Energy Homeostasis and Gastric Emptying in Ghrelin Knockout Mice


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ABSTRACT

To elucidate the role of endogenous ghrelin in the regulation of energy homeostasis and gastric emptying, ghrelin knockout mice (ghrelin\(^{-/-}\)) were generated. Body weight, food intake, respiratory quotient, and heat production (indirect calorimetry), and gastric emptying (\(^{14}\)C breath test) were compared between ghrelin\(^{-/+}\) and ghrelin\(^{-/-}\) mice. In both strains, the effect of exogenous ghrelin on gastric emptying and food intake was determined. Ghrelin\(^{-/-}\) mice showed some subtle phenotypic changes. Body weight gain and 24-h food intake were not affected, but interruption of the normal light/dark cycle triggered additional food intake in old ghrelin\(^{-/-}\) but not in ghrelin\(^{-/+}\) mice. Exogenous ghrelin increased food intake in both genotypes with a bell-shaped dose-response curve that was shifted to the left in ghrelin\(^{-/-}\) mice. During the dark period, young ghrelin\(^{-/-}\) mice had a lower respiratory quotient, whereas their heat production was higher than that of the wild-type littermates, inferring a leaner body composition of the ghrelin\(^{-/-}\) mice. Absence of ghrelin did not affect gastric emptying, and the bell-shaped dose-response curves of the acceleration of gastric emptying by exogenous ghrelin were not shifted between both strains. In conclusion, ghrelin is not an essential regulator of food intake and gastric emptying, but its loss may be compensated by other redundant inputs. In old mice, meal initiation triggered by the light/dark cue may be related to ghrelin. In young animals, ghrelin seems to be involved in the selection of energy stores and in the partitioning of metabolizable energy between storage and dissipation as heat.

Ghrelin, isolated from the rat stomach, is the endogenous ligand of the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). This G protein-coupled receptor, mainly expressed in the hypothalamus and pituitary gland, was already cloned in 1996 (Howard et al., 1996) and was first identified as the receptor for a family of synthetic peptides, growth hormone secretagogues, known to stimulate the secretion of growth hormone (GH) from the pituitary gland. Ghrelin is a 28-amino acid peptide with an \(n\)-octanoyl modification on Ser\(^3\), and this modification is essential for GH-releasing effects of ghrelin (Kojima et al., 1999).

Besides GH release, ghrelin is thought to play an important role in the regulation of the energy balance. Indeed, both central and peripheral administration of ghrelin stimulates food intake in a dose-dependent manner in rodents (Nakazato et al., 2001) and humans (Wren et al., 2001). The orexigenic effect of ghrelin is mediated via the vagal nerve (Date et al., 2002) and via hypothalamic centers. It results in body weight gain partly due to the increased food intake but also due to decreased fat utilization, resulting in adiposity (Tschop et al., 2000).

Surprisingly, the largest amount of ghrelin is found in the stomach, and ghrelin plasma levels are strongly reduced by gastrectomy (Ariyasu et al., 2001). This suggests a role for ghrelin in the regulation of the gastrointestinal system. Ghrelin is as yet the only peptide with significant sequence identity with motilin. Moreover, the receptors of motilin and ghrelin also share a marked sequence homology, and together they constitute a new subfamily within class A of rhodopsin like G protein-coupled receptors. It is generally accepted that motilin is involved in the regulation of the
migrating motor complex and that it can accelerate gastric emptying. Similar effects have now been observed with ghrelin and with synthetic growth hormone secretagogues.

Indeed, ghrelin accelerates gastric emptying in rodents and humans and is able to overcome postoperative ileus (Trudel et al., 2002; De Winter et al., 2004; Tack et al., 2005b) in a dose-dependent manner. Also, peptide (e.g., H-His-n-Trp-Ala-Trp-d-Phe-Lys-NH₂) and nonpeptide [e.g., capromorelin (2-amino-N-[(1R)-1-[(3zA)-3a-benzyl-2,3,3a,4,6,7-hexahydro-2-methyl-3-oxo-5H-pyrazolo[4,3-c]pyridin-5-yl]carbonyl]-2-benzyl-oxyethyl]-2-methylpropionamide)] growth hormone secretagogues show gastroprokinetic properties in mice and rats (Depoortere et al., 2005; Kitazawa et al., 2005). Recent studies also show that ghrelin induces premature interdigestive motility patterns in rats and humans (Fujino et al., 2003; Tack et al., 2005a). In humans, the effect is not mediated through the release of motilin (Tack et al., 2005a).

