Obesity in MENX rats is accompanied by high circulating levels of ghrelin and improved insulin sensitivity

Short title: MENX rats show islet hyperplasia and hyperghrelinemia

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Abstract:

Ghrelin, the natural ligand of the growth hormone secretagogue receptor (GHS-R1a), is mainly secreted from the stomach and regulates food intake and energy homeostasis. p27 regulates cell cycle progression in many cell types. Here, we report that rats affected by the multiple endocrine neoplasia syndrome MENX, caused by a p27 mutation, develop pancreatic islet hyperplasia containing elevated numbers of ghrelin-producing epsilon cells. The metabolic phenotype of MENX-affected rats featured high endogenous acylated and unacylated plasma ghrelin levels. Supporting increased ghrelin action, MENX rats show increased food intake, enhanced body fat mass, and elevated plasma levels of triglycerides and cholesterol. Ghrelin effect on food intake was confirmed by treating MENX rats with a GHS-R1a antagonist. At 7,5 months, MENX-affected rats show decreased mRNA levels of hypothalamic GHS-R1a, neuropeptide Y (NPY), and agouti-related protein (AgRP), suggesting that prolonged hyperghrelinemia may lead to decreased ghrelin efficacy. In line with ghrelin’s proposed role in glucose metabolism, we find decreased glucose-stimulated insulin secretion (GSIS) in MENX rats while insulin sensitivity is improved. In summary, we provide a novel, non-transgenic rat model with high endogenous ghrelin plasma levels and interestingly, improved glucose tolerance. This model might aid in identifying new therapeutic approaches for obesity and obesity-related diseases including type-2 diabetes.
**Introduction**

Currently, increased food intake and reduced physical activity have resulted in a dramatic increase in the development of obesity-associated disorders, such as type-2 diabetes, hyperlipidemia, and cardiovascular diseases. The increased prevalence of obesity has led to a growing interest in the fundamental understanding of energy homeostasis and metabolism. In the last two decades, several clinical and experimental studies in the field of obesity and obesity-associated diseases identified ghrelin as a peripheral peptide hormone exerting orexigenic effects via the hypothalamus [1, 2].

Ghrelin, the natural ligand of the growth hormone secretagogue receptor (GHS-R1a), was first discovered in 1999, and it is the only known circulating peripheral peptide hormone that stimulates food intake in humans and rodents [1, 3, 4]. Ghrelin is a 28-amino-acid peptide primarily released by X/A-like cells and P/D1 cells in the oxyntic glands of rat and human fundus, and secreted to a lesser extent by the pancreas, intestine, kidneys, liver, pituitary, and hypothalamus [5-11]. Pancreatic ghrelin-producing cells were described in 2002 [12] and later named epsilon cells. They represent a distinct hormone-producing cell population which is abundant in foetal and neonatal pancreas but poorly represented in adult pancreas in human, mice and rats [13]. In mice, ghrelin has been found localized within the glucagon-positive alpha cells at birth, while in rats this occurs only occasionally and in humans it has not been reported [13]. Acylated or active ghrelin (AG) has a unique fatty acid modification on the third amino acid residue (Ser3). This chemical modification, achieved by the enzyme ghrelin O-acyltransferase (GOAT), is essential for the binding of the hormone to the GHS-R1a receptor [3, 14]. However, the majority of circulating ghrelin lacks this acyl group and is called unacylated ghrelin (UAG).

The orexigenic properties of AG make it a promising target for the treatment of obesity and cachexia through either GHS-R1a activation or inhibition, respectively. Randomized,
placebo-controlled human studies on cancer-associated cachexia have shown an increase in food intake following ghrelin infusions [15].

Aside from its function as an appetite-stimulating peptide, ghrelin exerts several other hormonal, metabolic and cardiovascular activities [14, 16]. For instance, it enhances growth hormone, adrenocorticotropic hormone, prolactin and cortisol release, while it suppresses testosterone and luteinizing hormone secretion. Ghrelin has been also shown to accelerate gastric emptying, increase gastrointestinal tract motility, increase anti-lipolytic effect on adipose tissue, decrease colonic transit time, among other metabolic activities. Moreover, it may increase stroke volume, enhance systemic vasodilatation and decrease blood pressure [14, 16].

In recent years genetically engineered mouse models having loss- or gain-of-function mutations in the ghrelin axis were generated and have contributed to our understanding of the pleiotropic effects of ghrelin [17]. However, background-dependent predisposition to lean or fat phenotypes needs to be taken into account while interpreting the data obtained from these animal models. Currently, there are only a few mouse models with high endogenous ghrelin levels, and studies of these animals have generated inconsistent results concerning food intake, body weight gain, and energy expenditure [17].

MENX is a multiple endocrine neoplasia (MEN) syndrome in the rat caused by a spontaneous, homozygous loss-of-function mutation in the cyclin-dependent kinase inhibitor p27. This mutation leads to a rapid degradation of the encoded mutant p27 protein [18]. p27 is critical in maintaining quiescence in adult tissues by blocking cell cycle progression at the G1/S boundary. During embryonic development, p27 promotes cell cycle withdrawal of progenitor cells in various neuronal and endocrine tissues, including the anterior pituitary gland, thereby allowing cell differentiation [19-21]. Notably, we observed a persistent postnatal expression of embryonic progenitor cell markers in the pituitary gland of MENX-
affected rats [22], suggesting altered development and differentiation of neuroendocrine cells in these animals due to p27 deficiency.

