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Acylated ghrelin is not required for the surge
in pituitary growth hormone observed in pregnant mice

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Running title: Acylated ghrelin, growth hormone and pregnancy in mice

ABSTRACT

Objective

Ghrelin is produced by the stomach, hypothalamus and pituitary. It circulates as acylated ghrelin (AG, which stimulates growth hormone (GH) secretion) and unacylated ghrelin (UAG).

Acylation is mediated by the enzyme ghrelin *O*-acyltransferase (GOAT). In mice, pregnancy is associated with a marked increase in circulating pituitary GH. We investigated the role of AG and UAG in the surge of plasma GH concentrations in pregnant mice at the end of pregnancy.

Design

Using a mouse model generated on a C57BL/6 background (wild type, WT) in which the GOAT gene has been deleted (KO), we measured plasma AG, UAG and GH concentrations and tissue (stomach, pituitary and hypothalamus) preproghrelin and GOAT mRNA in non-pregnant (NP) and pregnant (P), WT and KO mice.

Results

GOAT deletion was associated with undetectable concentrations of AG. UAG concentrations were similar in all groups. In both WT and KO animals, mean GH concentrations increased 30 to 50 times during pregnancy. There was a tendency towards lower median GH concentrations in KO (301 ng/ml) compared to WT (428 ng/ml) mice ($p=0.059$). Preproghrelin expression was not affected by GOAT deletion or by pregnancy in the stomach. In contrast, pituitary and hypothalamic ghrelin gene expression were lower in KO-NP and KO-P mice compared to their WT counterparts.

Conclusion

The complete absence of ghrelin acylation, which is associated with undetectable AG concentrations, does not prevent the marked increase in pituitary GH concentrations observed in pregnant mice, suggesting that AG is not the major mediator of GH secretion during pregnancy.

KEY WORDS

acylated ghrelin, unacylated ghrelin, growth hormone, pregnancy, ghrelin *O*-acyltransferase, growth hormone secretagogue receptor type 1a, stomach, pituitary, hypothalamus

SPECIAL CHARACTERS

Microgram: μg

Nanogram: ng

Picogram: pg

INTRODUCTION

Growth hormone (GH) release from the anterior pituitary gland classically involves stimulation by growth-hormone-releasing hormone (GHRH) and inhibition by somatostatin from the hypothalamus [1].

Ghrelin is a 28 amino acid hormone mainly produced by the stomach in adult rodents and humans [2]. It circulates as acylated (AG) and unacylated ghrelin (UAG). Acylation is mediated by the enzyme ghrelin *O*-acyltransferase (GOAT) that attaches an octanoate group to the serine-3 residue of the peptide [3]. This is a key step that is required for the binding of AG to the GH secretagogue receptor (GHS-R1a) that mediates many of the actions ascribed to ghrelin [4].

Exogenous administration of AG (but not UAG) causes a marked increase in circulating GH, raising the possibility that similar to somatostatin and GHRH, AG could play a physiological role in GH regulation [5,6]. In addition, it has been recently demonstrated that in mice, vagotomy markedly lowered the GH response to exogenous AG administration suggesting the possibility of vagal stimulation in mediating AG's actions [7].

Plasma GH concentrations are differently affected by pregnancy in humans and in mice. In pregnant humans, maternal pituitary GH is gradually replaced by placental GH which has 91-99% identity with pituitary GH. Pituitary GH concentrations become almost undetectable during the 3rd trimester of pregnancy [8-10]. In mice, the placenta does not produce a GH-like protein but instead produces mouse placental lactogens 1 and 2, which have a low identity with pituitary GH [11]. Plasma concentrations of pituitary GH increase markedly during pregnancy and are 9 times higher in pregnant mice near term compared to non-pregnant mice. However, as the GH binding protein concentrations also markedly increase, the concentration of free GH is little affected by pregnancy [12].