To further define the endogenous role of ghrelin, we generated mice in which the ghrelin gene was deleted. The role of ghrelin in the regulation of the energy balance was investigated by comparing food intake, respiratory quotient, and heat production between young and old wild-type (ghrelin+/⁺) and ghrelin knockout (ghrelin⁻⁻) mice. Effects on gastrointestinal motility were analyzed by comparing gastric emptying parameters, as deduced from the [¹⁴C]octanoic acid method (SAS 9.1; SAS Institute, Cary, NC). The best fit was obtained after fitting to the Gompertz model; thus, this model was used to generate the targeting vector. A LacZ/Neo reporter/selection cassette was inserted as a SfiI fragment to replace a 285-bp ghrelin genomic fragment that includes the coding region of exon 2 up to exon 3 after yeast-mediated homologous recombination. The NotI-linearized vector was electroporated into 129 Sv/Evbrd(LEX1) em- bryonic stem cells, and G418-fialuridine-resistant embryonic stem cell clones were isolated and analyzed for homologous recombination by Southern blot analysis. Targeted embryonic stem cell clones were injected into C57BL/6(αlbin) blastocysts, and the resulting chimeras were mated to C57BL/6(αlbin) females to generate heterozygous animals. These were subsequently crossed to generate all three genotypes used in the reported studies. PCR was used to screen genotypes by using DNA isolated from mouse tail biopsy samples. Primers 5'-CTCGCGAGACAGACACCTG-3' and 5'-CAGTGCACTCAGTCTGGTCTC-3' amplified an 842-bp band from the wild-type allele, whereas primers 5'-CTCGCCAGACAGACACCTG-3' and 5'-GCAGGCAGCTGCTTCTACT-3' amplified a 630-bp band from the knockout allele. Quantitative RT-PCR analysis was used to show absence of the ghrelin transcript. Total RNA was isolated from different tissues using TRIzol (Invitrogen, Carlsbad, CA), and first strand cDNA synthesis was performed on 0.5 μg of total RNA using random hexamer primers and SuperScript II RT (Invitrogen). Quan- titative PCR was performed on an ABI Prism 7700 cycler (Applied Biosystems, Foster City, CA) using a TaqMan PCR kit. Serial dilu- tions of cDNA were used to generate standard curves of threshold cycles versus the logarithms of concentration for β-actin and ghrelin. A linear regression line calculated from the standard curves allowed the determination of transcript levels in RNA samples from mice. The ghrelin primer-probe pair (primer 5'-GGCAGCTCAGCCCTGGTC- CT-3' primer 5'-TGGCTCTTCTGATTCTTTCTTC-3', probe 5'-AGG- CCAGAGCCACAGAAAACGAGCA-3' 5'-5-carboxyfluorescein [3'5-carboxytetramethylrhodamine] relative to actin (primer 5'-CATCTGG- GCCTACGTGCCAC-3', primer 5'-GGCCGCGACTCATCGCTA-3', probe 5'-TGCTGGTCTGACATCCTGCTGAA-3' 5'-5-carboxyfluorescein [3'5-carboxytetramethylrhodamine] was used to assess expression levels.

**Immunohistochemistry.** Mice were deeply anesthetized (60–85 mg/kg pentobarbital, intraperitoneally) and perfused transcardially for 5 min with 0.9% NaCl (37°C) and then for 20 min with 4% paraformaldehyde (4°C). Stomach sections (14 μm) were cut with a cryostat and incubated for 2 h in 0.1 M phosphate-buffered saline containing 4% goat serum, 0.5% Triton X-100, and 0.3% Na₂CO₃ at 4°C. After incubation overnight with the rabbit anti-ghrelin antibody (dilution 1:500; kindly provided by Dr. P. Robberecht, Université Libre de Bruxelles, UK.), sections were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (dilution 1:50) (Jack- son ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h at 4°C. After three washes of 5 min in 0.1 M phosphate-buffered saline, the sections were mounted with Citifluor (Citifluor Ltd., London, UK).