It has been reported that germline ablation of p27 in mice causes pancreatic islet hyperplasia and glucose-induced hyperinsulinemia [23]. The aims of this study were to determine whether also MENX rats develop islet hyperplasia and to assess the associated metabolic phenotypes. We show here that MENX-affected rats develop pancreatic islet hyperplasia within their first year of life. Analysis of the endocrine pancreas revealed an unexpected large number of ghrelin-producing epsilon cells in the pancreatic islets. MENX rats accordingly show high endogenous levels of UAG and AG in combination with hyperphagia, dyslipidemia, and an increased white adipose tissue (WAT) mass. Interestingly, obese hyperghrelinemic MENX-affected rats show decreased glucose-stimulated insulin secretion but improved insulin sensitivity.

Research Design and Methods

Animals

The phenotype and genotype of MENX rats have been previously described [24]. In all studies, male MENX-affected rats (homozygous mut/mut) and their wild-type (wt/wt) littermates were group-housed under controlled conditions (temperature 23°C, 12-h/12-h light/dark cycle). The rats had access to standard rodent chow (Fa. Altromin) and water ad libitum. All experiments and procedures were approved by local authorities (AZ 55.2-1-54-2532-84-11) and complied with German animal protection laws. The group size in each experiment was n≥6.
**Immunohistochemistry**

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems) according to the manufacturer’s protocols. Staining of 2-μm-thick rat gastric corpus tissue sections was performed using antibodies against ghrelin (Phoenix Pharmaceuticals, H-031-31) (1:350) and GHS-R1a (Phoenix Pharmaceuticals, H-001-62) (1:500). Positively stained cells were visualized with DAB (Vector Laboratories). Images were recorded using an Olympus camera DP25 installed in an Olympus microscope BX43 with the Olympus LabSense software.

**Immunofluorescence**

Sections of rat pancreas were incubated in xylene (2 x 10 min) to remove the paraffin. After treatment with isopropanol (10 min), rehydration was achieved by successive transfer of the tissue through the following graded series of ethanol: 100% (two times for 5 min), 96% (5 min), and 70% ethanol (5 min). The slides were washed with TBS-T (5 min) and subsequently boiled in citrate buffer (Dako) (30 min) for epitope retrieval. Following a washing step in TBS-T (5 min), the tissue was blocked with 0.1% Sudan Black B in 70% ethanol (45 min) and washed in TBS-T (three times for 5 min). Staining was performed using primary antibodies against ghrelin (Phoenix Pharmaceuticals, H-031-31) (1:350), glucagon (Dako, A0565) (1:1500), Insulin (Dako, A0564) (1:750), Pancreatic Polypeptide (Abcam, ab77192) (1:150), and somatostatin (Santa Cruz Biotechnology, sc7819) (1:300). Primary antibodies were applied overnight at 4°C. After the removal of unbound antibodies, the tissue was incubated with secondary antibodies (anti-goat IgG (H+L) fluorescein (FITC)-conjugated (Jackson ImmunoResearch, 905-095-180) (1:200), anti-rabbit IgG (H+L) fluorescein (FITC)-conjugated (Invitrogen, F-2765) (1:200), and anti-guinea pig IgG (H+L) Alexa Fluor 555-conjugated (Invitrogen, A-21453) (1:200)) for 1 hour at room temperature (RT). The fluorescently labeled slides were washed with TBS-T (three times for 1 min), followed by
staining of nuclei with Hoechst 33342 bisBenzimid (Sigma-Aldrich). The tissue was subsequently washed in dH$_2$O and covered with VECTASHIELD® (Vector Laboratories). Cover glasses were mounted on the tissue and fixed with enamel. Images were recorded using an AxioImager Fluorescence Microscope (Carl Zeiss).

**ELISA of acylated ghrelin and total ghrelin**

Fasting blood was collected in EDTA tubes, and plasma was isolated by centrifugation. The plasma was acidified with HCl and treated with cOmplete® protease inhibitor cocktail (Roche, Germany). Active (EZRGRA-90K; Millipore, Billerica, MA) and total (EZRGRT-91K; Millipore) ghrelin in rat plasma was measured with the indicated ELISA assays according to the manufacturer’s protocols.

**RNA isolation and real-time qRT-PCR**

Rat tissues were snap-frozen in liquid nitrogen and stored at −80°C until use. For total RNA extraction from snap-frozen rat tissues, serial 20-µm tissue sections were cut and resuspended in RLT buffer (RNeasy Mini kit). Total RNA used for qRT-PCR was purified by using the RNeasy Mini Kit (Qiagen). We synthesized the first-strand cDNA from total mRNA using random hexamers and SuperScript II (Invitrogen). Quantitative RT-PCR for rat mRNA was performed with TaqMan primers and probes specific for rat *Agrp*, *Npy*, *Ghs-r1a*, *Lep*, and *Ghrl* genes, as well as for β2-microglobulin as an internal control (Applied Biosystems). The assays were performed as previously reported [18].

**Food intake experiments**

Daily food consumption of mut/mut and age-matched wt/wt rats was measured on five consecutive days (8 a.m.) at different ages, and the mean consumption of each individual rat was calculated.
Refeeding experiments

Mut/mut and wt/wt rats at 3.5 months of age were food-deprived for 24 h with water available ad libitum. Experiments were started at 8 am. Cumulative food intake was measured for each individual rat at 0.5 h, 1 h, 2 h, 4 h, 8h and 24 h after refeeding.

