change significantly, mean daily caloric intake throughout the treatment period in vehicle-injected wild-type control mice was lower (15.34 ± 0.47 kcal) than in mice injected with 10 μg GHRP-2 (18.5 ± 0.46 kcal,

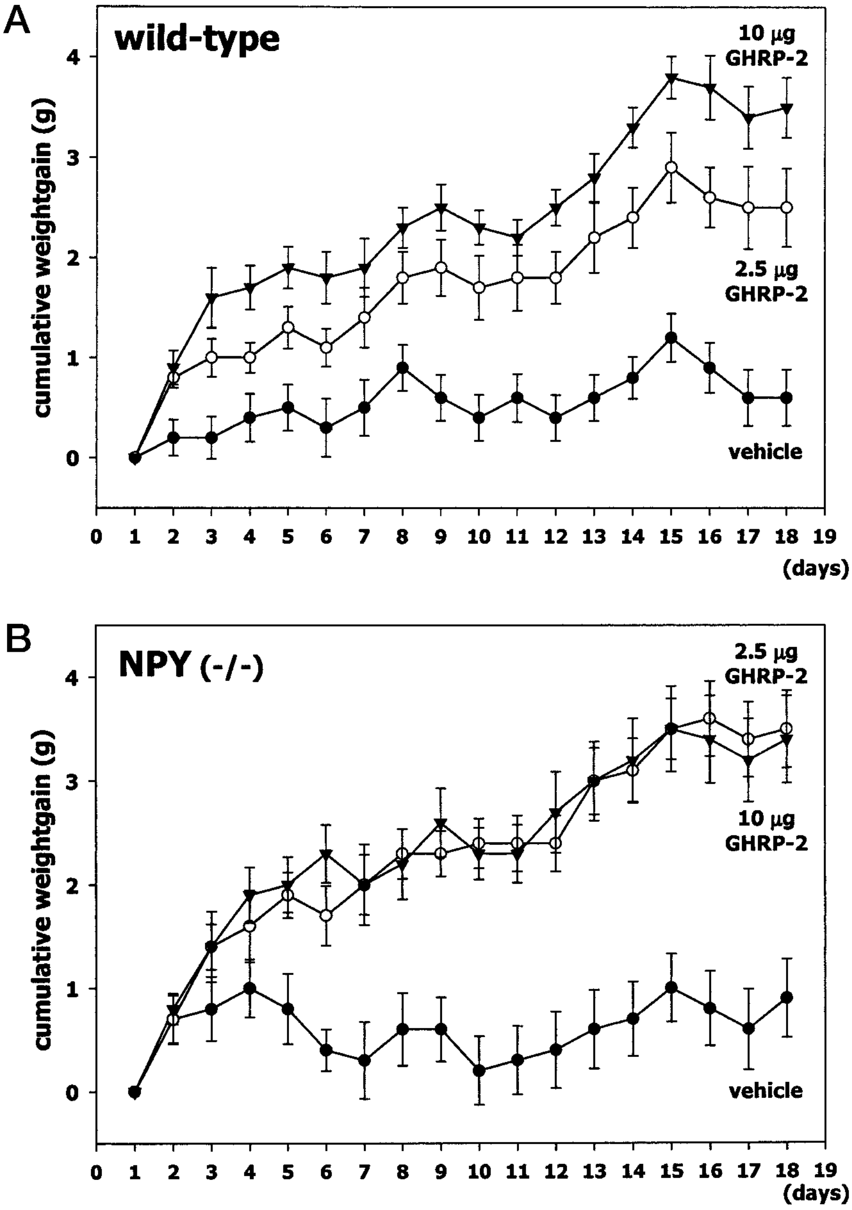

FIG. 1. The effect of GHRP-2 on body weight in mice (n = 84). Cumulative increase of body weight during a treatment period of 18 d was significant in wild-type mice (A) (n = 42, $P < 0.001$) as well as in 42 *Npy(−/−)* mice, (B) (n = 42, $P < 0.001$). One way ANOVA was used followed by Tukey test for multiple comparisons.