**Body Weight.** Body weight was measured every week or every 2 weeks (except between weeks 32 and 41) between 10:00 AM and 12:00 PM from week 12 until week 75 after birth. Initially, these data were fitted according to three different growth models: the Béntalanly model (Di Masso et al., 1990), the Gompertz model (Kid- well et al., 1969), and a logistic model (Pahl, 1969). The best fit was obtained after fitting to the Gompertz model; thus, this model was further used. The equation of this model is as follows,

\[ Y = A e^{B e^{-K t}} \]

where \( Y \) is body weight (grams), \( A \) is predicted mature body weight (grams), \( B \) is the integration constant, \( K \) is the intrinsic growth rate parameter, and \( t \) is time (weeks). Growth curve parameters were estimated with SAS procedure NLIN using the Marquardt search method (SAS 9.1; SAS Institute, Cary, NC).

**Food Intake.** Food intake in young mice during the light period (13 h) and the dark period (11 h) was measured daily for 1 week. Food intake analysis during 6 h in ad libitum fed mice was started at 10:00 AM. Each mouse was put in an individual cage, where it received a preweighed amount of food. Food intake was measured by subtracting the uneaten food at 30 min and 1, 2, 3, 4, 5, and 6 h. Cumulative food intake was calculated and plotted as a function of time.

To analyze the orexigenic effects of ghrelin in ad libitum fed mice, 200 μl of 0.9% saline or 1, 6, 15, 30, 50, or 75 nmol/kg ghrelin (Global Peptide Services, Ft. Collins, CO) or 15 or 30 nmol/kg des-octanoyl ghrelin (kindly provided by Dr. P. Robberecht, Université Libre de Bruxelles, Brussels, Belgium) was injected intraperitoneally immediately before the mice received the food. Mice were sensitized to ghrelin (kindly provided by Dr. P. Robberecht, Université Libre de Bruxelles, Ft. Collins, CO) or 15 or 30 nmol/kg des-octanoyl ghrelin and saline-injected mice as a function of the administered dose.

**Indirect Calorimetry.** Respiratory quotient (RQ) and heat production were measured for 24 h in ad libitum fed mice by using...
an open circuit indirect calorimetric unit. The respiration unit consists of six respiratory cells, placed two by two in three separate light- and temperature-controlled climatic chambers, a gas analyzer unit, and a data acquisition system (Buyse et al., 1998). The respiratory cells are made of stainless steel, with little insulation, and the temperature inside is measured by a platinum resistance temperature detector (Pt-100; Farnell In One, Grace-Hollogne, Belgium) (accuracy of 0.2°C). The paramagnetic O2 analyzer (ADC 02-823A) and the infrared CO2 analyzer (ADC D/8U/54/A) were calibrated before each measurement by using gas standards.

Mice were placed in the respiratory cells 24 h before the start of the experiment. After the adaptation period, gas exchanges (CO2 and O2) were measured continuously during 24 h for four consecutive days. Mice had free access to food and water, and 24-h food intake was followed. During this experiment, no exogenous ghrelin was administered.

O2 and CO2 concentrations from air samples coming out of each cell were measured for 60 s every 15 min during 24 h. The CO2 production and the O2 consumption were calculated from the differences between the gas concentrations of the outside fresh air and the cell air. RQ is the ratio of CO2 produced to the volume of O2 consumed. Heat production was calculated according to the formula of Romijn and Lokhorst (1961):

\[
\text{Heat production (kJ/h) } = 16.18 \text{ O}_2 \text{(l/h)} + 5.02 \text{ CO}_2 \text{(l/h)}
\]

**Gastric Emptying Studies: Breath Test.** Gastric emptying in mice was measured with the [14C]octanoic acid breath test as described by Kitazawa et al. (2005). Briefly, after an overnight fast (19 h, free access to water), mice were injected intraperitoneally, if applicable, with 0.9% saline or 1, 30, 75, and 125 mmol/kg ghrelin.