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4 The objective of this study was to investigate the role of AG and UAG in the surge in
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6 plasma GH concentrations in pregnant mice towards the end of pregnancy. Using a mouse model
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8 in which the GOAT gene has been deleted (GOAT KO), we demonstrate that the complete
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10 absence of ghrelin acylation, which is associated with undetectable AG concentrations, does not
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12 prevent the marked increase in pituitary GH concentrations observed in pregnant mice.
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19 MATERIALS AND METHODS

20 Animals and diet

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23 Eleven-12 week old female C57BL/6 wild-type (WT) and GOAT knock-out (KO) mice
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25 were used (Taconic, Hudson, NY, USA). GOAT KO mice are missing the gene that encodes for
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27 GOAT and are therefore unable to acylate ghrelin. They were generated on a C57BL/6
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29 background as previously published [13].
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33 All mice were put on a 12-h light/12-h dark cycle (dark cycle starting at 5:30 PM) and
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35 were fed a 9% fat enriched diet (# 5058, PicoLab® Mouse Diet 20, St. Louis, MO, USA) until
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37 pregnancy was achieved, before being switched to a 5.4% fat standard chow diet (# 5P76,
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39 Prolab® Isopro® RMH 3000, St. Louis, MO, USA) for the duration of the experiment. In
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41 addition to this, a small pellet of Love Mash™ (14.5% fat) (S3823P, BioServ, Frenchtown, NJ,
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43 USA) was added to the mating cages to promote reproduction of the mice. All experiments were
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45 approved by the University of British Columbia animal ethics committee (Protocol # A10-0030,
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47 A10-0041).
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53 Experimental procedure

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55 One male mouse was introduced into a cage consisting of two female mice and was
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57 removed the following day (Day 0). All mice, whether they became pregnant or failed to
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conceive, were fed ad-libitum and sacrificed at Day 18, 4 hours into their light cycle. Trunk blood was collected into chilled EDTA tubes. Blood was treated immediately with 100mM of p-hydroxymercuribenzoic acid (PHMB) to prevent ghrelin degradation. PHMB was selected as we previously demonstrated that, in contrast to other protease inhibitors, it did not interfere with absorbance readings [14]. Blood was then centrifuged and plasma was treated with 1N hydrochloric acid and stored at -20°C until assayed.

Hormone assays

Acylated ghrelin and UAG were measured using a double-antibody enzyme immunometric assay (EIA) (Cayman Chemical©, Ann Harbor, MI, USA). Inter-assay coefficients of variation [CV] were 11.4, 7.0 and 7.0% and intra assay CV were 11.2, 6.8 and 6.7% for concentrations of 2, 25 and 200 pg/mL respectively for AG (#10006307). Inter-assay CVs were 15.9, 5.5 and 4.3% and intra assay CVs were 15.9, 4.8 and 4.0% for concentrations of 2, 25 and 200 pg/mL respectively for UAG (#10008953).

Growth hormone concentrations were measured using an EIA (#EZRMGH-45K, EMD Millipore Corporation©, Billerica, MA, USA). Inter-assay CVs were 4.5, 3.2 and 4.9% for concentrations of 11, 5.6 and 5.2 ng/mL respectively. Intra-assay CVs were 2.3, 4.3, 2.1 and 1.7% for concentrations of 4.1, 2.7, 2.6 and 6.1 ng/mL respectively. Eight GH samples (5 in the WT and 3 in the KO groups) had values that were above the upper limit of detection of the assay and could not be repeated because of low plasma quantity (the concentration of the highest standard was used for statistical analysis).

Gene expression

After collection of the trunk blood, the stomach was excised and placed in a dish with cold 10X diethylpyrocarbonate (DEPC)-treated (D5758, Sigma-Aldrich®, St. Louis, MO, USA)

phosphate buffer saline (PBS) (P3813, Sigma-Aldrich®, St.Louis, MO, USA) solution.

Remnants of food were removed by flushing the lumen of the stomach with cold DEPC treated PBS solution and rinsed further another 3 times. The hypothalamus and pituitary gland were then excised and, along with the stomach, were snap frozen in liquid nitrogen and stored at -80°C.