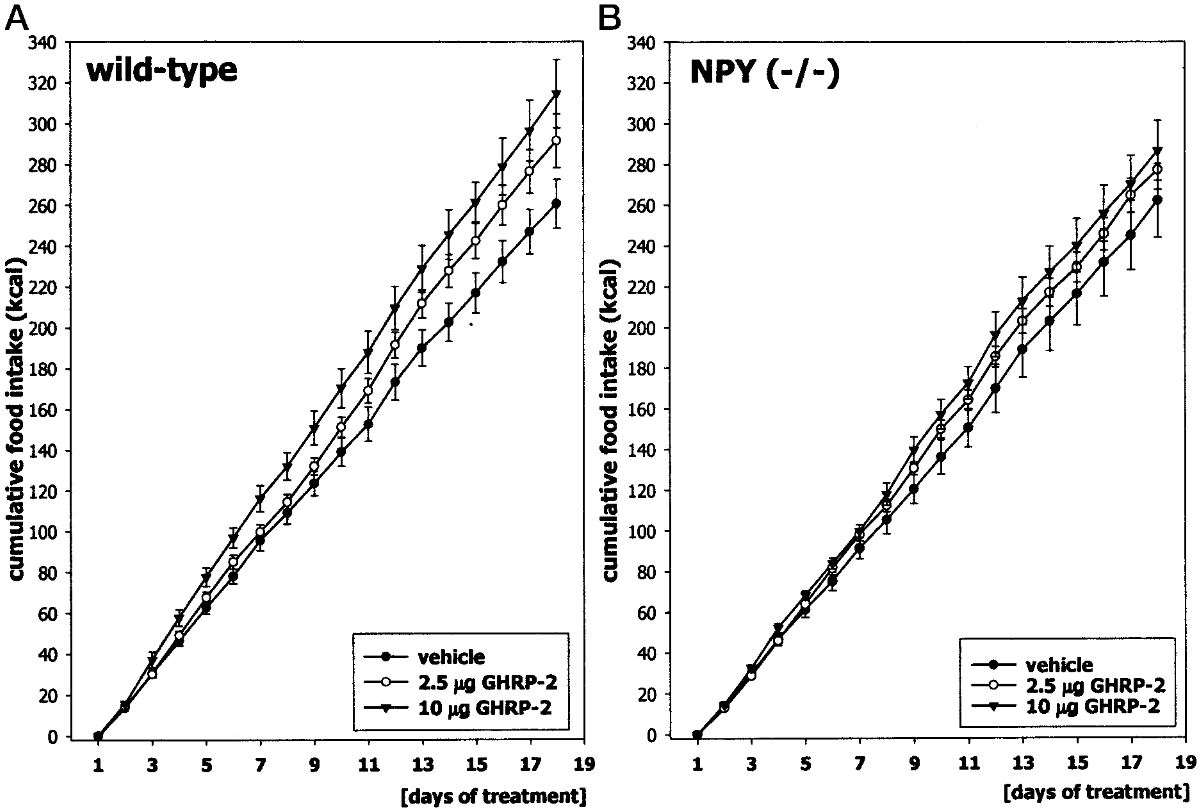

FIG. 2. The effect of GHRP-2 on food intake in wild-type mice (A) and *Npy(−/−)* mice (B). Although there was a trend for GHRP-2 mediated hyperphagia, 24-h food intake was not significantly different ($P = 0.089$).

$P = 0.001$). Mean daily food intake in vehicle-injected *Npy(−/−)* mice was also significantly lower (15.42 ± 0.40 kcal) than in *Npy(−/−)* mice treated with 10 μg GHRP-2 (17.3 ± 0.68 kcal, $P = 0.05$). In a separate experiment, a significant increase of food intake was found when measured acutely (6 h after GHRP-2 injection) in *Npy(−/−)* mice (average increase: +35.0%, n = 20, $P = 0.039$*)* as well as in wild-type mice (average increase: +36.8%, n = 20, $P = 0.001$, both after 10 μg GHRP-2).

GHRP-2 increases fat mass and bone mass but does not change lean mass

Analysis of body composition by DXA (n = 54) (Fig. 3) before and after 18 d of treatment with GHRP-2 revealed a significant increase of body fat in wild-type mice (increase after treatment with 2.5 μg GHRP-2: 3.57 ± 0.8 g, $P = 0.15$; increase after treatment with 10 μg GHRP-2: 4.42 ± 0.5 g, $P = 0.02$) and *Npy(−/−)* mice (increase after treatment with 2.5 μg GHRP-2: 4.26 ± 0.6 g, $P = 0.01$; increase after treatment with 10 μg GHRP-2: 4.42 ± 0.7 g, $P = 0.01$), compared with vehicle-treated controls (wild-type mice: 1.77 ± 0.6 g, *Npy(−/−)* mice: 1.48 ± 0.6 g). However, GHRP-2 did not cause any significant changes in lean mass ($P > 0.63$).

The relatively short 18-d treatment produced a significant increase in bone mass of animals treated with the ghrelin receptor agonist: (wild-type mice: 110 ± 33 mg [2.5 μg GHRP-2, $P = 0.13$], 144 ± 17 mg [10 μg GHRP-2, $P = 0.017$], 38 ± 23 mg [control], *Npy[−/−]* mice: 129 ± 25 mg [2.5 μg GHRP-2, $P = 0.085$], 151 ± 22 mg [10 μg GHRP-2, $P = 0.018$], 60 ± 18 mg [control]) (Fig. 3).

GHRP-2 increases the RQ

RQ, measured on d 18 (24-h measurement period), was increased following treatment with GHRP-2 in wild-type mice (0.933 ± 0.013 [10 μg GHRP-2], 0.88 ± 0.013 [vehicle], average RQ [24 h], $P = 0.001$, n = 5 each group). The increase of RQ in *Npy(−/−)* mice did not reach statistical significance (0.95 ± 0.03 [10 μg GHRP-2], 0.88 ± 0.08 [vehicle], $P = 0.1$, n = 5 each group) (Fig. 4, A and B). However, EE (Fig. 4, C and D) did not differ between any of the treated groups and the controls ($P > 0.05$). Energy derived from fat was calculated as 24.0 kcal/kg per day in vehicle-treated wild-type mice and 60.2 kcal/kg per day in wild-type mice treated with 10 μg GHRP-2.

Effect of GHRP-2 on plasma IGF-I, corticosterone, insulin, and glucose

Serum levels of IGF-I, insulin, corticosterone, and glucose were unchanged between the treatment groups on d 18 (n = 84, data not shown).