A baseline breath sample (5 min) was taken 23 min after the injection, and 30 min later, the [14C]octanoic-labeled test meal was given to the mice. Before the start of the experiment, fasted mice were trained twice weekly at a fixed time schedule for 2 weeks (2- or 3-day interval) to eat spontaneously the test meal (without radioactive marker) within 60 s. Sampling of exhaled breath was performed every 5 min during the first 30 min and then every 15 min for the next 3.5 h. From the 14CO2 excretion curve, two parameters, t1/2(tot) (time at which 50% of the total amount of 14CO2 was excreted) and t1/2(tot) (initial delay in gastric emptying due to the time required for the stomach to grind the meal into fine particles) were calculated as described previously (Kitazawa et al., 2005).

The time interval between two breath tests was set at 3 to 4 days. To test the effect of exogenous ghrelin on gastric emptying, each group of mice first underwent a control breath test (saline injection) followed by two consecutive breath tests with increasing doses of ghrelin and again a control breath test. The effect of ghrelin was compared with the mean of the control breath tests given before and after the injection of ghrelin.

**Statistical Analysis.** Data are presented as mean ± S.E.M. Growth curve parameters, 24-h food intake, and gastric emptying parameters were compared with an unpaired t test. Cumulative food intake, respiratory quotient, and heat production were analyzed by two-way analysis of variance, with one repeated measure factor (time). The effect of exogenous ghrelin on food intake and on gastric emptying was analyzed by two-way analysis of variance, with two repeated measure factors. In case of significant factor effects, tests with contrasts were performed to locate pairs of factor levels with significant differences in the examined variables. Data were analyzed with Statistica 6.0 (StatSoft, Tulsa, OK), and significance was accepted at the P < 0.05 level. Dose-response curves to exogenous ghrelin were fitted according to a Gaussian distribution (Prism 4.0; GraphPad Software Inc., San Diego, CA).

**Results**

**Generation of the Ghrelin**⁻/⁻ **Mice**

The homologous recombination resulted in deletion of the coding region of exon 2 and exon 3, with insertion of the LacZ reporter gene (Fig. 1A). The targeting vector consisted of 2.6- and 4.0-kb homologous regions of genomic DNA at 5’ and 3’ of the selection cassette, respectively. In mice, loss of the wild-type ghrelin allele was confirmed by PCR analysis (Fig. 1B). Loss of expression of the ghrelin transcript in the knock-out mice was confirmed by quantitative RT-PCR performed on total RNA isolated from stomach, jejunum, brain, and blood from wild-type, heterozygous, and homozygous ghrelin−/− animals (n = 3 for each genotype). Clearly, the ghrelin transcript was absent in all tissues derived from the homozygote ghrelin−/− (Fig. 1C).

**Immunohistochemistry**

An immunohistochemical staining for ghrelin was performed on sections of the mouse oxyntic gland, which is the main source of ghrelin, to confirm the deletion of the ghrelin gene in the ghrelin−/− mice. In the oxyntic mucosa of ghrelin−/− mice, ghrelin-positive cells could be easily detected (Fig. 2A), whereas no immunoreactivity for ghrelin could be observed in sections from the ghrelin−/− mice (Fig. 2B).

**Body Weight and Food Intake Studies**

**Evolution of Body Weight.** Body weight (n = 11 for each genotype) was followed between 12 and 75 weeks of age (Fig. 3). The data were fitted to the Gompertz growth model, and the growth curve parameters were calculated. The mature body weight of the ghrelin−/− mice (36.85 ± 1.45 g) was not significantly different from the mature weight of the ghrelin−/− mice (41.19 ± 2.45 g). In addition, the intrinsic growth rate parameter was the same for both genotypes (ghrelin−/+ and ghrelin−/−, 0.10 ± 0.01 versus ghrelin−/−, 0.09 ± 0.02). However, because the two curves seemed to diverge with increasing age, mice were divided for further observations into two subgroups: young mice (15–25 weeks old) and old mice (50–65 weeks old).

