Energy Homeostasis and Gastric Emptying in Ghrelin Knockout Mice


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Abbreviations: GH, growth hormone; GHRP-6, growth hormone releasing peptide 6; GHS-R, growth hormone secretagogue receptor; RQ, respiratory quotient; HP, heat production; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, proopiomelanocortin; GABA, gamma-amino butyric acid, MC3/4R, melanocortin 3/4 receptor
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 24h 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 RQ, while 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 appears to be involved in the selection of energy stores and in the partitioning of metabolisable energy between storage and dissipation as heat.
INTRODUCTION

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 ghrelin’s GH releasing effects (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 man (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 man and is able to overcome postoperative ileus (Tack et al., 2005; Trudel et al., 2002; De Winter et al., 2004) in
a dose-dependent manner. Also peptide (e.g. GHRP-6 (H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$)) and non-peptide (e.g. capromorelin (2-amino-N-[(1R)-1-[(3aR)-3a-benzyl-2,3,3a,4,6,7-hexahydro-2-methyl-3-oxo-5H-pyrazolo[4,3-c]pyridin-5-yl]carbonyl]-2-benzyloxy)ethyl]-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 man (Fujino et al., 2003; Tack et al., 2004). In man the effect is not mediated through the release of motilin (Tack et al., 2004).

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 $^{14}$C octanoic breath test, between the two genotypes. In addition the effect of exogenous administration of ghrelin on food intake and gastric emptying was determined in ghrelin$^{+/+}$ and ghrelin$^{-/-}$ mice in order to elucidate whether the ghrelin/GHS-R pathway was still functional.
MATERIALS AND METHODS

Animals

All animals (male) were housed in a temperature-controlled environment (20-22°C) under a 13h-11h light-dark cycle with lights on at 7 a.m. and lights off at 8 p.m. Young animals were 15 to 25 weeks old, old mice were 50 to 65 weeks old. Standard commercial mouse chow (4352 Muracon G, Nutreco Belgium NV, Belgium) (4.5% fat, 4% cellulose, 21% proteins, 1.404 kcal/g) and tap water was available ad libitum. The Ethical Committee for Animal Experiments of the Catholic University of Leuven approved all experiments.

Generation of ghrelin⁻/⁻ mice

Ghrelin knockout (ghrelin⁻/⁻) mice were developed by Lexicon Genetics Incorporated (The Woodlands, TX). A genomic clone spanning 12.4 kb of the ghrelin gene including exons 2 to exon 4, isolated by screening of the 129SvEvBrd derived lambda pKOS genomic library (Wattler et al., 1999), 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 129Sv/Evbrd(LEX1) embryonic 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(albino) blastocysts, and the resulting chimeras were mated to C57BL/6(albino) females to generate heterozygous animals. These were subsequently crossed to generate all three genotypes employed in the resorted studies. PCR was used to screen genotypes by using DNA isolated from mouse tail biopsy samples. Primers 5’CCTGGCAGACAGACGACCTG3’ and 5’CAGGTCAGTCAAGTCTGTCTC’ amplified a 842bp band from the wild type allele while
primers 5’CCTGGCAGACAGACCCATG3’ and 5’GCAGCGCATCGCCTTCTATC3’ amplified a 630bp 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 total RNA using random hexamer primers and SuperscriptII RT (Invitrogen, Carlsbad, CA). Quantitative PCR was performed on a ABI Prism 7700 cycler (Applied Biosystems, Foster City, CA) using a Taqman PCR kit. Serial dilutions of cDNA were used to generate standard curves of threshold cycles versus the logarithms of concentration for ß-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’GGCAGGCTCCAGCTTCT3’ primer 5’TGGCTTCTTGGATTCCCTTCTC3’, probe 5’ AGCCCAAGGCACGAAAGCCCA3’ [5’]FAM [3’]TAMRA) relative to actin (primer 5’ CATCTTGGCCTCAGCTCCAC3’, primer 5’ GGGCGGACTCATCGTACT3’, probe 5’ TGCTTGCTGATCCACATCTGGA3’ [5’]FAM [3’]TAMRA) was used to assess expression levels.

