Mice with Hyperghrelinemia are Hyperphagic, Glucose Intolerant and have Reduced Leptin Sensitivity.

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ABSTRACT

Objective: Ghrelin is the only known peripheral hormone to increase ingestive behaviour. However, its role in the physiological regulation of energy homeostasis is unclear since deletion of ghrelin or its receptor does not alter food intake or body weight in mice fed a normal chow diet. We hypothesized over expression of ghrelin in its physiological tissues would increase food intake and body weight.

Research Design and Methods: We used bacterial artificial chromosome transgenesis to generate a mouse model with increased ghrelin expression and production in stomach and brain. We investigated the effect of ghrelin over expression on food intake and body weight. In addition, we measured energy expenditure and determined glucose tolerance, glucose stimulated insulin release and peripheral insulin sensitivity.

Results: Ghrelin transgenic mice exhibited increased circulating bioactive ghrelin which was associated with hyperphagia, increased energy expenditure, glucose intolerance, decreased glucose stimulated insulin secretion and reduced leptin sensitivity.

Conclusion: This is the first report of a transgenic approach suggesting ghrelin regulates appetite under normal feeding conditions and provides evidence that ghrelin plays a fundamental role in regulating β-cell function.
Ghrelin is a 28-aa peptide which is expressed at high levels in the stomach. It is the endogenous ligand for the growth hormone secretagogue receptor (GHSR) and increases growth hormone secretion from the pituitary (1). Ghrelin also increases food intake and adiposity suggesting a role in the control of energy homeostasis (2). Consistent with this, plasma ghrelin levels have been shown to increase prior to a meal and during fasting (3). Ghrelin circulates in two forms, the biologically active octanoylated form and the des-octanoyl form which is thought to be biologically inactive (4). Recent data shows that Ghrelin O-Acyltransferase (GOAT) a membrane-bound enzyme is responsible for octanoylation of the serine-3 residue of ghrelin and confers biological activity (5;6).

Despite unequivocal pharmacological data, the evidence for a physiological role for ghrelin in the control of appetite is much less clear. Mice with targeted deletion of either ghrelin or the GHSR exhibit an essentially normal metabolic phenotype when fed a regular chow diet, suggesting ghrelin may have a redundant role in the regulation of food intake (7;8). However, when fed on a high fat diet these mice are resistant to diet induced obesity (DIO), exhibiting reduced adiposity and increased energy expenditure (9;10). More recent data suggests these knockout models are not resistant to DIO when backcrossed to a pure C57BL6 genetic background. Despite this, calorie restriction in the pure bred mice did result in lower blood glucose in both knockout models (11). The conflicting food intake and body weight data from transgenic models has made defining a key role for endogenous ghrelin in the control of appetite difficult. However, the data does consistently suggest ghrelin may be important in the control of glucose homeostasis.

Ghrelin gain-of function models have not produced the expected hyperphagic and obese phenotype (12-14). However, these models did not exhibit increases in plasma bioactive ghrelin. Reed et al. developed a model in which ghrelin was over expressed in the brain but not the stomach (15). In one transgenic line circulating bioactive ghrelin was found to be increased but this was not associated with hyperphagia. The lack of an obese phenotype in these mice was attributed to developmental compensation, alterations in peripheral versus central nervous system ghrelin concentrations and or alterations in diurnal patterns of ghrelin release.

The production of bioactive ghrelin critically depends on its octanoylation by GOAT. In order to physiologically over express bioactive ghrelin the ghrelin transgene must be expressed in tissues which also produce GOAT, the stomach and small intestines. We used the ghrelin promoter to drive ghrelin over expression and generated mice with increased circulating levels of bioactive ghrelin. We then investigated the phenotype of these mice.