Effects on mRNA expression levels of NPY and AGRP (RT-PCR)

Treatment with GHRP-2 did not alter hypothalamic NPY mRNA levels of wild-type animals after the final treatment

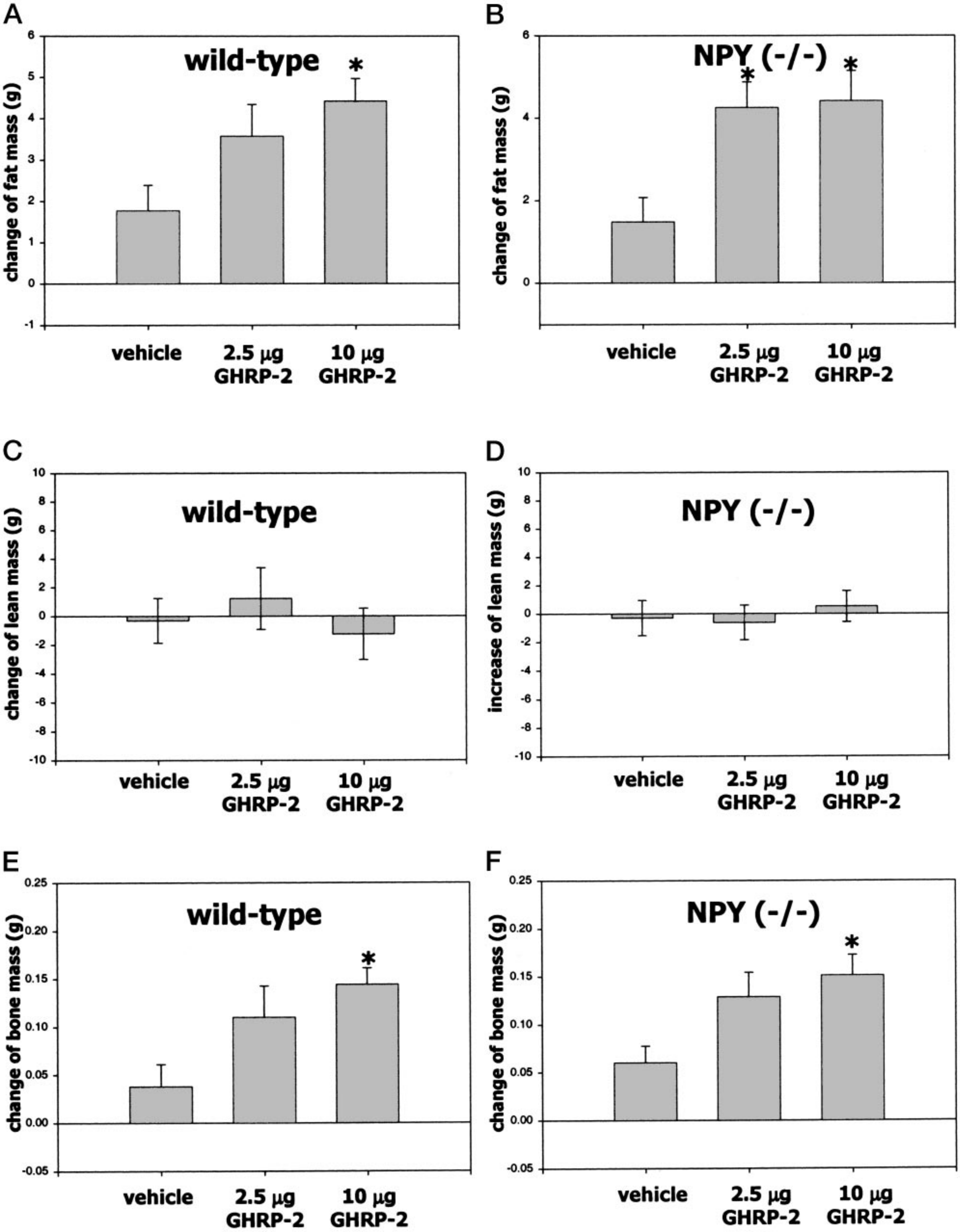

FIG. 3. Change of body composition after 18 d of treatment with GHRP-2 over 18 d in wild-type mice and *Npy(−/−)* mice (each treatment group: n = 9) measured by DXA. An increase of fat mass ($P < 0.02$) (A, B) and bone mass ($P < 0.02$) (E, F) was found in all GHRP-2 treated groups. No change in lean mass was observed (C, D). One-way ANOVA was used, followed by Tukey test for multiple comparisons.