GHS-R1a antagonist treatment

Mut/mut and wt/wt rats at 3.5 months of age were food-deprived for 24 h with water available ad libitum. They were injected intraperitoneally with ghrelin antagonist [D-Lys³]-GHRP-6 (12 mg/kg body weight) (Bachem Switzerland, H-3108) or vehicle (saline) at 8 am. Cumulative food intake was measured for each individual rat at 0.5h, 1h, 2h, 4h, 8h and 24 h after injection.

Preparation of gastric corpus tissue for ghrelin ELISA

The gastric corpus of 7.5 months old mut/mut and wt/wt rats was quickly removed from euthanized animals and snap frozen. Serial 10 μM sections of each tissue were obtained using a cryotome and were lysed in cold lysis solution (RIPA buffer [R0278; Sigma], 10mM HCl, cOmplete® protease inhibitor cocktail [Roche, Germany]). The lysates were centrifuged at 15.000 rpm 4°C for 15 minutes, and supernatants were collected and used for total ghrelin measurements with an ELISA kit [EZRGRT-91K; Millipore] and for total protein content measurement to normalize the ELISA results.

Oral and intraperitoneal glucose tolerance test

For the intraperitoneal glucose tolerance test (i.p.GTT) and oral glucose tolerance test (oGTT), a tail-tip blood sample was obtained from fasting rats (overnight 16 h; t0). The blood glucose concentration was immediately measured by using a glucometer (Contour; Bayer Health Care) after D-glucose solution (40%) was injected intraperitoneally (i.p.GTT) or
administered by oral gavage (2 g/kg; oGTT). Additional blood samples for glucose and insulin measurements were obtained 15, 30, 60, 90, and 120 min post-injection.

**Intraperitoneal insulin tolerance test**

For the intraperitoneal insulin tolerance test (i.p.ITT), a tail-tip blood sample was obtained from fasting rats (6 h; t0). The blood glucose concentration was immediately measured using a glucometer (Contour; Bayer Health Care) after insulin solution (Berlinsulin® H; Berlin-Chemie AG) was injected intraperitoneally (0.75 IU/kg; 0.375 IU/kg). Additional blood samples for glucose measurements were obtained 15, 30, 60, 90, and 120 min post-injection.

**Statistical analyses**

Data are presented as the mean ± SEM. Statistical significance between two series of data was determined by Student’s t test. \( P<0.05 \) was considered statistically significant.

**Results**

**Enhanced ghrelin immunoreactivity in hyperplastic pancreatic islets of MENX rats**

Previous studies in transgenic mice showed that deletion of the gene encoding p27 (Cdkn1b) leads to pancreatic beta-cell hyperplasia [23, 25]. In the present study, we observed that 7.5-month-old MENX-affected (mut/mut) rats displayed a greater than two-fold increase in islet-cell mass compared with age-matched unaffected wild-type (wt/wt) rats (Fig. 1 A,B). To determine whether this phenotype represents an early or a late event, we also studied the pancreatic islets of 14 days old mut/mut and wt/wt rats. Already at this early age mut/mut rat have an increased islet-cell mass when compared with wt/wt rats (Fig. 1B). To analyze the cellular composition of the hyperplastic islets in mut/mut rats, glucagon-producing alpha cells, insulin-producing beta cells, somatostatin-producing delta cells, pancreatic polypeptide-
producing PP cells, and ghrelin-producing epsilon cells were examined by immunohistochemistry or immunofluorescence using antibodies against the respective hormones. The results show not only beta cell hyperplasia, but also an increase in the number of alpha, delta, and PP cells in the endocrine pancreas of mut/mut rats compared with wt/wt littermates at both 14 days and 7.5 months (Fig. 1 C-J and Supplementary Figure 1). This result suggests an altered development and differentiation of endocrine pancreatic cells due to defective p27 function. Further, we found abnormally high numbers of ghrelin-positive epsilon cells in the islets of adult mut/mut rats (Fig. 1 K). In 14 days old mut/mut and wt/wt rats, ghrelin was not found localized with glucagon (Supplementary Figure 1).

Previous studies in rats described large numbers of ghrelin-producing cells at birth, occasional ghrelin-positive cells at up to one month of age, and virtually no positive cells in the endocrine pancreas of adult animals [26, 27]. The overrepresentation of ghrelin-positive cells in mut/mut rat pancreata together with the fact that they are usually not found in adult wt/wt rats lead us to hypothesize that pancreatic islet-derived ghrelin may play a physiologically meaningful role in these animals.

**Plasma ghrelin levels, food intake, and body weight in MENX rats**

Based on the observation that mut/mut rats contain high numbers of ghrelin-positive epsilon cells in their islets, we assessed possible differences in fasting plasma AG and UAG levels between mut/mut and wt/wt rats at various ages (2.5, 5.5, and 7.5 months). We found that at 5.5 months of age, the mut/mut rats had increased ghrelin levels compared with the age-matched wt/wt rats (AG $p=0.023$; UAG $p=0.002$). The amount of ghrelin was even higher in the 7.5-month-old rats and resulted in endogenous hyperghrelinemia (Fig. 2 A,B). To assess the physiological relevance of this endogenous hyperghrelinemia, we determined the daily food intake and body weight changes in both the mut/mut and wt/wt rats over time. AG levels were slightly elevated in 2.5 months old mut/mut rats compared to wt/wt littermates (not
significant) and highly elevated at the subsequent time points (5.5 and 7.5 months; \( p < 0.01 \) and \( p < 0.001 \), respectively). In parallel with the increment in AG levels in mut/mut rats, we found a significant increase in food intake combined with a significant body weight gain in these rats when compared with the wt/wt animals, especially between the ages of 3 and 6 months (Fig. 2 C,D).