RESEARCH DESIGN AND METHODS
Generation of ghrelin Tg mice. We identified a BAC containing the ghrelin gene RP23-441K11 (Invitrogen, Huntsville, AL, USA). Tg mice were created using standard pronuclear injection techniques. F0 mice were mated with CBA/C57Bl6 mice and transgenic lines were maintained separately. Mice were maintained in cages under controlled temperature (21-23°C) and light (eleven hours light/thirteen hours dark) with ad libitum access to food (RM1 diet, SDS UK Ltd.) and water. Animal procedures performed were approved under the British Home Office Animals Scientific Procedures Act 1986.

Body weight, food intake, indirect calorimetry and body composition. Mice were singly housed from weaning and food
intake and body weight measured. Body composition of sixteen week old mice was calculated using the method of Salmon and Flatt (16). Metabolic parameters were obtained using the open-circuit Oxymax comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH) as previously described (17).

**Glucose tolerance test and Insulin tolerance test.** An intraperitoneal glucose tolerance test (IP-GTT) was performed in conscious sixteen week old mice. Following an 18hr fast D-glucose (2g/kg) was administered i.p. and blood glucose was measured by tail bleeds at 0, 15, 30, 60, 120 and 150 post glucose administration. For glucose stimulated insulin release blood glucose and insulin levels were measured at 0, 15, 30 and 60 mins post glucose. The insulin tolerance test (ITT) was performed similarly except mice were fasted for 4 hours prior to i.p. administration of Humulin (1.5U/Kg). Plasma glucose was measured using the Accesia Contour blood glucose monitoring system (Bayer Health Care, UK).

**Measurement of circulating hormones.** Whole blood was collected by cardiac puncture from fasted 16 week old mice. Mouse plasma insulin and leptin concentrations were determined using reagents and methods from Crystal Chem Inc. (Downers Grove, IL). Plasma corticosterone was measured using a radioimmunoassay kit from MP Biomedicals, Inc. (Orangeburg, NY). Plasma IGF-I levels were measured using reagents and methods from Immunodiagnostic Systems Ltd. (Bolden, UK). Octanoylated ghrelin concentrations were analysed using an elisa kit from LINCO research (St. Charles, Missouri).

**Northern blot analysis and quantitative PCR.** Tissues from 16 week old mice were snap frozen and RNA extracted using TRI reagent. Northern blot analysis was used to determine UCP-1 mRNA expression in BAT and ghrelin mRNA expression in stomach. Real-time quantitative PCR analysis was performed using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using the ABI Prism 7900 Sequence Detection System according to the protocols provided by the manufacturer (Applied Biosystems, Melbourne, Australia). The relative mRNA transcript levels were calculated according to the $2^{-\Delta\Delta CT}$ method, with $\Delta\Delta CT$ being the difference in cycle threshold values between the target mRNA and the 18S internal control.

**Peripheral administration of ghrelin and PYY 3-36.** At 16 weeks of age ad libitum fed mice were injected i.p. with either saline or ghrelin (0.3nmol/g) in a randomised blinded cross-over design. A recovery period of two days was allowed between each study day. Mice were injected i.p. in the early light phase at 09:00h. Food intake was measured at 1 hour post injection. At 16 weeks of age, in a randomised blinded cross-over design, mice were injected i.p. with either saline or leptin (3μg/g). Before each study day mice were fasted for twenty-four hours and leptin was administered at 09:00, food intake was measured at 1 and 4 hours post injection.

**Statistical analysis.** Values are the mean ± sem unless otherwise stated. Differences in cumulative food intake through time were compared across experimental groups using generalized estimating equation curve analysis (Stata 9.1; Statacorp, College Station, TX). For analysis of the effect of peripheral administration of ghrelin and leptin a paired student’s $t$ test with a Bonferroni’s correction was used. All other comparisons were made using an un-paired Student’s $t$ test. $P$ values $< 0.05$ were considered significant.

**RESULTS**

**Circulating bioactive ghrelin levels are increased in transgenic mice.** We identified a bacterial artificial chromosome (BAC) which contained the ghrelin gene and its
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Phenotypic characterisation of ghrelin over expressing mice.