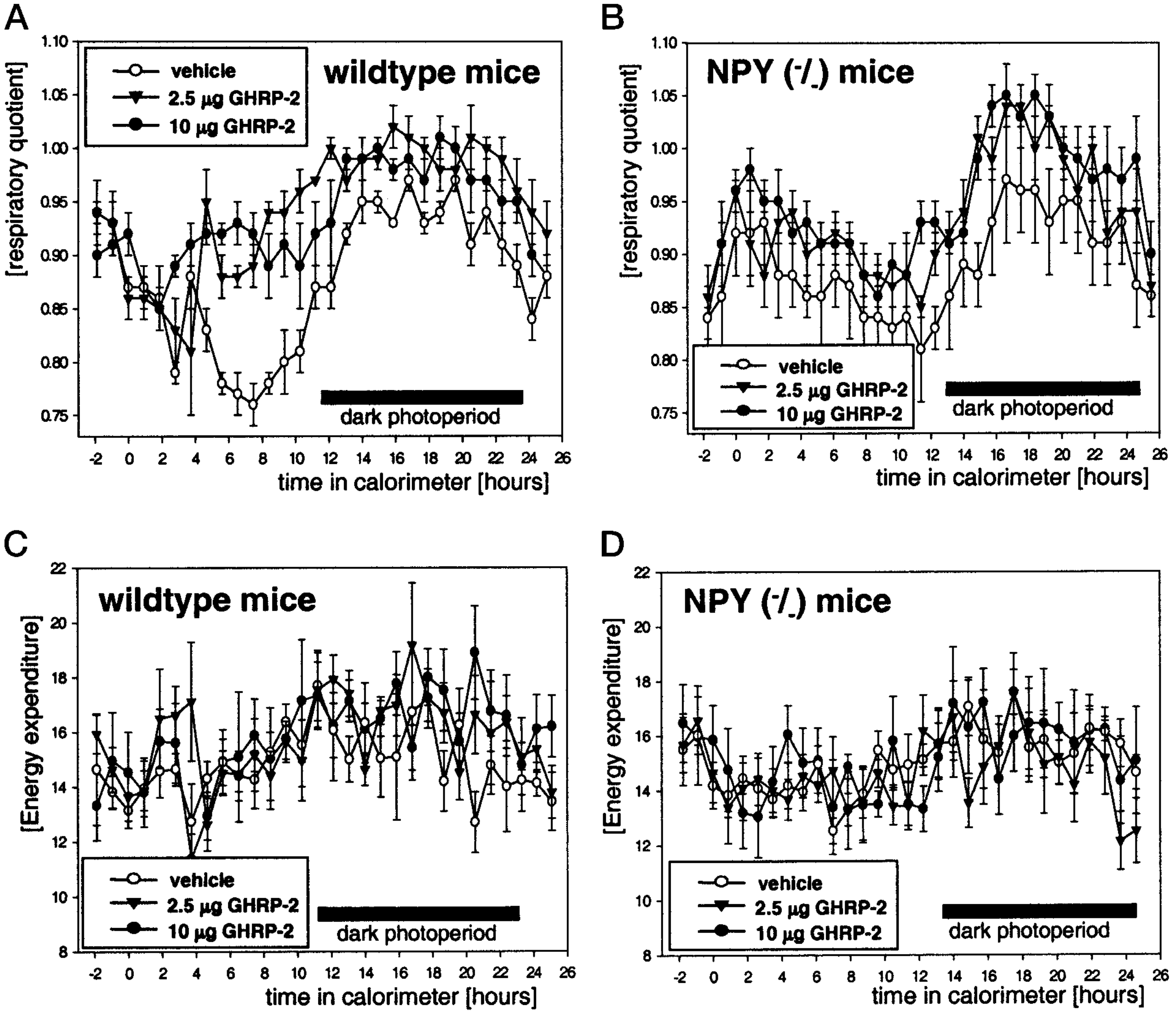

FIG. 4. Treatment with GHRP-2 significantly increased RQ in wild-type mice (n = 15, $P = 0.02$) (A) but only induced a trend for GHRP-2-mediated increase of RQ in *Npy(−/−)* mice (n = 15, $P > 0.8$) (B). One-way ANOVA was used, followed by Tukey test for multiple comparisons. EE (C, D) was not altered by GHRP-2 treatment ($P > 0.5$).

(n = 8 for each group) ($P = 0.63$). Relative hypothalamic NPY mRNA levels (NPY/G3PDH) were 1.17 ± 0.13 U (controls), 1.06 ± 0.09 U (2.5 μg GHRP-2), and 1.19 ± 0.08 U (10 μg GHRP-2) (Fig. 5A). No NPY mRNA was detected in *Npy(−/−)* mice or in minus-RT controls from either genotype.

Treatment with GHRP-2 tended to increase AGRP mRNA expression in wild-type mice after 18 d of treatment. Relative levels of hypothalamic AGRP mRNA in wild-type mice were 1.46 ± 0.18 U (controls), 3.24 ± 0.82 U (2.5 μg GHRP-2), and 3.27 ± 0.97 U (10 μg GHRP-2) (n = 24, $P = 0.16$) (Fig. 5B). Hypothalamic AGRP mRNA levels were also increased approximately 2-fold in *Npy(−/−)* mice animals treated with GHRP-2. Relative levels of hypothalamic AGRP mRNA in *Npy(−/−)* mice were 1.33 ± 0.19 U (controls), 2.80 ± 0.33 U (2.5 μg GHRP-2, $P = 0.019$) and 2.98 ± 0.47 U (10 μg GHRP-2, $P = 0.008$), (Fig. 5C). No differences in basal AGRP expression was observed between wild-type mice and *Npy(−/−)* mice ($P = 0.63$, n = 16).