**Food Intake in Young and Old Mice Fed Ad Libitum. Normal light cycle phase.** Normal circadian patterns of spontaneous food intake were observed for the two genotypes. Food intake of young ghrelin−/− mice during the 13-h light period and the 11-h dark period amounted to 1.18 ± 0.08 and 3.87 ± 0.14 g, respectively, and it did not differ significantly from that of the age-matched wild-type littermates both during the light (ghrelin−/+), 0.67 ± 0.10 g versus ghrelin−/−, 0.80 ± 0.11 g) and the dark period (ghrelin−/+, 4.05 ± 0.16 g versus ghrelin−/−, 4.32 ± 0.15 g) (Fig. 4A). In addition, food intake in old ghrelin−/− mice did not differ from that of the wild-type littermates both during the light (ghrelin−/+), 0.67 ± 0.10 g versus ghrelin−/−, 0.80 ± 0.11 g) and the dark period (ghrelin−/+), 4.05 ± 0.16 g versus ghrelin−/−, 4.32 ± 0.15 g) (Fig. 4B).

**Artificial light cycle phase.** Cumulative food intake followed during 6 h at times of minimal feeding (10:00 AM to 4:00 PM) in old ghrelin−/− mice fed ad libitum did not differ significantly from the food intake of the age-matched ghrelin−/+ mice (Fig. 5A). However, when mice were artificially stimulated to eat food by turning the lights off at 10:00 AM for 6 h, cumulative food intake during the first 4 h was significantly increased in the old ghrelin−/+ mice but not in the ghrelin−/− mice (Fig. 5B). This was mainly due to an increased food intake during the first 30 min and second 30...
min after the start of the experiment (Fig. 5C). This effect was only apparent in the aged group because interruption of the light/dark cycle did not affect cumulative food intake in young mice (Fig. 6).

**Effect of Exogenous Administration of Ghrelin on Food Intake.** To determine whether the ghrelin/GHS-R pathway was still functional in the absence of ghrelin, food intake after intraperitoneal administration of different doses (1–75 nmol/kg) of ghrelin was compared between young ghrelin+/+ and wild-type mice. Results show the difference in cumulative food intake after 6 h between ghrelin- and saline-injected mice as a function of the administered dose. The dose-response relationship to ghrelin was bell-shaped in both genotypes (Fig. 7). The curve of the ghrelin−/− mice was shifted to the left and had a more narrow activity range than that of the ghrelin+/+ mice. The optimal dose to stimulate food intake in ghrelin−/− mice (10.8 ± 4.9 nmol/kg) was 3.3-fold lower than in ghrelin+/+ mice (35.9 ± 17.5 nmol/kg).

To investigate whether the octanoyl modification of ghrelin on Ser3 is essential for the orexigenic properties of ghrelin, both genotypes were injected with a dose of des-octanoyl ghrelin that was close to the maximal effective dose of ghre-
lin. Des-octanoyl ghrelin did not stimulate food intake [cumulative food intake after 6 h (Δ NaCl)] in ghrelin+/+ mice (30 nmol/kg: ghrelin, 0.53 ± 0.09 g versus des-octanoyl ghrelin, 0.00 ± 0.11 g) or in ghrelin+/− mice (15 nmol/kg: ghrelin, 0.35 ± 0.15 g versus des-octanoyl ghrelin, −0.27 ± 0.14 g).

**RQ and HP**

To obtain more information on the proportion of fat and carbohydrate oxidation, CO₂ production and O₂ consumption were determined. From their ratio, the RQ was obtained,
whereas HP was calculated as described under Materials and Methods.

No differences in RQ value were found between both genotypes during the light period (ghrelin$^{+/+}$, 1.02 ± 0.008; ghrelin$^{-/-}$, 1.00 ± 0.007; two groups of five mice for each genotype, age 23 weeks), but during the dark period, the RQ value of the ghrelin$^{-/-}$ mice (1.06 ± 0.008) was significantly ($P < 0.001$) lower than that of the ghrelin$^{+/+}$ mice (1.10 ± 0.009) (Fig. 8A). This effect disappeared with aging because in old mice the RQ value did not differ between both genotypes either during the light (ghrelin$^{+/+}$, 0.98 ± 0.007 versus ghrelin$^{-/-}$, 1.02 ± 0.008; age 54 weeks) or the dark phase (ghrelin$^{+/+}$, 1.02 ± 0.004 versus ghrelin$^{-/-}$, 1.06 ± 0.008; two groups of five mice for each genotype; Fig. 8C).