Over expression of ghrelin does not affect the growth hormone axis but inhibits the hypothalamo-pituitary adrenal (HPA) axis. Administration of a single dose of ghrelin is known to stimulate growth hormone release (18). To study the possible effects of ghrelin over expression on the growth hormone axis we measured IGF-1 as a surrogate marker for growth hormone and longitudinal growth. At sixteen weeks of age there were no differences in circulating IGF-1 levels between Tg and Wt littermates (Wt 774±49ng/ml vs Tg 799±80ng/ml). In addition, there was no difference in nose to anus length between genotypes (Wt 7.52±0.09 cm vs Tg 7.57±0.10 cm n=10). Together these data suggest over expression of bioactive ghrelin did not affect the HPA axis; central administration of ghrelin increases corticosterone release whilst intravenous administration inhibits corticosterone release in rats with high basal corticosterone levels (19;20). We observed a significant reduction in corticosterone levels in Tg mice (Wt 77.06±8.05 ng/ml vs Tg 47.3±6.34 ng/ml, n = 12-15, P < 0.05).

Bioactive ghrelin over expression increases both food intake and energy expenditure. Chronic pharmacological administration of ghrelin significantly increases food intake and body weight. However, previous studies of genetic over expression of ghrelin have not produce the expected hyperphagic and obese phenotype. To determine the effects of over expression of bioactive ghrelin on energy homeostasis we monitored food intake and body weight of mice fed on regular chow. Tg mice exhibited hyperphagia compared to wild type controls. Daily food intake was increased in both male and female mice from six weeks of age (males Wt 98.8± 2.2 kj/day vs Tg 107.0±3.3 kj/day, n=6, p< 0.05; females Wt 51.25±1.25 kj vs Tg 56.13±1.9 kj, n=6,
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p<0.05) until the end of the study at sixteen weeks of age (males Wt 99.5 ± 1.2 kj/day vs Tg 114.3±5.0 kj/day n=6 p<0.05; females 54.25±1.75 kj/day vs 61.63±2.13 kj/day n=6 p<0.05, Figure 2A). In accordance with increased daily food intake, cumulative food intake was also increased by 13% between five and sixteen weeks of age in Tg mice (males Wt 7440±68 kj vs Tg 8480±379 kj; females Wt 4087±115 kj vs Tg 4544±148 kj n=6 p<0.05, Figure 2C). This increase in food intake would be expected to significantly increase both weight gain and adiposity. However, the increased energy intake did not result in increased weight gain in either male or female Tg mice (Figure 2B and D). In addition, there was no change in adiposity or lean mass between the two groups (Figure 2E). Consistent with this finding, circulating leptin levels were unaltered between Tg and Wt mice (Wt 2.08±0.68 ng/ml vs. Tg 2.66±0.63 ng/ml, Figure 2F). The hyperphagia without increased body weight was indicative of increased energy expenditure. To determine the mechanism by which Tg mice remained lean in the face of increased food intake we measured uncoupling protein-1 (UCP-1) expression levels in brown adipose tissue (BAT) as a surrogate marker of energy expenditure. UCP-1 expression levels were found to be significantly increased in Tg mice compared to Wt controls (Wt 4.42±0.45 (AU) vs. Tg 5.62±0.5 (AU), Figure 3A) suggesting that energy expenditure was increased in Tg mice. This conclusion was supported by indirect calorimetry which indicated oxygen consumption was increased during both the light and dark cycle (Figure 3B). Energy expenditure was found to be increased by 15% in Tg mice compared to Wt controls (hourly average VO₂ wt 5458±114 ml/hr/kg vs tg 6207±367 ml/hr/kg n=6-8, females p<0.01, Figure 3C). In addition, we measured locomotor activity and found no differences in either dark or light cycle locomotor activity between genotypes (Figure 3D). This suggested the normal body weight observed in the Tg mice was a consequence of increased basal metabolic rate rather than an increase in locomotor activity. Exogenous ghrelin administration has been shown to decrease fat utilization whilst mice with targeted deletion of ghrelin have increased fat utilization when fed on a high fat diet. Our ghrelin over expressing mice did not show an altered respiratory exchange ratio suggesting chronic exposure to ghrelin does not alter nutrient partitioning in our model (Figure 3E). *Bioactive ghrelin over expression decreases glucose stimulated insulin release.* Pharmacological administration of ghrelin inhibits glucose stimulated insulin release whilst both knockout and over expression models of the ghrelin system exhibit changes in glucose homeostasis (15;21;22). Our transgenic mice had similar fasting plasma glucose concentrations to Wt litter mates (Wt 5.1±0.4 mmol/l vs Tg 5.5±0.4 mmol/l, mean±sem n=6). Despite similar plasma glucose levels Tg mice had elevated fasting plasma insulin levels, although this was not statistically significant (Wt 0.37±0.1 ng/ml, n=9, Tg 0.61±0.17 ng/ml, n=5 p=0.2). We carried out intraperitoneal glucose tolerance tests (IP-GTT) to further explore the effects of ghrelin on glucose homeostasis. Transgenic mice were glucose intolerant with significantly increased plasma glucose concentrations at 30, and 60 min (Figure 4A) following glucose (2g/kg) injection compared to Wt controls (AUC Wt 1253±58 vs 1769±158 Tg n=6, p<0.01, Figure 4B). This was indicative of either increased insulin resistance or impaired glucose stimulated insulin release. To determine the mechanism we measured glucose stimulated insulin release and performed insulin tolerance tests (ITT). Following insulin administration there were no differences in plasma glucose concentrations between genotypes (AUC Wt 5301±388 vs Tg 5740±438 n=6-10, Figure 4C.
Ghrelin transgenic mice are glucose intolerant and D). Glucose stimulated insulin release was significantly inhibited in Tg mice during an IP-GTT (glucose and insulin % baseline, Figure 4E and F, insulin AUC Wt 25116±6182 vs. Tg 11202±2136, Figure 4G). These results suggest ghrelin inhibits glucose stimulated insulin release but has no effect on insulin sensitivity.