To better understand the effect of the high ghrelin levels in mut/mut rats on their feeding behavior, we also performed re-feeding experiments where rats were food-deprived, fed and then the food intake was measured at various time points thereafter (30 min, 1h, 2h, 4h, 8h, and 24h). In parallel, AG and UAG levels were measured in all rats after fasting and 2h post-re-feeding. We used 3,5 months old animals because at this age the mut/mut rats show a significant increase in body weight compared with wt/wt rats (Fig. 2 D). The results show that up to 4 h post-re-feeding both mut/mut and wt/wt rats eat similar amounts of food, but after 8h the mut/mut animals start eating significantly more than the unaffected ones and the difference in food intake further increases at the 24h time point (Supplementary Figure 2A).

We also observed that, after fasting, UAG and AG levels are similarly elevated in both rat groups (mut/mut and wt/wt), in agreement with the physiological role of ghrelin (Supplementary Figure 2B, C). After re-feeding, the levels of both UAG and AG decrease in all rats, as expected. However, at 2h post-re-feeding, we could already measure a significantly higher level of UAG and a slightly elevated level of AG (not significant) in mut/mut rats when compared with the wt/wt ones (Supplementary Figure 2B, C). These results show that ghrelin levels fluctuate in mut/mut rats with feeding, as in wt/wt rats, but in the former group the hormone levels are increased.

To verify whether the high levels of endogenous ghrelin are responsible for the feeding response of mut/mut rats, we treated these animals with a GHS-R1a antagonist able to block ghrelin signaling, i.e. [D-Lys]-GHRP-6. Eight mut/mut rats were food-deprived for 24h and
then injected with [D-Lys]-GHRP-6 or saline solution (control group). Food intake was measured at 30 min, 1h, 2h, 4h, 8h and 24h after injection. We found that, up to the 8h time point, [D-Lys]-GHRP-6 significantly decreased food intake in the control wt/wt rats, attesting that this compound efficiently blocks ghrelin signaling (Fig. 5A). Similarly, mut/mut rats treated with the GHS-R1 antagonist showed a significant reduction in food intake up to 8h post-treatment (Fig. 5B). Twenty-four hours after antagonist administration, no changes in food intake could be detected in both rat groups as the compound is probably no longer active. The reduction in food intake for up to 8h post-treatment was significantly higher in wt/wt versus mut/mut rats probably because of a higher amount of circulating ghrelin in the latter. Altogether, this data confirms that the elevated ghrelin levels in mut/mut rats are responsible for their feeding response.

**Ghrelin-producing cells in the gastric corpus mucosa of MENX rats**

Ghrelin is predominantly secreted from X/A-like oxyntic gland cells in the gastric corpus mucosa of the rat stomach [9]. To determine whether the number of ghrelin-producing cells changes in affected rats, we stained the gastric corpus tissue of mut/mut and wt/wt rats using an anti-ghrelin antibody (Fig. 3 A). Interestingly, the mut/mut rats with high circulating ghrelin levels showed a significant decrease in the number of ghrelin-positive cells in the gastric corpus mucosa compared with the wt/wt rats (Fig. 3 B). Concordantly, the ghrelin content of the gastric corpus of wt/wt rats was higher than that of mut/mut rats (Fig. 3C). Ghrelin gene expression in the gastric corpus was not significantly different between the mut/mut and wt/wt animals. These findings support the hypothesis that the high levels of circulating ghrelin in mut/mut rats originates from the endocrine pancreas.

**Ghrelin receptor (GHS-R1a) expression in the gastric corpus mucosa of MENX rats**
Previous studies showed that GHS-R1a is expressed in both rat and human gastric tissues, among other tissues. Similar to other studies [28], we also demonstrated a high expression of GHS-R1a in the gastric oxyntic glands in the wt/wt rats, particularly in the glands distributed along the middle and basal regions of the stomach corpus region (Fig. 3 C). Interestingly, immunostaining against GHS-R1a consistently showed a significant decrease in receptor abundance in the gastric corpus mucosa of 7.5-month-old mut/mut rats compared with that in the age-matched wt/wt rats (Fig. 3 D).

Gene expression profiling of gastric corpus mucosa tissue also revealed a decrease in GHS-R1a in 7.5-month-old mut/mut rats compared with wt/wt rats (Fig. 4 A), potentially as a consequence of high endogenous ghrelin levels in mut/mut rats.

**GHS-R1a, NPY, and AgRP expression in the hypothalamus of MENX rats**

To assess whether the elevated plasma ghrelin levels in mut/mut rats coincide with enhanced activation of orexigenic hypothalamic neurocircuits, we investigated the expression of GHS-R1a and the ghrelin target genes NPY and AgRP in the hypothalamus of these animals over time. At the age of 5.5 months, MENX-affected rats showed a significant increase in GHS-R1a and NPY expression compared with age-matched wt/wt animals, whereas no difference was observed for AgRP (Fig. 5 A, C). Interestingly, at 7.5 months of age, GHS-R1a, NPY, and AgRP transcripts were significantly decreased in the arcuate nucleus of mut/mut rats, suggesting desensitization of the ghrelin system (Fig. 5 B,D,F) due to persistently high plasma ghrelin levels.