MT-II, an agonist at melanocortin-receptor, abolishes induction of weight gain by GHRP-2 in Npy(−/−) mice

To test *in vivo* whether AGRP, the endogenous antagonist to melanocortin hypothalamic receptors, would be the critical mediator for the effects of GHRP-2 on body composition, we treated three groups of wild-type mice and three groups of *Npy(−/−)* mice with vehicle, GHRP-2 (10 μg/d, sc), or a combination of GHRP-2 (10 μg/d, sc) and MT-II for a period of 6 d. Consistent with our previous study, ghrelin receptor agonist treatment increased body weight in both genotypes (Fig. 6). Coadministration of MT-II was unable to prevent GHRP-2-induced weight gain in wild-type mice. However, in *Npy(−/−)* mice, coadministration of MT-II prevented GHRP-2 induced weight gain. In wild-type mice, body weight increased by 0.9 ± 0.21 g (vehicle-treated animals), 3.0 ± 0.24 g (10 μg/d GHRP-2), and 2.4 ± 0.20 g (10 μg/d GHRP-2 + 20 μg/d MT-II). In *Npy(−/−)* mice, body weight increased by 0.7 ± 0.22 g (vehicle-treated animals), 2.2 ±

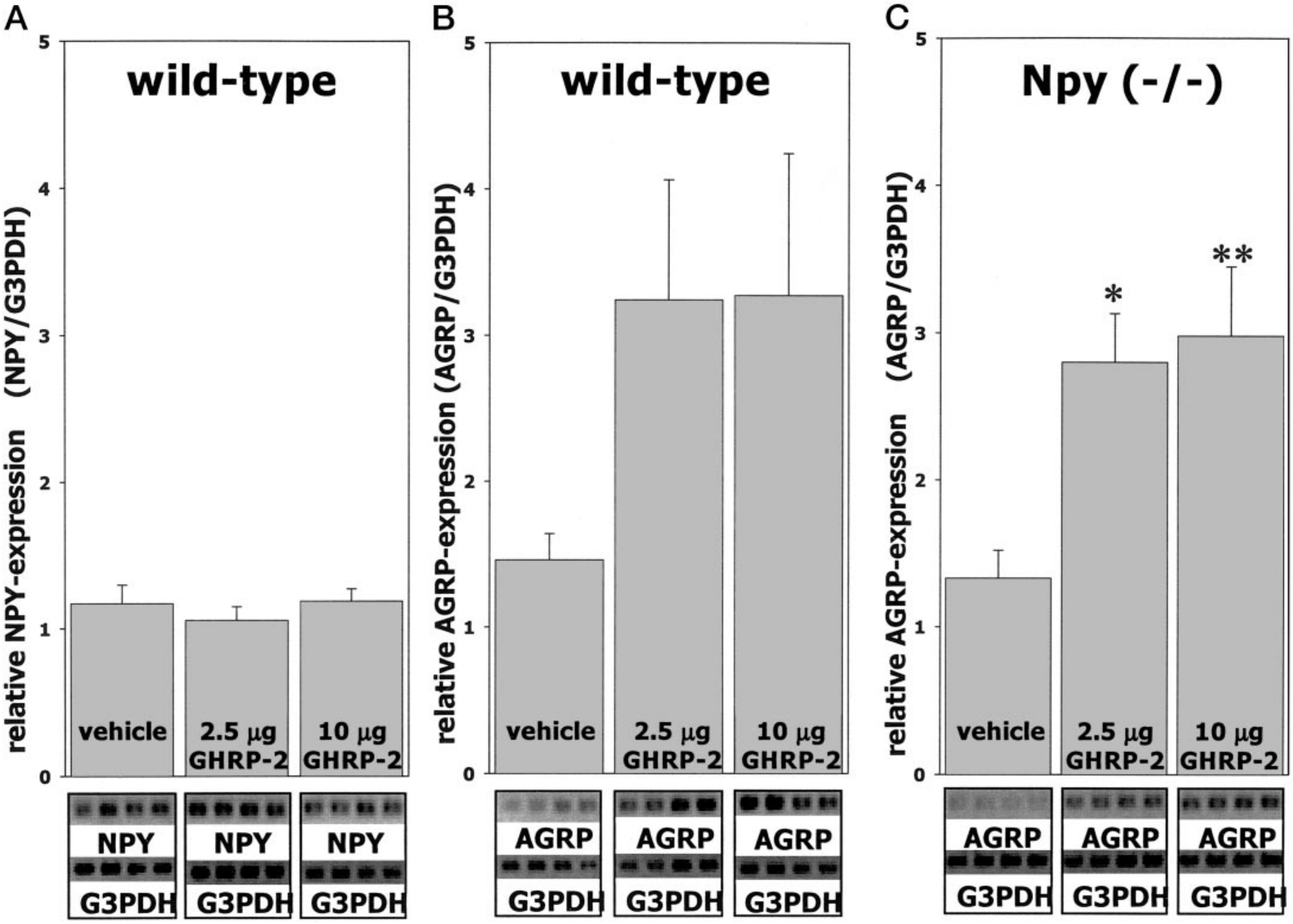

FIG. 5. GHRP-2 treatment did not alter hypothalamic levels of NPY mRNA (n = 24, $P > 0.63$) (A). But in *Npy(−/−)* mice, GHRP-2 stimulated AGRP mRNA expression (n = 24, $P = 0.008$) (C). An increase of hypothalamic AGRP mRNA levels in wild-type animals was also detected, but that increase was not significant (n = 24, $P > 0.16$), (B). One-way ANOVA was used, followed by Tukey test for multiple comparisons. Representative bands of gels for amplifications of PCR products are shown below each graph.