**Bioactive ghrelin over expressing mice are equally sensitive to exogenous ghrelin but have reduced leptin sensitivity.** To determine if over expression of ghrelin attenuated the response to exogenously administered ghrelin we measured 1 hour food intake following i.p injection of ghrelin (0.3nmol/g). Ghrelin was equally potent at increasing 1 hour food intake in both genotypes (Wt 0.02±0.01g vs 0.11±0.03g saline vs ghrelin mean±sem n=6, p<0.01, Tg 0.03±0.01g vs 0.12±0.03g saline vs ghrelin mean±sem n=6, p<0.01, Figure 5A).

Within hypothalamic feeding centres ghrelin and leptin have been shown to be functional antagonists (23). To determine if ghrelin over expression altered leptin sensitivity we measured the effect of peripherally administered leptin (3μg/g) on food intake. Tg mice were less sensitive to the anorexigenic effect of leptin than Wt controls. Leptin significantly reduced food intake 0-1 hours post administration in Wt but not in Tg mice compared to saline (saline vs leptin Wt 0.54±0.12g vs 0.22±0.05g p<0.05, Tg 0.69±0.15g vs 0.62±0.12g p=ns mean±sem n=6, Figure 5B). Four hours post administration leptin significantly reduced food intake in both genotypes (saline vs leptin Wt 1.08±0.15g vs 0.57±0.12g, Tg 1.33±0.17g vs 0.99±0.12g mean±sem n=6, p<0.05, Figure 5C). However, the magnitude of the reduction in food intake was less in Tg than Wt mice. Leptin reduced 4 hour food intake by approximately half in Wt animals but only by one quarter in transgenic animals. These results suggest Tg mice are less sensitive to the effects of leptin but equally sensitive to ghrelin.