**Body composition, leptin expression, as well as plasma cholesterol and triglyceride levels in MENX rats**

Earlier reports showed that chronic ghrelin administration increases body fat mass in rodents and humans [1]. Furthermore, UAG and AG were found to stimulate lipid accumulation in
adipose tissue [29]. The analysis of WAT revealed that epididymal-, peritoneal-, and inguinal fat pad mass was significantly increased in hyperghrelinemic mut/mut rats compared with age-matched wt/wt rats (Fig. 6 A), but this fat accumulation was independent of the common hypothalamic orexigenic pathways (see Fig. 5 D, F). This observation is in line with previous reports indicating that ghrelin’s orexigenic action in the hypothalamus is independent from its effect in promoting lipogenesis in the adipose tissue [30].

Additionally, fasting plasma cholesterol and triglyceride levels showed a highly significant elevation in mut/mut rats compared with wt/wt rats (Fig. 6 B, C), suggesting that endogenous hyperghrelinemia is associated with increased fat mass and dyslipidemia in mut/mut rats.

Leptin is primarily produced by the adipocytes in WAT, usually in direct proportion to the amount of WAT. Gene expression profiling of epididymal and peritoneal fat tissue revealed significantly lower levels of leptin in mut/mut rats (Fig. 6 D, E). These results are in agreement with plasma leptin measurements in fasting rats, which showed a trend toward a decrease in circulating leptin in mut/mut compared with wt/wt rats.

**Suppressed glucose-stimulated insulin secretion (GSIS) in MENX rats**

As illustrated above, mut/mut rats showed elevated AG and UAG levels at 5.5 months, and these levels further increased at 7.5 months. Previous studies have reported that AG can act as a negative regulator of glucose-induced insulin secretion (GSIS) [31, 32].

Consistent with these publications, mut/mut rats showed a significant decrease in GSIS during i.p.GTT compared with age-matched wt/wt rats (Fig. 7 A-F). This phenotype was already observed at 5.5 months of age when AG and UAG levels are already significantly elevated (Fig. 7 A,B).

To determine whether the route of glucose administration may influence the outcome of the GTT test, oGTT tests on 7.5-month-old mut/mut and wt/wt rats were performed (Fig. 7 G-J).
The mut/mut rats also showed suppressed insulin secretion during the oGTT test compared with the age-matched wt/wt animals (Fig. 7 G-J). Together, these tests suggest that the high plasma levels of AG in mut/mut rats are able to inhibit GSIS \textit{in vivo}.

**Enhanced insulin sensitivity in MENX rats**

As described above, mut/mut rats exhibit low insulin secretion with simultaneous unchanged blood glucose levels during GTT tests. These data prompted us to perform i.p.ITTs to determine whether the mut/mut animals with high circulating AG and UAG levels display a change in insulin sensitivity in comparison to wt/wt animals (Fig. 8 A-E).

In contrast to ghrelin’s proposed effect on impairing glucose metabolism [11], we observed a significant increase in insulin sensitivity in the 7.5-month-old mut/mut rats compared with the age-matched wt/wt animals (Fig. 8 B, C). As depicted in Fig. 8 A and C, the mut/mut animals showed increased insulin sensitivity already at 5.5 months of age. To gather additional evidence supporting different insulin sensitivity in mut/mut and wt/wt rats, i.p.ITT tests were performed in 5.5-month-old rats with half the dose of insulin we used in the previous experiments. This low amount of insulin caused a strong reduction in plasma glucose in the mut/mut but not in wt/wt rats (Fig. 8 D, E). Taken together, these data indicate that obese mut/mut rats with high circulating AG and UAG levels show a suppressed GSIS but interestingly improved insulin sensitivity.

**Discussion**

Here, we present a new, non-transgenic animal model with high endogenous levels of AG and UAG in combination with hyperphagia, dyslipidemia, and an increased WAT mass. We observed that rats affected by the MENX syndrome develop pancreatic islet hyperplasia.
This observation is consistent with recent reports describing beta cell hyperplasia in p27\(^{-/-}\) mice [25, 33]. In our model, MENX-affected rats show an increase not only in the number of beta cells but also in that of alpha, delta, and PP cells, leading to an increase in total islet mass.

One interesting observation derived from studies of islet cell hyperplasia is the highly increased number of ghrelin-producing epsilon cells present in the endocrine pancreas of adult mut/mut rats with a simultaneous decrease in X/A-like cells in the gastric corpus mucosa, which is normally the major source of ghrelin. An inverse relationship between the number of ghrelin-producing cells in the pancreas and in the stomach has been documented in rats during development [13], with the ghrelin cells in the pancreas being more abundant in late prenatal and early postnatal development when the density of the ghrelin cells in the stomach is still low. In our rat model, the density of the pancreatic and gastric ghrelin cells is also reversed, and we speculate that the elevated circulating ghrelin levels in mut/mut rats are due to the increase in pancreatic ghrelin-producing cells. Although most of the circulating ghrelin comes from the stomach, pancreatic islets-derived ghrelin has been shown to be secreted and to play a role in controlling glucose levels in rodents [31]. *In vitro*, pancreatic islets isolated from wild-type rats were found to produce and secrete active ghrelin, which downregulates insulin release following glucose stimulation [32]. The same research group later demonstrated that islet-originated ghrelin suppresses insulin secretion in perfused rat pancreata, where islet circulation is well preserved, and that the concentration of ghrelin is higher in pancreatic veins than in arteries of wild-type rats *in vivo* [31]. Additionally, the findings that gastrectomy in human reduces circulating ghrelin to a level of 35% [34], and that rats subjected to sleeve gastrectomy with duodenal-jejunal bypass also show moderate decrease in fasting ghrelin levels [35] suggest that a minor but substantial amount of the hormone originates from organs other than the stomach, including the pancreas. Altogether, pancreas-derived ghrelin is secreted into circulation, and, in addition to its role as a physiological regulator of GSIS, may
also affect other metabolic functions. The elevated number of epsilon cells in the pancreas of adult mut/mut rats is contradictory to previous publications in which few such cells were present in the endocrine pancreas of adult rats and humans [26], and suggests an altered development and differentiation of neuroendocrine cells in the pancreas of mut/mut animals due to the lack of functional p27. In agreement with previous studies reporting that in rats glucagon and ghrelin only occasionally co-localize around birth [13], in the pancreatic islets of 2 weeks old mut/mut rats the ghrelin-positive cells do not co-express glucagon.