0.21 g (10 μg/d GHRP-2), and 1.0 ± 0.24 g (10 μg/d GHRP-2 + 20 μg/d MT-II). According to these results, agonistic action at the central melanocortin receptors prevents the induction of weight gain by GHRP-2 in *Npy(−/−)* mice ($P = 0.002$, n = 10 each group) but not in wild-type mice ($P > 0.07$, n = 10 each group). In *Npy(−/−)* mice, no significant difference in weight gain between untreated controls and mice treated with a combination of GHRP-2 and MT-II was found ($P = 0.37$, each group n = 10).

Discussion

We recently demonstrated that ghrelin, an endogenous GHS, is capable of stimulating adiposity in rodents when administered either peripherally or centrally (22). These findings were confirmed later by others who focused on specific central mechanisms for such adiposity. Indeed, Asakawa *et al.* (26) demonstrated that ghrelin-mediated hyperphagia is a consequence of its ability to stimulate NPY neurons. We now demonstrate that the ghrelin receptor agonist GHRP-2 is capable of stimulating adiposity even in *Npy(−/−)* mice. NPY and AGRP are orexigenic neuropeptides that dramatically induce positive energy balance and are colocalized in neurons of the medial arcuate nucleus (34). These NPY/AGRP neurons express GHS-Rs, and peripheral administration of ghrelin or ghrelin receptor agonists activates early gene expression in these neurons (17–21, 35). Therefore, treatment of *Npy(−/−)* mice with ghrelin or its receptor agonists cannot only test whether the anabolic actions are exclusively mediated by NPY but can provide a model to study metabolic physiology of AGRP stimulation in absence of NPY influences.

GHRP-2 is the most potent ghrelin receptor agonist of the GHRP family (36). Although no direct comparisons to ghrelin have been reported, we used GHRP-2 in this study because of the short half-life of ghrelin (22). Our study clearly demonstrates that chronic peripheral treatment with GHRP-2 stimulated weight gain in both wild-type and *Npy(−/−)* mice. Moreover, the observed increase in body weight was primarily a consequence of increased fat mass. Because GH increases lean mass and decreases fat mass (37, 38), the accumulation of fat mass is unlikely a consequence of GHRP-2-mediated GH secretion. First, GH does not