**DISCUSSION**

Bioactivity of ghrelin is conferred by octanoylation of its serine-3 residue. This reaction is catalysed by the enzyme GOAT. Thus bioactive ghrelin is only produced in GOAT expressing tissues. In the mouse, GOAT is expressed exclusively in the gastrointestinal tract (5;6). To increase bioactive ghrelin concentrations, the ghrelin transgene should ideally be expressed in tissues which produce endogenous ghrelin and GOAT. To do this we chose to drive ghrelin transgene expression using its own promoter. The approach successfully increased both stomach and plasma concentrations of bioactive ghrelin. Our data is the first report of the targeted over expression of bioactive ghrelin in its physiological sites of production the stomach and hypothalamus.

Exogenously administered ghrelin has been shown to have powerful effects on food intake (2;24). Despite the overwhelming pharmacological evidence, data from transgenic models has not supported the expected role for ghrelin in the control of appetite, leading to the suggestion that ghrelin is not a critical regulator of appetite. On the contrary our data suggest it is an important regulator of food intake. Over expression of bioactive ghrelin in our model causes hyperphagia, increased energy expenditure and glucose intolerance. The increased food intake suggests ghrelin may physiologically regulate appetite under normal feeding conditions.

The hyperphagia observed in our Tg mice was in contrast to the phenotype described for mice with non-specific neuronal over expression of ghrelin (15). These mice are reported to exhibit an increase in plasma octanoylated ghrelin without an increase in feeding behaviour. A likely explanation for
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this difference is the non-specific nature of the CNS over expression. Results from this model are likely to be pharmacological since physiologically only a few neurones in the hypothalamus produce ghrelin. The hyperphagic phenotype is also in contrast to mice with targeted deletion of the ghrelin gene (ghr\(^{-}\)) or its receptor. These mice have normal growth rates and appetites when fed on regular chow (25). A possible explanation for this is that knockout mice are susceptible to developmental compensation which can mask the true role of the gene in question.

To fully interpret transgenic studies it is important to consider the genetic background of the model. This is particularly true for mice with targeted deletion of genes. In which it has been suggested that c57bl/6j genetic traits co-segregate in the null mice whilst 129Sv traits are more influential in the Wt controls (7). This can lead to misinterpretation of results since c57bl/6j mice are more obesity prone than 129Sv mice. However in classical Tg mice there is no evidence that the transgene co-segregates with a particular background and therefore the Tg and Wt littermates are likely to be of similar genetic background. Backcrossing our model to a pure C57bl/6j background may indeed increase the magnitude of the observed phenotype.

Our Tg mice were hyperphagic but did not have an increase in body weight which suggested they had increased energy expenditure. Tg mice were found to have increased oxygen consumption and BAT UCP-1 expression, both of which are surrogate markers of energy expenditure. This is in contrast to the pharmacological administration of ghrelin which decreases energy expenditure (26). It is possible that the increased metabolic rate in Tg mice is due to an indirect effect of ghrelin which manifests itself following chronic exposure. For example, circulating corticosterone levels were suppressed in our Tg mice. Since corticosterone is known to suppress energy expenditure the increased metabolic rate observed in our Tg mice may be attributed to an indirect effect of their attenuated corticosterone levels (27).

It is generally accepted that ghrelin activates the HPA axis and this is thought to occur at the level of the hypothalamus (19). In contrast corticosterone levels were reduced in our Tg mice. However, when given intravenously, GH-secretagogues (GHS) reduce corticosterone in rats with high basal corticosterone levels (20). It has therefore been suggested that high circulating levels of glucocorticoids feedback to reduce the ACTH response to GHS. A similar mechanism could account for the reduced corticosterone in our Tg mice. Chronic exposure to ghrelin may cause attenuation of the HPA axis. Pharmacologically ghrelin is a powerful growth hormone secretagogue acutely. We found the growth hormone axis of our Tg mice to be normal, IGF-1 levels and linear growth were unaltered between genotype. A potential explanation for this is that the growth hormone axis becomes less sensitive to ghrelin following chronic exposure. In agreement with this others have found that chronic ghrelin or growth hormone secretagogue treatment does not affect circulating levels of growth hormone, IGF-1 or linear growth in rodents (28;29). In addition the finding that targeted deletion of the growth hormone secretagogue receptor or ghrelin also failed to produce any alteration in the growth hormone axis (7;8).