After measuring plasma ghrelin levels, we found that mut/mut rats had increased amounts of AG and UAG compared with the wt/wt animals. The elevated ghrelin levels in the mut/mut rats were associated with hyperphagia and an increased body weight in 5.5-month-old mut/mut rats. At the age of 7.5 months, the mut/mut rats exhibited a high level of dyslipidemia associated with increased WAT. The ghrelin receptor GHS-R1a was initially characterized in the pituitary and hypothalamus, where it is highly expressed and mediates GH release and appetite regulation [36, 37]. GHS-R1a is also expressed in numerous peripheral tissues, including the gastric enteric nervous plexus in rats and humans [28]. Given that the effects of ghrelin are mediated by binding to its receptor, we assessed GHS-R1a expression in the stomach of affected and unaffected rats. We found a significant downregulation of GHS-R1a in the gastric corpus mucosa of the 7.5-month-old hyperghrelinemic mut/mut rats compared with the wt/wt rats. These data suggest the existence of a possible link between chronically elevated ghrelin levels and decreased GHS-R1a expression in the gastric mucosa of mut/mut rats. The fact that stomach cells express both ghrelin and its receptor suggests that ghrelin may potentially act in a paracrine manner to self-regulate its own secretion.

GHS-R1a is mainly present on the NPY/AgRP-expressing neurons in the arcuate nucleus of the hypothalamus, where it mediates ghrelin’s orexigenic action [38]. Consistent with data
obtained by injecting ghrelin into wild-type Sprague-Dawley rats [39], 5.5-months-old MENX-affected rats showed a significant increase in NPY expression, probably due to the high circulating ghrelin levels. Interestingly, at the age of 7.5 months, MENX-affected rats showed a significant decrease in GHS-R1a, NPY, and AgRP expression. This reduction could potentially be an indicator of ghrelin resistance. This phenomenon was already described in diet-induced obese (DIO) mice, which show decreased hypothalamic GHS-R1a expression. In these mice, diet induced obesity is accompanied with downregulation of both NPY and AgRP and central (intracerebroventricular) ghrelin injection was unable to effect NPY and AgRP expression [40]. However, other mechanisms such as compensatory effects or a negative feedback could also explain the time-dependent decrease in ghrelin-regulated gene expression in the hypothalamus of our rats.

If the elevated ghrelin is responsible for the feeding behavior of mut/mut rats, we would expect that a blockade of ghrelin signaling with a GHS-R1a antagonist would reduce food intake. This is exactly what we observed following administration of the antagonist [D-Lys]-GHRP-6 to mut/mut rats. The control group of wt/wt rats also showed a similar response to the antagonist, but the reduction of food intake was more pronounced in wt/wt than in mut/mut rats likely because of the higher ghrelin levels in the latter.

Previous studies on type 2 diabetic db/db mice showed that deletion of the gene encoding p27 ameliorated hyperglycemia by increasing pancreatic islet mass and maintaining compensatory hyperinsulinemia, effects that were attributable to the stimulation of pancreatic beta cell proliferation [23, 25]. In the present study, we demonstrated a very similar hyperplastic phenotype of the pancreatic islets in the mut/mut rats. For this reason, we would have expected high insulin levels following the glucose challenge during the oGTT/i.p.GTT tests in the mut/mut rats, but the opposite was the case; the mut/mut animals exhibited a severe inhibition of GSIS, probably due to the high levels of circulating AG. Interestingly,
the i.p.ITT performed on obese mut/mut rats showing decreased GSIS revealed significantly improved insulin sensitivity. Data concerning the relationship between ghrelin levels and insulin sensitivity are still controversial. In contrast to our data, it has been demonstrated that ghrelin administration to humans and rodents is accompanied by glucose intolerance [32, 41-46]. Vice versa, ablation of ghrelin was found to improve glucose disposal and insulin sensitivity [31, 47]. Tong et al. have shown in healthy humans, that a continuous ghrelin infusion suppresses GSIS but also that glucose disappearance is then reduced [44]. However, in agreement with our findings, it has been shown that continuous ghrelin administration enhances insulin sensitivity mainly by acting on peripheral tissues, thereby increasing glucose disposal. For example, Barazzoni et al. have shown that sustained ghrelin administration to rats had an insulin-sensitizing effect on skeletal muscles by enhancing muscle AKT activation [48]. Furthermore, Heijboer et al. have demonstrated that muscle and adipose tissue-specific glucose uptake were significantly higher (by a factor of 3-4) in mice treated with ghrelin [49]. Similarly, Patel et al. found that ghrelin potentiates insulin-stimulated glucose uptake in isolated white adipocytes [50]. The later published data are in agreement with data from our mutant rats displaying hyperghrelinemia and lower GSIS, but unchanged glucose concentrations compared to wild-type controls. We therefore speculate that hyperghrelinemic mut/mut rats display improved insulin sensitivity and lower GSIS due to improved peripheral tissue insulin sensitivity. The functional relationship between high ghrelin levels and GSIS in mut/mut rats warrants further investigation.