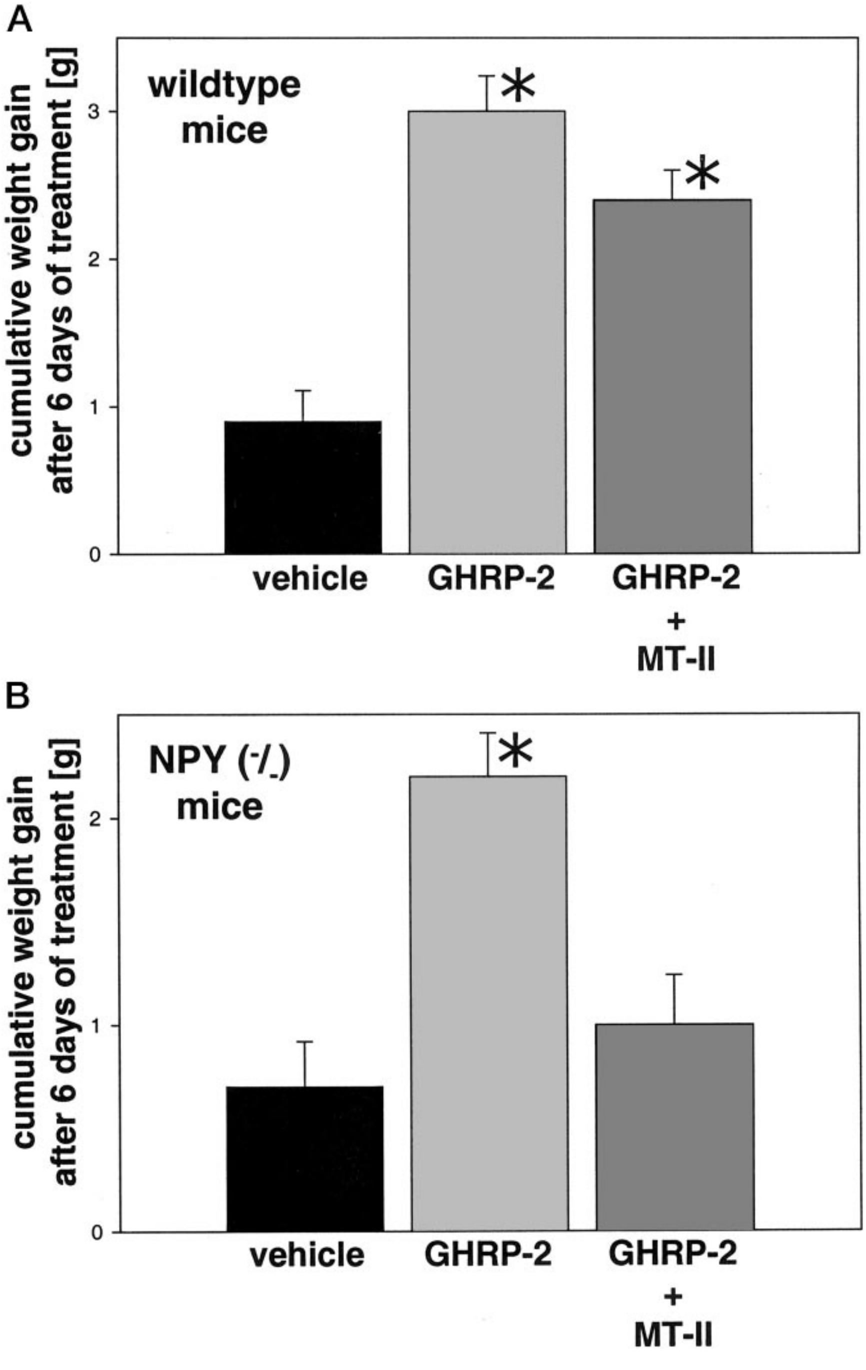

FIG. 6. MT-II blocks GHRP-2 induced adiposity during a treatment of 6 d in the absence (B), but not in the presence (A) of NPY. (*, Significant difference from vehicle).

change RQ but increases EE in mice (22). In addition, ghrelin increases body weight in dwarf rats in the absence of GH (22). Thus, GHRP-2-induced adiposity is owing to direct hypothalamic effects of GHRP-2 rather than mediation via the GH-IGF-I axis. We acknowledge that ghrelin and its receptor agonists do stimulate GH release from the pituitary (1, 7, 8, 10–12). Further, we recognize the requirement of adequate nutrition for GH-mediated anabolism (39, 40). We therefore propose that ghrelin, as an endogenous ligand of the GHS-R, provides that component-linking regulation of energy balance with growth and repair. The fact that ghrelin and its receptor agonists appear to stimulate effects opposite to those expected for GH but at the same time stimulate GH release might indicate that ghrelin actions are not only caused by multiple exogenous and endogenous ligands but also may be mediated by multiple receptors or at least multiple receptor subtypes (41).

NPY mRNA levels were unaltered at the end of the treatment period (d 18). These results are consistent with recent *in situ* hybridization studies demonstrating that NPY mRNA levels are unchanged in rats after chronic treatment with the ghrelin receptor agonist GHRP-6 (21). We hypothesize that NPY might be responsible for mediation of ghrelin-induced early orexigenic effects, and AGRP mediates chronic effects on energy balance and body composition. No significant orexigenic effects of GHRP-2 were observed in the present study by measuring food intake every 24 h. However, significant stimulation of food intake was found when analyzed 4 h after injection, indicating an acute, transient character of orexigenic effects of GHRP-2.

According to the first law of thermodynamics, it may appear incongruous that administration of GHRP-2 or ghrelin increases body fat mass without neither significantly increasing (daily) food intake nor markedly decreasing (24 h) energy expenditure. However, as shown in this and other rodent studies, ghrelin as well as some of its receptor agonists do effectively increase body weight and body fat mass. We confirmed that GHSs have a short-lasting, acute orexigenic potency (23–26) that may however not be obvious when food intake is measured once daily and the compound is administered peripherally (22). Short-term measurement reveals acutely raised energy intake and continuous as well as central administration exerts incomparably stronger effects on rodent feeding. Furthermore feeding is not a 100% efficient process. We speculate that ghrelin and its receptor analogues may enhance efficiency of assimilating calories from food. Therefore, experiments designed to complement calorimetry studies by measuring calories consumed as well as those in urine and feces are needed. Analysis of short-term effects of ghrelin receptor agonists on energy expenditure might reveal changes that have been overlooked by 24-h monitoring using indirect calorimetry. Cumulative accretion of calories gained by repetitive short periods with increased food intake and decreased energy expenditure might be responsible for the GHS-induced changes in body weight and body fat mass. An additional switch from fat utilization to carbohydrate utilization as indicated by a GHS-induced increase in RQ might further contribute to the additional energy gain to be stored in fat.

Because GHRP-2 stimulated adiposity in *Npy(−/−)* mice without significantly altering caloric intake, caloric expenditure or the level of lipid utilization, it might have increased the overall efficiency of calories ingested. Such enhanced metabolic efficiency was recently reported in the melanocortin-3 receptor gene disrupted mouse (42, 43). These mutants do not present with either hyperphagia or increased oxygen consumption but become obese with age. Because AGRP antagonizes αMSH at hypothalamic melanocortin receptors (44, 45), we speculate that GHRP-2 is a potent stimulator of AGRP in *Npy(−/−)* mice and can induce a functional antagonism at the MC-R.