Ghrelin has been shown to alter glucose homeostasis in humans and rats (21;30). It powerfully inhibits glucose stimulated insulin release (31). In support of this, ghrelin deletion has been shown to improve glucose tolerance during an IP-GTT by amplifying glucose stimulated insulin release (22). Moreover, mice with ghrelin neuronal over expression develop age related glucose intolerance (15). Our Tg mice were glucose
Ghrelin transgenic mice are glucose intolerant due to an inhibition of glucose stimulated insulin release. Our data suggested ghrelin over expression did not alter insulin sensitivity. However this was tested using a relatively high dose of insulin. It is therefore possible that there are subtle effects on insulin sensitivity which would not be detectable at the doses of insulin used. Lower doses of insulin or the use of hyperinsulinaemic/euglycemic clamp studies would provide firmer evidence of the effect of ghrelin over expression on insulin sensitivity. These results suggest ghrelin has an important role in regulating β-cell function and glucose homeostasis. Indeed, the weight of evidence supporting the role of ghrelin in the regulation of β-cell function could indicate a more physiologically important function in the control of glucose homeostasis than appetite regulation.

Ghrelin and leptin are known to have opposing effects on the orexigenic neurones expressing neuropeptide Y and agouti related protein within the arcuate nucleus of the hypothalamus (32;33). It has been suggested that ghrelin and leptin may act as functional antagonists at this neuronal population to control energy homeostasis. Consistent with this hypothesis Tg mice were as sensitive to exogenous ghrelin as WT mice but less sensitive to the anorexigenic effects of leptin.

From our data we conclude ghrelin has important physiological roles in the control of energy homeostasis. Chronic over expression of bioactive ghrelin increases food intake but does not alter long term body weight gain because of a paradoxical increase in energy expenditure. We also found that ghrelin plays an important role in β-cell function by inhibiting glucose stimulated insulin release.

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REFERENCES

1. Kojima,M, Hosoda,H, Date,Y, Nakazato,M, Matsuo,H, Kangawa,K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660, 1999
2. Tschop,M, Smiley,DL, Heiman,ML: Ghrelin induces adiposity in rodents. Nature 407:908-913, 2000
3. Asakawa,A, Inui,A, Kaga,T, Yuzuriha,H, Nagata,T, Ueno,N, Makino,S, Fujimiya,M, Niijima,A, Fujino,MA, Kasuga,M: Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337-345, 2001
4. Bednarek,MA, Feighner,SD, Pong,SS, McKee,KK, Hreniuk,DL, Silva,MV, Warren,VA, Howard,AD, Van Der Ploeg,LH, Heck,JV: Structure-function studies on the new growth
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hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem 43:4370-4376, 2000

5. Yang, J, Brown, MS, Liang, G, Grishin, NV, Goldstein, JL: Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132:387-396, 2008

6. Gutierrez, JA, Solenberg, PJ, Perkins, DR, Willency, JA, Knierman, MD, Jin, Z, Witcher, DR, Luo, S, Onyia, JE, Hale, JE: Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci U S A 105:6320-6325, 2008

7. Sun, Y, Ahmed, S, Smith, RG: Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23:7973-7981, 2003

8. Sun, Y, Wang, P, Zheng, H, Smith, RG: Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci U S A 101:4679-4684, 2004

9. Wortley, KE, del Rincon, JP, Murray, JD, Garcia, K, Iida, K, Thorner, MO, Sleeman, MW: Absence of ghrelin protects against early-onset obesity. J Clin Invest 115:3573-3578, 2005

10. Zigman, JM, Nakano, Y, Coppari, R, Balthasar, N, Marcus, JN, Lee, CE, Jones, JE, Deysher, AE, Waxman, AR, White, RD, Williams, TD, Lachey, JL, Seeley, RJ, Lowell, BB, Elmquist, JK: Mice lacking ghrelin receptors resist the development of diet-induced obesity. J Clin Invest 115:3564-3572, 2005