Another possible explanation for our findings is that the improved insulin sensitivity might be caused by the elevated UAG plasma concentration in mut/mut rats. This effect was once described by Gauna et al. [51] in humans, where AG injection caused a decrease in insulin sensitivity while the co-injection of AG and UAG led to an improvement in insulin sensitivity. Alternatively, we could speculate that in MENX rats the genetic mutation in p27
affects ghrelin-independent signaling mechanisms able to override the negative effect of ghrelin on glucose metabolism. Further studies are required to clarify this issue.

It is well known that dyslipidemia associated with obesity is a strong risk factor for the development of cardiovascular diseases and type 2 diabetes mellitus [52]. Because cardiovascular disease has been the leading cause of death worldwide since the 1970s and its frequency is likely to increase, it is imperative to fully understand the mechanisms leading to this metabolic lipid phenotype. As previously mentioned, MENX rats develop dyslipidemia associated with an increased WAT mass and, therefore, represent a model with which to elucidate open questions concerning dyslipidemia development. It has been reported that receptor-mediated uptake and accumulation of plasma cholesterol into the endocrine pancreas reduces GSIS and therefore may represent a link between obesity and type 2 diabetes mellitus [53, 54]. These findings are in accordance with our MENX rat model, in which mut/mut rats showed decreased GSIS probably due to high plasma AG levels and further reinforced by high plasma triglyceride and cholesterol levels.

Altogether, the MENX rat provides a meaningful animal model for ghrelin and obesity research. A substantial benefit of this experimental model is the endogenous origin of the elevated plasma ghrelin levels without the need of external administration of the hormone at supraphysiological doses or of genetic modifications of the animals [17, 55, 56].

The main focus of future studies will be to use this new animal model to shed light on the role of UAG, especially concerning insulin secretion and sensitivity. This research could help to identify new therapeutic approaches for insulin resistance, and type 2 diabetes.

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Author Contribution:

T.W.: planned and performed the experiments, wrote the manuscript, researched the data
M.Bielohuby: performed the experiments and contributed to discussion
T.M.: reviewed/editing the manuscript and contributed to discussion
M.Bidlingmaier: researched the data and contributed to discussion
N.S.P.: planned the experiments, reviewed/editing the manuscript

References


Figure Legends:

**Fig. 1** High ghrelin expression in normal and hyperplastic pancreatic islets in mut/mut rats. (A) Hematoxylin-eosin-stained paraffin-embedded pancreas tissue from 7.5-month-old wt/wt and mut/mut rats. (B) Quantification of the pancreatic islet area of 14 days (d) old and 7.5 months (mo) old rats. The mut/mut rats showed a significant increase in pancreatic endocrine tissue when compared with age-matched the wt/wt rats at both time points. Values represent the mean ± SEM from 8 rats in each group. **p≤0.01; ***p≤0.001. (C-J) Immunofluorescent staining of paraffin-embedded pancreas tissue from 7.5-month-old (C,E,G,I) wt/wt and (D,F,H,J) mut/mut rats. Antibodies against (C,D) insulin, (E,F) glucagon, (G,H) pancreatic polypeptide, and (I,J) somatostatin were used. (G,H) The insets in panels G and H show an enlarged view of single pancreatic polypeptide-positive cells (white arrows) in the endocrine pancreas. (K) Immunofluorescent staining of paraffin-embedded pancreas tissue from 7.5-month-old rats using an antibody against ghrelin revealed higher ghrelin expression in pancreatic islets of the mut/mut rats versus the wt/wt rats.

**Fig. 2** High plasma ghrelin levels together with increased food intake and body weight gain in the mut/mut rats. (A,B) Fasting (A) total and (B) active plasma ghrelin levels of the wt/wt and age-matched mut/mut rats at 2.5, 5.5, and 7.5 months of age. (C) Daily food intake was determined for each individual rat at 2.5, 5.5, and 7.5 months of age. (D) Body weight changes in the wt/wt and mut/mut rats were measured at the indicated time points. The mut/mut rats showed a significant increase in fasting total and active ghrelin, food intake, and body weight gain compared with the age-matched wt/wt rats over time. Values represent the mean ± SEM of n≥8 rats in each group. #p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001.
Fig. 3 Reduced number of ghrelin-producing cells and of GHSR-1a expression in the gastric corpus mucosa of mut/mut rats. (A) Ghrelin-producing cells were detected with an anti-ghrelin antibody in paraffin-embedded gastric corpus tissue from 7.5 months old wt/wt and mut/mut rats. (B) Quantification of the number of ghrelin-positive cells in the gastric corpus mucosa. Values represent the mean ± SEM of n≥8 rats in each group. (C) Total ghrelin content in gastric corpus tissue of 7.5 months-old wt/wt and mut/mut rats. Values represent the mean ± SEM of n≥6 rats in each group. (D) GHS-R1a was detected with an anti-GHS-R1a antibody in paraffin-embedded gastric corpus tissue from 7.5 months old wt/wt and mut/mut rats. The mut/mut rats showed a significant reduction in GHS-R1a in the gastric corpus mucosa compared with the wt/wt rats. (E) GHS-R1a expression in the gastric corpus tissue was measured by real-time PCR and normalized to β2-microtubulin. Gastric corpus tissue from 7.5 months old mut/mut rats (n=8) showed a significant decrease in GHS-R1a expression compared with that from age-matched wt/wt rats (n=8). The box plots show 25th to 75th percentiles (box) and 5th to 95th percentiles (whiskers). The line in the box represents the median. *p≤0.05; **p≤0.01.