**Immunohistochemistry**

Mice were deeply anaesthetized (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 2h in 0.1M PBS containing 4% goat serum, 0.5% Triton-X-100 and 0.3% NaN3 at 4°C. After incubation overnight with the rabbit anti-ghrelin antibody (kindly provided by Dr. Tomasetto, Strasbourg, France; dilution 1:500), sections were washed and incubated with FITC-conjugated goat anti-rabbit IgG (dilution 1:50) (Jackson ImmunoResearch Lab, PA, USA) for 2h at 4°C. After 3 washes of 5 min in 0.1M PBS, the sections were mounted with Citifluor (Citifluor, UK).
**Body weight**

Body weight was measured every week or every two weeks (except between week 32 and 41) between 10 and 12 a.m. from week 12 until week 75 after birth. Initially these data were fitted according to 3 different growth models: the Bertalanffy model (Di Masso et al., 1990), the Gompertz model (Kidwell 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

\[ Y = Ae^{-(Be^{-Kt})} \]  

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

**Food intake**

Food intake in young mice during the light period (13h) and the dark period (11h) was measured daily for 1 week.

Food intake analysis during 6h in ad libitum fed mice was started at 10 a.m. Each mouse was put in an individual cage, where it received a pre-weighed amount of food. Food intake was measured by subtracting the uneaten food at 30 min, 1, 2, 3, 4, 5 and 6 hours. 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 saline (0.9%) or 1, 6, 15, 30, 50 or 75 nmol/kg ghrelin (Global Peptide Services, Colorado, USA) or 15 or 30 nmol/kg des-octanoyl ghrelin (kindly provided by Dr Robberecht, ULB, Brussels, Belgium) was injected intraperitoneally immediately before the mice received the food. Mice were sensitized to intraperitoneal injections before the start of the experiment. These results are presented as the difference in cumulated food intake after 6h between ghrelin or des-octanoyl ghrelin and saline injected mice as a function of the administered dose.
Indirect calorimetry

Respiratory quotient (RQ) and heat production (HP) were measured for 24 hours 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 analyser 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 Pt resistance temperature detector (Pt-100, Farnell In One, Grace-Hollogue, Belgium) (accuracy of 0.2°C). The paramagnetic O₂ analyser (ADC 02-823A) and the infrared CO₂ analyser (ADC D/8U/54/A) were calibrated before each measurement by using gas standards.

Mice were placed in the respiratory cells 24h before the start of the experiment. After the adaptation period, gas exchanges (CO₂ and O₂) were measured continuously during 24h for 4 consecutive days. Mice had free access to food and water and 24h 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 minutes during 24h. 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).

\[ \text{Heat production (kJ/h)} = 16.18 \text{ O}_2 (l/h) + 5.02 \text{ CO}_2 (l/h) \] (2)

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 (19h, free access to water) mice were injected intraperitoneally, if applicable, with saline (0.9%) or ghrelin (1, 30, 75 and 125 nmol/kg).
A baseline breath sample (5 min) was taken 23 min after the injection and 30 min later the $^{14}$C 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 two weeks (two or three days 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 3.5h. From the $^{14}$CO$_2$ excretion curve 2 parameters $t_{\text{half}}$ (time at which 50% of the total amount of $^{14}$CO$_2$ was excreted) and $t_{\text{lag}}$ (initial delay in gastric emptying due to the time required for the stomach to grind the meal into fine particles) were calculated as described earlier (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 2 consecutive breath tests with increasing doses of ghrelin and again a control breath test. The effect of ghrelin was compared to 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, 24h 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 ANOVA analysis, with one repeated measures factor (time). The effect of exogenous ghrelin on food intake and on gastric emptying was analyzed by two-way ANOVA analysis, with two repeated measures 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, Inc, 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 (GraphPad Prism 4.0 Software, 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 (Figure 1A). The targeting vector consisted of 2.6kb and 4.0kb 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 (Figure 1B). Loss of expression of the ghrelin transcript in the knockout 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−/− (Figure 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 (Figure 2A), while no immunoreactivity for ghrelin could be observed in sections from the ghrelin−/− mice (Figure 2B).