11. Sun, Y, Butte, NF, Garcia, JM, Smith, RG: Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology 149:843-850, 2008

12. Ariyasu, H, Takaya, K, Iwakura, H, Hosoda, H, Akamizu, T, Arai, Y, Kangawa, K, Nakao, K: Transgenic mice overexpressing des-acyl ghrelin show small phenotype. Endocrinology 146:355-364, 2005

13. Asakawa, A, Inui, A, Fujimiya, M, Sakamaki, R, Shinfuku, N, Ueta, Y, Meguid, MM, Kasuga, M: Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18-24, 2005

14. Iwakura, H, Hosoda, K, Son, C, Fujikura, J, Tomita, T, Noguchi, M, Ariyasu, H, Takaya, K, Masuzaki, H, Ogawa, Y, Hayashi, T, Inoue, G, Akamizu, T, Hosoda, H, Kojima, M, Itoh, H, Toyokuni, S, Kangawa, K, Nakao, K: Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. J Biol Chem 280:15247-15256, 2005

15. Reed, JA, Benoit, SC, Pfluger, PT, Tschop, MH, D'Alessio, DA, Seeley, RJ: Mice with chronically increased circulating ghrelin develop age-related glucose intolerance. Am J Physiol Endocrinol Metab 294:E752-E760, 2008

16. Salmon, DM, Flatt, JP: Effect of dietary fat content on the incidence of obesity among ad libitum fed mice. Int J Obes 9:443-449, 1985

17. Liu, YL, Semjonous, NM, Murphy, KG, Ghatel, MA, Bloom, SR: The effects of pancreatic polypeptide on locomotor activity and food intake in mice. Int J Obes (Lond) 2008

18. Wren, AM, Small, CJ, Ward, HL, Murphy, KG, Dakin, CL, Taheri, S, Kennedy, AR, Roberts, GH, Morgan, DG, Ghatel, MA, Bloom, SR: The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325-4328, 2000

19. Stevanovic, D, Milosevic, V, Starcevic, VP, Severs, WB: The effect of centrally administered ghrelin on pituitary ACTH cells and circulating ACTH and corticosterone in rats. Life Sci 80:867-872, 2007
20. Thomas, GB, Fairhall, KM, Robinson, IC: Activation of the hypothalamic-pituitary-adrenal axis by the growth hormone (GH) secretagogue, GH-releasing peptide-6, in rats. *Endocrinology* 138:1585-1591, 1997

21. Dezaki, K, Hosoda, H, Kakei, M, Hashiguchi, S, Watanabe, M, Kangawa, K, Yada, T: Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* 53:3142-3151, 2004

22. Sun, Y, Asniciar, M, Saha, PK, Chan, L, Smith, RG: Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. *Cell Metab* 3:379-386, 2006

23. Kohno, D, Gao, HZ, Muruya, S, Kikuyama, S, Yada, T: Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 52:948-956, 2003

24. Wren, AM, Seal, LJ, Cohen, MA, Brynes, AE, Frost, GS, Murphy, KG, Dhillo, WS, Ghatei, MA, Bloom, SR: Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86:5992, 2001

25. Sun, Y, Ahmed, S, Smith, RG: Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* 23:7973-7981, 2003

26. Yasuda, T, Masaki, T, Kakuma, T, Yoshimatsu, H: Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. *Neurosci Lett* 349:75-78, 2003

27. Strack, AM, Bradbury, MJ, Dallman, MF: Corticosterone decreases nonshivering thermogenesis and increases lipid storage in brown adipose tissue. *Am J Physiol* 268:R183-R191, 1995

28. Wren, AM, Small, CJ, Abbott, CR, Dhillo, WS, Seal, LJ, Cohen, MA, Batterham, RL, Taheri, S, Stanley, SA, Ghatei, MA, Bloom, SR: Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540-2547, 2001

29. Tschop, M, Statnick, MA, Suter, TM, Heiman, ML: GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* 143:558-568, 2002