The HP of the young ghrelin$^{-/-}$ mice was significantly higher than the HP of the ghrelin$^{+/+}$ mice both during the light (ghrelin$^{+/+}$, 23.67 ± 0.30 kcal/kg × h; ghrelin$^{-/-}$, 35.16 ± 1.25 kcal/kg × h; $P < 0.01$) and dark phase (ghrelin$^{+/+}$, 26.85 ± 0.30 kcal/kg × h; ghrelin$^{-/-}$, 41.14 ± 1.11 kcal/kg × h; $P < 0.01$) (Fig. 8B). This was not related to a difference in food consumption. In contrast to the young mice, the HP of the old ghrelin$^{-/-}$ mice was not significantly different from that of old ghrelin$^{+/+}$ mice (Fig. 8D).

**Gastric Emptying Studies**

**Gastric Emptying of Young and Old Mice.** To investigate the gastropokinetic effects of ghrelin, gastric emptying in both young (age 15 weeks) and old (age 60 weeks) ghrelin$^{-/-}$ mice was analyzed. Figure 9 shows the average $^{14}$CO$_2$ excretion curves obtained from both genotypes. No significant differences in gastric emptying parameters between mice of both genotypes were observed. The $t_{\text{half}}$ value was 90.72 ± 5.71 min for the ghrelin$^{+/+}$ mice and 91.90 ± 2.03 min for the ghrelin$^{-/-}$ mice, whereas the $t_{\text{lag}}$ value was 42.17 ± 1.93 and 39.71 ± 3.02 min, respectively ($n = 6$ for each genotype). In addition, in old mice, no difference in gastric emptying parameters was observed between the two genotypes; however, $t_{\text{half}}$ (ghrelin$^{+/+}$ mice, 66.60 ± 5.76 min; ghrelin$^{-/-}$ mice, 66.00 ± 3.00 min; $n = 8$–9 for each genotype) and $t_{\text{lag}}$ (ghrelin$^{+/+}$, 35.20 ± 3.51 min; ghrelin$^{-/-}$, 31.02 ± 1.62 min) were significantly shorter than in the young mice, indicating that gastric emptying was accelerated in older animals.

**Effect of Exogenous Administration of Ghrelin on Gastric Emptying.** Peripheral injection of ghrelin significantly accelerated gastric emptying at a dose of 30 and 75
nmol/kg in young (age 25 weeks) ghrelin\(^{-/-}\) mice (\(n = 8\)). This is reflected in a decrease of \(t_{\text{half}}\) of 11.7 ± 2.8% (\(P < 0.01\)) and 17.1 ± 4.2% (\(P < 0.001\)) and of \(t_{\text{lag}}\) of 24.9 ± 2.7% (\(P < 0.001\)) and 20.3 ± 4.7% (\(P < 0.01\)), respectively. Because the higher dose (125 nmol/kg) had no effect, the dose-response curve for ghrelin was bell-shaped. From the fitting, it was estimated that the maximum effect for \(t_{\text{half}}\) occurs at 57.25 ± 27.04 nmol/kg (Fig. 10A) and for \(t_{\text{lag}}\) occurs at 51.73 ± 23.73 nmol/kg (Fig. 10B).

A similar bell-shaped dose-response relationship to exogenous ghrelin was observed in age-matched ghrelin\(^{-/-}\) mice (\(n = 8\)). The maximal effect was estimated to occur for \(t_{\text{half}}\) at 48.72 ± 25.27 nmol/kg and for \(t_{\text{lag}}\) at 54.71 ± 30.17 nmol/kg (Fig. 10).