Body weight and food intake studies

Evolution of body weight

Body weight (n=11 for each genotype) was followed between 12 to 75 weeks of age (Figure 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). Also the
intrinsic growth rate parameter was the same for both genotypes (ghrelin<sup>+/+</sup>: 0.10±0.01 versus ghrelin<sup>−/−</sup>: 0.09±0.02) However as the two curves appeared to diverge with increasing age, mice were divided for further observations into two subgroups, young (15-25 weeks) and old (50-65 weeks) mice.

**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<sup>−/−</sup> mice during the 13h light period and the 11h dark period amounted to 1.18±0.08 g and 3.87±0.14 g respectively, and did not differ significantly from that of ghrelin<sup>+/+</sup> mice (1.25±0.07 g and 3.93±0.10 g) (Figure 4A). Also food intake in old ghrelin<sup>−/−</sup> mice did not differ from that of the wild-type littermates both during the light (ghrelin<sup>+/+</sup>: 0.67±0.10 g vs ghrelin<sup>−/−</sup>: 0.80±0.11 g) and the dark period (ghrelin<sup>+/+</sup>: 4.05±0.16 g vs ghrelin<sup>−/−</sup>: 4.32±0.15 g) (Figure 4B).

*Artificial light cycle phase*

Cumulative food intake followed during 6h at times of minimal feeding (10 a.m.-4 p.m.) in old ghrelin<sup>−/−</sup> mice fed ad libitum did not differ significantly from the food intake of the age-matched ghrelin<sup>+/+</sup> mice (Figure 5A). However, when mice were artificially stimulated to eat food by turning the lights off at 10 a.m. for 6h, cumulative food intake during the first 4h was significantly increased in the old ghrelin<sup>+/+</sup> mice but not in the ghrelin<sup>−/−</sup> mice (Figure 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 (Figure 5C).

This effect was only apparent in the aged group as interruption of the light-dark cycle did not affect cumulative food intake in young mice (Figure 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 cumulated food intake after 6h 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 (Figure 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 Ser\(^3\) is essential for ghrelin’s orexigenic properties, both genotypes were injected with a dose of des-octanoyl ghrelin that was close to the maximal effective dose of ghrelin. Des-octanoyl ghrelin did not stimulate food intake (cumulated food intake after 6h (\(\Delta\) NaCl)) nor in ghrelin\(^{+/+}\) mice (30 nmol/kg: ghrelin: 0.53±0.09 g vs des-octanoyl ghrelin: 0.00±0.11 g), nor in ghrelin\(^{-/-}\) mice (15 nmol/kg: ghrelin: 0.35±0.15 g vs des-octanoyl ghrelin: -0.27±0.14 g).

Respiratory quotient (RQ) and heat production (HP)

In order to obtain more information on the proportion of fat and carbohydrate oxidation, CO\(_2\) production and O\(_2\) consumption were determined. From their ratio the RQ was obtained, while HP was calculated as described in the Methods section.

No differences in RQ value were found between both genotypes during the light period (ghrelin\(^{+/+}\): 1.02±0.08, ghrelin\(^{-/-}\): 1.00±0.007, 2 groups of 5 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) (Figure 8A). This effect disappeared with aging because in old mice the RQ value did not differ between both
genotypes neither during the light (ghrelin+/+: 0.98±0.007 versus ghrelin−/−: 1.02±0.008, age: 54 weeks) nor during the dark phase (ghrelin+/+: 1.02±0.004 versus ghrelin−/−: 1.06±0.008) (2 groups of 5 mice for each genotype, Figure 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) (Figure 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 (Figure 8D).