30. Broglio, F, Arvat, E, Benso, A, Gottero, C, Muccioli, G, Papotti, M, van der Lely, AJ, Deghenghi, R, Ghigo, E: Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J Clin Endocrinol Metab* 86:5083-5086, 2001

31. Salehi, A, Dornonville de la, CC, Hakanson, R, Lundquist, I: Effects of ghrelin on insulin and glucagon secretion: a study of isolated pancreatic islets and intact mice. *Regul Pept* 118:143-150, 2004

32. Bagnasco, M, Tulipano, G, Melis, MR, Argiolas, A, Cocchi, D, Muller, EE: Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regul Pept* 111:161-167, 2003

33. Cowley, MA, Smart, JL, Rubinstein, M, Cerdan, MG, Diano, S, Horvath, TL, Cone, RD, Low, MJ: Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480-484, 2001
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Figure 1. Total and bioactive ghrelin levels are increased in transgenic mice. Ghrelin stomach mRNA expression was significantly increased in Tg mice compared to WT litter mates (A). In addition, both total ghrelin (B) and octanoylated ghrelin (C) were significantly increased in stomach extracts. Plasma total ghrelin concentrations were increased in male mice (D). Fasting plasma octanoylated ghrelin concentrations were increased in both female (E) and male (F) mice. Ghrelin expression was found exclusively in the hypothalamus and stomach of Tg and Wt mice (G). The results are presented as mean±SEM n=6, *p<0.05, **p<0.01 Tg and WT controls at sixteen weeks of age. RQ represents relative quantification compared to control stomach.
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Figure 2. Over expression of bioactive ghrelin increases food intake. Cumulative food intake was measured from 5 to sixteen weeks of age in both male (A) and female (C) mice fed on regular chow. Body growth curves from 5 to sixteen weeks of age for male (B) and female (D) mice. Body composition (E) and plasma leptin concentrations (F) were measured in sixteen week old male mice. Wild Type closed circles, Transgenic open diamonds. The results are presented as mean±SEM n= 6-8, *=p<0.05 **=p<0.01 Tg and WT controls. Dashed lines Tg solid lines Wt.
Figure 3. Over expression of bioactive ghrelin increases energy expenditure. Brown adipose tissue UCP-1 mRNA expression in sixteen week old mice (A) and average oxygen consumption measured during one 24h period (B) and average hourly oxygen consumption (C) using the CLAMS in sixteen week old mice. Average ambulatory activity estimated as X beam breaks during either the light or dark period (D). Respiratory exchange ratio was calculated as VCO2/VO2 (E). The results are presented as mean±SEM n= 6-8, *=p<0.05 **=p<0.01 Tg and WT controls.
Ghrelin transgenic mice are glucose intolerant

Figure 4. Over expression of bioactive ghrelin attenuates glucose stimulated insulin release. Following an 18h fast sixteen week old male mice were injected with glucose (2g/Kg). Plasma glucose (A) was measured and area under the curve (AUC) calculated (B). Plasma glucose was measured during an ITT (insulin 1.5U/Kg) (C) and the area under the curve calculated (D). Following IP-GTT plasma glucose (E) and insulin (F) were measured and AUC for insulin release calculated (G). The results are presented as mean±SEM n=6-10, *=p<0.05 **=p<0.01 Tg and WT controls. Dashed lines Tg solid lines Wt.
Ghrelin transgenic mice are glucose intolerant

Figure 5. Ghrelin over expressing transgenic mice are sensitive to ghrelin but have reduced leptin sensitivity. Following i.p injection of ghrelin (0.3nmol/g) or saline to fed sixteen week old male Tg and Wt mice 0-1 hour food intake was measured (A). Following i.p. administration of leptin (3μg/g) or saline to fasted sixteen week old male Tg and Wt mice 0-1hour food intake was measured (B) and 0-4 hour (C). The results are presented as mean±SEM n= 9-10, *=p<0.05 saline versus ghrelin or leptin treatment.