Hypothalamic AGRP mRNA was significantly increased in *Npy(−/−)* mice and tended to be increased in wild-type mice following chronic treatment with GHRP-2. This statistical difference between both phenotypes may reflect a compensatory mechanism involving AGRP in *Npy(−/−)* mice, but we cannot exclude the possibility that this difference is just caused by more variability in the wild-type mice. Ghrelin also increases hypothalamic expression of AGRP after an acute intracerebroventricular injection in wild-type rats, as shown by Northern blotting (46). Our findings provide quantitative data for a GHS-R-mediated increase of hypothalamic AGRP mRNA when NPY expression is absent, suggesting that AGRP may compensate for the absence of NPY signaling. Indeed, GHRP-2 administration to *Npy(−/−)* mice appears to provide a perfect means to study AGRP-mediated physiology distinct from the influence of hypothalamic NPY systems. The complete disruption of GHS-R-ligand mediated weight gain in *Npy(−/−)* mice using the MT-II (47), a melanocortin receptor agonist that is opposed by AGRP at the melanocortin receptors further supports the important role of AGRP in stimulating adiposity resulting from GHS-R activation. Based on this result, we concluded that no pathway apart from NPY or the melanocortin receptors is mediating ghrelin/ghrelin receptor agonist-induced adiposity. That GHRP-2 induced weight gain was not completely blocked by MT-II in wild-type animals likely indicates that NPY comediates other anabolic effects after GHS-R activation. AGRP actions are very long lasting and persist for up to 1 wk after a single administration (48). An acute increase in hypothalamic NPY mRNA levels after a single intracerebroventricular ghrelin injection has recently been shown using *in situ* hybridization (24). However, NPY levels are negatively regulated by increased levels of stored energy (28). Thus, NPY mRNA may be attenuated in wild-type mice that have accrued fat mass, possibly explaining why NPY mRNA was not stimulated after 18 d of GHRP-2 treatment. However, stimulation of AGRP mRNA by GHRP-2 appears to be maintained and this apparent lack of feedback regulation is responsible, at least in part, for the increased fat mass. Extended time course studies are needed to fully understand these mechanisms.

Npy(−/−) mice appeared to be slightly more sensitive to GHRP-2-mediated weight gain than were wild-type-mice (Fig. 1). A significant weight gain was observed in the latter group only at the 10-μg dose, but this level of obesity was stimulated by the lower dose (2.5 μg) of GHRP-2 in mutant mice. The increased adiposity did not primarily result from enhanced stimulation of hyperphagia (energy intake), decreased EE or reduced utilization of lipid. In fact, the wild-type mice were observed to reduce lipid utilization by oxidizing carbohydrates to a greater extent than the *Npy(−/−)* mice. It has been reported that ghrelin receptor agonists may induce an insulin resistant state in rats (49) and humans (50). Zarjevski *et al.* (51) demonstrated that chronic central administration of NPY would result in an insulin-resistant state even when the NPY-treated rats were not permitted to overeat. Thus, wild-type mice may be less sensitive to GHRP-2 stimulated glucose oxidation because they are more insulin resistant than their *Npy(−/−)* counterparts. Further studies are necessary to test this hypothesis.

Recent clinical findings have shown that treatment with the oral ghrelin receptor agonist MK-677 increases markers of bone formation and bone resorption in obese young males (52). We observed an increase in bone mass after treatment with ghrelin receptor agonists. In addition, longitudinal bone growth and bone mineral density were increased in rats after treatment with ghrelin receptor agonist, ip amorelin (53, 54). The present study confirms these findings and suggests that GHRP-2 treatment could be an effective means to stimulate bone mineral content. Further, we demonstrate that the increase in bone density is independent of NPY but is paralleled by an increase in hypothalamic AGRP mRNA expression. The work of Ducy *et al.* (55, 56) suggests the interesting perspective that bone metabolism might be controlled by central regulatory structures to a greater extent than endocrine researchers had recognized before. They speculate that decreases in bone density of ob/ob mice after leptin replacement may involve central/hypothalamic mechanisms. To our opinion, the melanocortin system may participate in that mechanism and from this perspective, the GHRP-2 induced ($n = 42$, $P < 0.05$) increase in bone mass and a parallel increase in hypothalamic AGRP mRNA levels offer an interesting hypothesis of explaining GHS-induced effects on bone. However, although a hypothalamic role in mediating effects of GHRP-2 on bone offers an intriguing new perspective, the possibility that an increase in bone mass is a secondary effect caused by GHRP-induced GH-secretion cannot be ruled out at the current moment.

In summary, we have shown that GHRP-2 dose dependently increases body weight by decreasing fat utilization and increasing caloric intake in mice after daily sc administration. This effect has been reproduced in the presence, as well as in the absence, of NPY. In fact, *Npy(−/−)* mice were more sensitive to GHRP-2-stimulated weight gain than wildtype mice suggesting distinct roles for NPY and AGRP. Further, only the weight gain of NPY-mutant mice could be blocked by simultaneous injection of an MC-R agonist (MT-II), indicating the principal role of the observed AGRP mRNA increase. Such weight gain results primarily from increase in body fat. In addition, a significant increase in bone mass caused by GHRP-2 treatment was observed after only 3 wk of treatment.

Ghrelin, GHRP-2, and other synthetic receptor agonists could offer an interesting treatment concept for the therapy of cachexia, including stimulation of weight gain, food intake, and possibly bone mass. In clinical studies, the nonpeptide, MK-677 was shown to reverse catabolism (57). GHRP-2 was successfully tested in a combined treatment regimen with TRH in patients with protracted critical illness (58). Weight loss, reduced appetite, and increased energy expenditure as well as loss of bone mass are hallmarks of catabolic syndromes caused by senescence (59), HIV (60), or cancer (61). Thus, these therapeutic agents acting on neuroendocrine regulators of energy balance such as AGRP and the melanocortin system could offer a promising perspective for the therapy of cachexia and critical illness.

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