**Gastric emptying studies**

**Gastric emptying of young and old mice**

To investigate the gastroprokinetic 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 14CO2 excretion curves obtained from both genotypes. No significant differences in gastric emptying parameters between mice of both genotypes were observed. Half-excretion time (t_{half}) was 90.72±5.71 min for the ghrelin+/+ mice and 91.90±2.03 min for the ghrelin−/− mice, while t_{lag} was 42.17±1.93 min and 39.71±3.02 min (n=6 for each genotype). Also in old mice no difference in gastric emptying parameters was observed between the two genotypes but t_{half} (ghrelin+/+ mice: 66.60±5.76 min, ghrelin−/− mice: 57.80±3.00 min, n=8-9 for each genotype) and t_{lag} (ghrelin+/+: 35.20±3.51 min, ghrelin−/−: 31.02±1.62 min) was 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
$t_{\text{half}}$ with 11.7±2.8% (P<0.01) and 17.1±4.2% (P<0.001) and of $t_{\text{lag}}$ with 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 (Figure 10A) and for $t_{\text{lag}}$ at 51.73±23.73 nmol/kg (Figure 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 (Figure 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 knock-out mice are not dwarf, 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) compensate 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 POMC 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 MC3R-/-;MC4R-/- double knockout mice as well as in NPY-/- and AgRP-/- single knockout mice confirmed that AgRP and NPY are obligatory mediators of the orexigenic effect of ghrelin and imply 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 pro-melanin-concentrating hormone resulted in hypophagia and reduced body weight (Shimada et al., 1998). The lack of anticipated feeding phenotypes when orexigenic factors 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 towards 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 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 metabolisability of gross energy might be different between these genotypes, hence a similar metabolisable 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 metabolisable energy intake. As young ghrelin−/− mice have a higher heat production compared to 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 towards a leaner body composition in ghrelin−/− mice compared to normal mice, though 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 weeks 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 weeks 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 wildtype littermates. Indeed, an RQ value above
unity is indicative for de novo fatty acid synthesis (Ferrannini, 1988). As 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_{half} and t_{lag}, 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 ghrelin’s effect 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 ghrelin’s starvation signal and the effect on motility are relayed to the brain via vagal afferents (Masuda et al., 2000; Date et al., 2002; Fujino et al., 2003), while the effect on motility may also depend upon activation of peripheral receptors (Depoortere et al., 2003; Depoortere et al., 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 metabolisable energy between storage and dissipation as heat, leading to an altered body composition.
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FIGURE LEGENDS

**Figure 1:** (A) Targeted disruption of the ghrelin gene. Structure of the wild type allele, targeting vector and recombinant locus. Black boxes represent exons, ATG indicates the startcodon in exon 2. (B) PCR analysis of mouse genomic DNA. The wild type (left panel) and targeted (right panel) allele give a 842 and 791 bp PCR product respectively and identify ghrelin^{+/+} (lane 1), ghrelin^{-/-} (lane 2) and ghrelin^{+/+} (lane 3) animals (C) Expression of the ghrelin transcript in stomach, jejunum, brain and blood was abolished in the homozygous knockout mouse as determined by quantitative RT-PCR.

**Figure 2:** Immunohistochemical staining for ghrelin in sections of the mouse oxyntic gland in ghrelin^{+/+} (A) and ghrelin^{-/-} mice (B).

**Figure 3:** Evolution of body weight of 12 to 75 weeks old ghrelin^{+/+} (■) and ghrelin^{-/-} (▲) mice. Data were fitted to the Gompertz model, which is represented by the line. Results are mean±SEM of 11 mice from each genotype. No significant differences between ghrelin^{+/+} and ghrelin^{-/-} mice were found.

**Figure 4:** 24h food intake during normal light cycle conditions (13h light, 11h dark) in young (A) and old (B) mice. The amount of chow consumed did not differ between ghrelin^{+/+} (filled column) and ghrelin^{-/-} (open column) mice. Each column is the mean±SEM of at least 5-7 mice from each genotype.

**Figure 5:** Cumulative (A, B) and periodic (C) food intake in ad libitum fed old ghrelin^{+/+} (■) and ghrelin^{-/-} (▲) mice during 6 h of minimal feeding (10 a.m. – 4 p.m.) with lights on (A) and lights artificially turned off (B, C). Results are the mean±SEM of 6-9 mice from each genotype. * : P<0.05, ** : P<0.01; *** : P<0.001 indicate significant differences between ghrelin^{+/+} and ghrelin^{-/-} mice.
Figure 6: Cumulative food intake followed during 6h in young ad libitum fed ghrelin\(^{+/+}\) (●) and ghrelin\(^{-/-}\) (▲) mice when lights were artificially turned off between 10 a.m. and 4 p.m.. No significant differences between ghrelin\(^{+/+}\) and ghrelin\(^{-/-}\) mice were found.