### Discussion

In this study, ghrelin\(^{-/-}\) mice were generated to investigate the role of endogenous ghrelin in the regulation of the energy balance and gastric emptying. Despite the fact that ghrelin and its receptor are widely expressed in the brain and in several peripheral tissues and that numerous studies have demonstrated an effect of exogenous ghrelin on food intake, fat utilization, growth hormone secretion, and gastric motility (Murray et al., 2003), our study shows that neither young nor old ghrelin\(^{-/-}\) mice show major phenotypic abnormalities. The knockout mice are not dwarf and show normal increases in body weight gain and food intake. This is in agreement with previous studies performed in young mice only (Sun et al., 2003; Wortley et al., 2004). These results suggest that ghrelin is not a critical endogenous orexigenic factor and/or that other mechanism(s) compensates for the loss of ghrelin. It has been demonstrated that ghrelin activates arcuate NPY/AgRP neurons by binding to presynaptic terminals of NPY neurons to increase the secretion of NPY, AgRP, and GABA. This altered neuropeptide secretion then modulates the activity of postsynaptic secondary order neurons in the paraventricular nucleus, the dorsomedial nucleus, and the lateral hypothalamic area (e.g., orexin neurons) to stimulate food intake, whereas activation of proopiomelanocortin neurons by GABA inhibits the anorectic melanocortin signaling pathway (Dickson and Luckman, 1997; Kamegai et al., 2000; Nakazato et al., 2001; Cowley et al., 2003; Seoane et al., 2003). Lack of response to feeding stimulation by ghrelin in AgRP\(^{-/-}\), NPY\(^{-/-}\) and melanocortin 3 receptor\(^{-/-}\), melanocortin 4 receptor\(^{-/-}\) double knockout mice, and NPY\(^{-/-}\) and AgRP\(^{-/-}\) single knockout mice confirmed that AgRP and NPY are obligatory mediators of the orexigenic effect of ghrelin and implies that inhibition of melanocortin signaling is required for this effect (Chen et al., 2004). Others have shown that ghrelin-induced feeding was also suppressed in orexin knockout mice, thereby linking the ghrelin pathway to orexin neurons (Toshinai et al., 2003). Thus, fine-tuning of these pathways or redundant inputs from non-AgRP and non-NPY pathways may compensate for the loss of ghrelin. It is interesting to note that single or double knockout models of other orexigenic peptides such as NPY and AgRP do not show obvious feeding or body weight deficits (Erickson et al., 1996; Qian et al., 2002). In fact, among the generally accepted orexigenic factors, only deletion of promelanin-concentrating hormone resulted in hypophagia and reduced body weight (Shimada et al., 1998). The lack of anticipated feeding phenotypes when orexigenic fac-
tors are inactivated may reflect a greater degree of redundancy in pathways responsible for stimulating and sustaining feeding behavior than in pathways signaling satiety, such as leptin.

However, our study showed that when the normal light/dark cycle was interrupted by turning the lights artificially off, food intake in old ghrelin+/– but not in ghrelin–/– mice was stimulated. This difference was absent in the young ghrelin–/– mice. Enhanced ghrelin signaling or a sudden increase in plasma ghrelin levels could trigger this event. Sanchez et al. (2004) showed a sharp rise in blood ghrelin levels just before the onset of the dark period, which is the active feeding period of rodents. Together with our findings, this suggests that ghrelin may function as an endogenous meal-initiating signal that is triggered by the dark/light cue. The fact that this effect does not occur in young animals suggests the involvement of compensatory mechanisms that are lost during aging. Also in humans, it was shown (Cummings et al., 2001) that ghrelin plays a role as a meal initiator because plasma ghrelin levels of healthy volunteers increase before the meal. However, this seemed to be independent of time- or food-related cues (Cummings et al., 2004).

Recent studies in GHS-R-null mice showed that the effect of ghrelin on GH secretion and on food intake is mediated by the GHS-R (Sun et al., 2004). To elucidate whether the ghrelin/GHS-R pathway was still functional in the absence of ghrelin, the effect of exogenous administration of ghrelin on food intake was evaluated. In both genotypes, ghrelin stimulated food intake with a bell-shaped dose-response relationship. There are many reasons for bell-shaped dose-response curves. Desensitization is one of the possibilities, especially because the ghrelin receptor is susceptible to rapid desensitization (Orkin et al., 2003; Camina et al., 2004). Another possibility is that, at higher doses of ghrelin, anorectic pathways are activated as a kind of feedback mechanism. In ghrelin–/– mice, the dose-response curve was shifted toward lower concentrations and had a more narrow activity range. This suggests an up-regulation and/or a change in sensitivity of the GHS-R involved in the regulation of food intake, but it may also suggest that the receptor is more vulnerable to desensitization.