Fig. 4 Effect of the GSH-R1a antagonist (D-Lys³)-GHRP-6 on cumulative food intake in food deprived rats. Food intake was measured in 3.5 months old (A) wt/wt and (B) mut/mut rats at different time points (up to 24 h) following intra-peritoneal injection of 12 mg/kgBW (D-Lys³)-GHRP-6 or vehicle (saline). (C) Reduction of cumulative food intake in wt/wt and mut/mut rats following administration of (D-Lys³)-GHRP-6 as above. #p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001.

Fig. 5 Persistently high circulating ghrelin levels cause ghrelin resistance in mut/mut rats. Hypothalamic expression of (A,B) Ghsr-1a, (C,D) NPY, and (E,F) AgRP at (A,C,E) 5.5 months and (B,D,F) 7.5 months of age. 7.5-month-old mut/mut rats showed a significant
reduction in hypothalamic Ghs-r1a, NPY, and AgRP compared with the wt/wt rats. Values represent the mean ± SEM of n≥6 rats in each group. #p>0.05 *p≤0.05; **p≤0.01.

**Fig. 6** Obesity associated with high plasma cholesterol and triglyceride levels and suppressed leptin expression in the white adipose tissue (WAT) of mut/mut rats. (A) The 7.5-month-old wt/wt and mut/mut rats were sacrificed, and epididymal (=Epi), peritoneal (=Peri), and inguinal (=Ing) fat pads were weighted. The mut/mut rats showed a significant increase in WAT mass compared with the wt/wt animals. Values represent the mean ± SEM of n≥8 rats in each group. (B) Cholesterol and (C) triglyceride values were determined using fasting blood plasma (overnight 16 h) from 7.5-month-old wt/wt and mut/mut rats. Values represent the mean ± SEM of n≥8 rats in each group. Leptin expression in (D) epididymal and (E) peritoneal fat was measured by real-time PCR and normalized to β2-microtubulin. Both the epididymal and peritoneal fat from mut/mut rats showed a significant decrease in leptin mRNA compared with that from the wt/wt rats. All box plots show 25th to 75th percentiles (box) and 5th to 95th percentiles (whiskers). The line in the box represents the median.**p≤0.01; ***p≤0.001.

**Fig. 7** High circulating ghrelin levels attenuate GSIS in mut/mut rats. (A,C) Intraperitoneal and (G) oral glucose tolerance tests (i.p.GTT/oGTT) were performed, and (B,D,H) plasma insulin was measured. (E,F,I,J) The corresponding area under the curve (AUC) analysis results are presented. i.p.GTTs were performed at an age of (A+B) 5.5 months and (C+D) 7.5 months. (G,H) oGTTs were performed at 7.5 months. Plasma insulin levels were significantly reduced in mut/mut animals both at 5.5 and 7.5 months of age. Values represent the mean ± SEM of 8 rats in each group. #p>0.05, *p≤0.05, ***p≤0.001.
**Fig. 8** High circulating ghrelin levels may increase the insulin sensitivity in mut/mut rats. Intraperitoneal insulin tolerance tests (i.p.ITTs) were performed at (A+D) 5.5 and (B) 7.5 months of age. (C) Area under the curve (AUC) analysis corresponding to panels A and B is presented. (D) i.p.ITT with a low-dose (0.375 IU/kg) insulin injection at 5.5 months of age. (E) AUC analysis corresponding to panel D is presented. Values represent the mean ± SEM of n≥6 rats in each group. *p≤0.05; ***p≤0.001.
Figure 2:
Figure 3:

A

Wt/wt

Mut/mut

B

C

D

Wt/wt

Mut/mut

E

209x297mm (300 x 300 DPI)
Figure 4:

A  Wt/wt

B  Mut/mut

C

Reduction of food intake (%)
Figure 5:

A

B

C

D

E

F

209x297mm (300 x 300 DPI)
Figure 6:

A.

B.

C.

D.

E.
Figure 7:

(A) 5.5 mo  
(B) 5.5 mo  
(C) 7.5 mo  
(D) 7.5 mo  
(E) AUC Glucose  
(F) AUC Glucagon  
(G) 7.5 mo  
(H) 7.5 mo  
(I) AUC Glucose  
(J) AUC Glucagon

209x297mm (300 x 300 DPI)
Figure 8:

A

B

C

D

E

209x297mm (300 x 300 DPI)
Online Supplemental Data: Supplementary Figures

Supplementary Fig. 1. Hormone expression in pancreatic islets of 14 days old mut/mut and wt/wt rats. (A-H) Immunofluorescent staining of paraffin-embedded pancreas tissue from (A,C,E,G) wt/wt and (B,D,F,H) mut/mut rats. Antibodies against (A,B) insulin, (C,D) glucagon, (E,F) pancreatic polypeptide, and (G,H) somatostatin were used. (I-L) Immunofluorescent staining of paraffin-embedded pancreas tissue using an antibody against (I,K) ghrelin or antibodies against (J,L) ghrelin and glucagon together.

Supplementary Fig. 2. Fasting and re-feeding experiment. (A) Cumulative food intake was measured in food deprived 3.5 months old wt/wt and mut/mut rats at the indicated time points after refeeding. (B,C) In parallel to A, total (B) and active (C) plasma ghrelin levels were measured after fasting and 2h post-re-feeding. *, p<0.05; **p<0.01; ***p<0.001.