Figure 7: Dose-dependent effects of ghrelin on food intake in young ghrelin\(^{+/+}\) (●) and ghrelin\(^{-/-}\) (▲) mice. Mice were injected intraperitoneally with saline or increasing doses (1-75 nmol/kg) of ghrelin and the effect on food intake was followed. Results are expressed as the cumulated food intake after 6 hours and represent the difference between saline and ghrelin injected mice. Results are the mean±SEM of 6 mice from each genotype. * : P<0.05, ** : P<0.01; *** : P<0.001 indicate a significant increase compared to saline treatment.

Figure 8: Respiratory quotient (RQ) (A, C) and heat production (HP) (B, D) measured during 24h in ad libitum fed young (A,B) and old (C,D) ghrelin\(^{+/+}\) (●) and ghrelin\(^{-/-}\) (▲) mice using an open circuit indirect calorimetric system. Results are the mean±SEM of 2 groups of 5 individuals for each genotype. Only a significant difference in RQ (lights-off period) (P<0.001) and in HP (P<0.01) between young ghrelin\(^{+/+}\) and ghrelin\(^{-/-}\) mice was found.

Figure 9: Gastric emptying in young (A) and old (B) ghrelin\(^{+/+}\) (●) and ghrelin\(^{-/-}\) (▲) mice as determined by the \(^{14}\)CO\(_2\) octanoic breath test. Results show typical CO\(_2\) excretion curves obtained after ingestion of a solid meal enriched with \(^{14}\)C octanoic acid. No difference in the gastric emptying parameters, t\(_{\text{half}}\) and t\(_{\text{lag}}\), were found between the two genotypes although emptying was accelerated in the old mice. Results are the mean±SEM of 6-9 mice from each genotype.

Figure 10: Dose-dependent effects of ghrelin on gastric emptying in young ghrelin\(^{+/+}\) (●) and ghrelin\(^{-/-}\) (▲) mice. Mice were injected with saline or increasing doses of ghrelin (1-125 nmol/kg) 30 min before a solid meal enriched with \(^{14}\)C octanoic acid was ingested. The effect on gastric emptying parameters t\(_{\text{half}}\) (A) and t\(_{\text{lag}}\) (B) was determined. Results are expressed as a percentage decrease in time compared with the injection of saline before and after the
injection of ghrelin. Results are the mean±SEM of 6 mice from each genotype. * : P<0.05, ** : P<0.01; *** : P<0.001 indicate a significant decrease compared with saline treatment.
Figure 1

A

Wild type allele

Targeting vector

Recombinant allele

ATG

IRES LacZ MC1Neo

B

M 1 2 3

M 1 2 3

C

Fold change

stomach +/+ stomach +/- stomach +/- jejunum +/+ jejunum +/- jejunum +/- brain +/+ brain +/- brain +/- blood +/+ blood +/- blood +/-
Figure 2
Figure 4

A

B

Food intake (g)

+/

+/−

light

dark

light

dark

Food intake (g)
Figure 5

A

Cumulative food intake (g)

Time (h)

B

Cumulative food intake (g)

Time (h)

C

Periodic food intake (g/h)

Time (h)

[Graphs showing cumulative and periodic food intake over time for different genotypes (+/+ and -/-).]
Figure 6

Cumulative food intake (g)

Time (h)

+/-

-/-
Figure 7
Figure 9

A

\[ {}^{14}\text{CO}_2 \text{-excretion (\%dose/h)} \]

Time (min)

B

\[ {}^{14}\text{CO}_2 \text{-excretion (\%dose/h)} \]

Time (min)

■ +/+  ▲ -/-
Figure 10

A

B

$T_{\text{half}}$ (% decrease)

Dose (nmol/kg)

$T_{\text{last}}$ (% decrease)

Dose (nmol/kg)

[Graphs showing dose-response relationship with symbols for +/- and -/-]