To gain more insight in the metabolic characteristics of the ghrelin–/– mice, RQ and HP were determined by using indirect calorimetry. Because no differences in food consumption were observed, gross energy intake was similar for both genotypes. There are no indications (e.g., absence of difference in gastric emptying rate) that the metabolizability of gross energy might be different between these genotypes; hence, a similar metabolizable energy intake can be inferred. This energy can be retained in the body or alternatively must be dissipated as heat. Thus, the total amount of heat produced plus the amount of energy retained must equal metabolizable energy intake. Because young ghrelin–/– mice have a higher heat production compared with the ghrelin+/– mice, it follows that ghrelin–/– mice must retain a lower amount of the metabolized energy. Given their similar body weights, the lower energy retention suggests that the ghrelin–/– mice have a leaner body composition (higher lean-to-fat ratio). Another group (Wortley et al., 2004) indeed observed a tendency toward a leaner body composition in ghrelin–/– mice compared with normal mice, although only when fed on a high-fat diet. Sun et al. (2003) did not find any changes in body composition between ghrelin–/– and ghrelin+/– mice fed on a standard diet, although it should be pointed out that this study was performed in 8-week-old mice. Our study clearly indicates that age-related changes may play an important role since changes in RQ and heat production were observed in 23- but not in 54-week-old ghrelin+/+ and ghrelin–/– mice, suggesting that the role of ghrelin dampens with age. Supportive for our hypothesis of a leaner body composition is that in the fed state young ghrelin–/– mice have a lower RQ than the corresponding wild-type littermates. Indeed, an RQ value above unity is indicative for de novo fatty acid synthesis (Ferrannini, 1988). Because the RQ values of the ghrelin–/– mice did not exceed unity to the same extent as the ghrelin+/+ mice, a relatively lower de novo lipogenesis can be inferred in the ghrelin–/– mice. This supports previous reports on the adipogenic effects of exogenous ghrelin in mice (Tschop et al., 2000).

The effect of genetic deletion of ghrelin on gastric emptying was evaluated by using the [14C]octanoic breath test, as described and validated by Kitazawa et al. (2005). No changes in gastric emptying parameters t_{1/2} and t_{max} between young or old ghrelin+/+ and ghrelin–/– mice were found. This may suggest that endogenous ghrelin does not play a critical role in gastric emptying or that the effect of ghrelin is compensated by other mechanisms. Exogenous ghrelin accelerated gastric emptying in ghrelin–/– mice in a bell-shaped, dose-dependent manner, but in contrast to the effect on food intake no shift in the dose-response curve of the ghrelin–/– mice was observed. This suggests that the effects on food intake and gastric emptying are mediated via different pathways. Previous studies have shown that the starvation signal and the effect on motility by ghrelin are relayed to the brain via vagal afferents (Masuda et al., 2000; Date et al., 2002; Fujino et al., 2003), whereas the effect on motility may also depend upon activation of peripheral receptors (Depoortere et al., 2003, 2005; Kitazawa et al., 2005; Xu et al., 2005). Selective up- and/or down-regulation of central and peripheral receptors may determine the final effect of exogenous ghrelin on food intake and gastric emptying.

In conclusion, ghrelin is not a critical endogenous factor or has a redundant role in the regulation of food intake and gastric emptying in young and old mice. Instead, the primary orexigenic effect of ghrelin in old mice may be to function as an endogenous meal-initiating signal, and this effect is triggered by the light/dark cue. Metabolism studies revealed that in young animals, ghrelin is likely to be involved in the preference of metabolic fuel oxidation and in the partitioning of metabolizable energy between storage and dissipation as heat, leading to an altered body composition.

References
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