RNA was isolated from the stomach, hypothalamus and pituitary gland using a mixed isolation method that allows for total protein and total RNA isolation. One microgram of RNA (per sample) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcriptase Kit (#4368814, Applied Biosystems™, Foster City, CA). Quantitative real-time PCR reactions were performed using a ViiA™ Real-Time PCR system. Preproghrelin cDNA was amplified with TaqProbe 2x qPCR Mastermix- ROX (#Mastermix-P, Applied Biological Materials™, Richmond, BC) using a TaqMan probe specific for the gene (Assay IDs: Mm01200389_m1 (GOAT) and Mm00612524_m1 (preproghrelin), Life Technologies™, Burlington, ON). We used the comparative Ct method to determine the mRNA expression of preproghrelin relative to β -actin (a housekeeping gene that was not influenced by our experimental conditions (ie. KO mice and/or pregnancy). The normalized values of these samples ($\Delta Ct = Ct$ of preproghrelin – Ct of β -actin) were then compared to a calibrator consisting of pooled RNA from the WT-NP group to determine the fold-expression of preproghrelin in each of the groups. Preproghrelin expression per nanogram of cDNA was higher in the stomach calibrator compared to the hypothalamus and pituitary calibrator (5035-fold higher compared to pituitary and 6190-fold higher compared to hypothalamus).

Statistical analysis

Except as otherwise noted, data are expressed as median (range) or median (25th – 75th percentile). As many of the data were not normally distributed (Shapiro-Wilk test of normality)

and/or had unequal variances (Levene's Test of Homogeneity), non-parametric tests for independent samples (Kruskal-Wallis) were used to assess the differences between the various experimental groups, followed by Mann-Whitney U tests for the post-hoc analyses between each individual group. Wilcoxon signed-rank test was used to assess the changes in weight during pregnancy. A p value less than 0.05 was considered statistically significant. Data were analyzed with SPSS version 18.0 (2009, Chicago, IL).

RESULTS

Body weights were similar between WT and KO mice at Day 0 ($p>0.05$) (Table 1). In NP mice, median body weight at the time of sacrifice was also similar between the two groups ($p>0.05$). Over the 18 days of the experiment, median (range) weight increased by 1.5g (0.5-1.6)($p=0.01$) in WT-NP mice but not in KO-NP mice. In pregnant mice, weight at 18 days of gestation as well as weight gain during pregnancy (+14.3g [13.7-14.5] in WT-P and +13.0g [13.2-13.7] in KO-P) were similar in both groups ($p>0.05$). The number of fetuses were similar in both groups (8 [4-10] in WT-P and 7 [5-10] in KO-P, $p>0.05$).

Figure 1 shows the plasma concentrations of AG, UAG and GH in WT and KO, NP and P mice. As expected, GOAT deletion (KO mice) was associated with undetectable concentrations of AG. In WT animals, there was no statistically significant difference in AG between NP and P mice at 18 days of gestation ($p=0.36$). Overall, UAG concentrations were similar in all 4 groups ($P=0.73$). In both WT and KO animals, mean GH concentrations increased 30 to 50 times during pregnancy. There was a trend towards lower median [interquartile] GH concentrations in KO (301 [208-355] ng/mL) compared to WT (428 [320-445] ng/ml) mice ($p=0.059$).

Figure 2 describes median (interquartile) preproghrelin (A) and GOAT (B) gene expression in the stomach, pituitary and hypothalamus. Preproghrelin expression was not affected by GOAT deletion or by pregnancy in the stomach. In contrast, there was a statistically significant difference in pituitary and hypothalamus preproghrelin gene expression (both $P=0.001$ by Kruskal-Wallis). Pregnancy caused a 69% increase in pituitary preproghrelin mRNA in WT mice only ($p=0.001$). In the pituitary, preproghrelin gene expression tended to be lower in KO-NP ($p=0.075$) and KO-P ($p=0.048$) mice compared to their WT counterparts. In the hypothalamus, preproghrelin gene expression was lower in both KO-NP and KO-P mice compared to their WT counterparts ($p=0.004$ for NP and $p=0.013$ for P mice) (Fig 2A). As expected, GOAT mRNA was not detectable in KO animals. In WT animals, pregnancy did not significantly affect GOAT mRNA in the stomach or hypothalamus (Fig 2B). In our hands, pituitary GOAT mRNA was below the limit of detection in WT animals.

DISCUSSION

We demonstrate that the physiological surge in pituitary GH during pregnancy in mice is not prevented by the absence of ghrelin acylation secondary to GOAT deletion. To our knowledge, this is the first study investigating the role of the GOAT-ghrelin axis in GH secretion during pregnancy in mice.

When discussing the role of AG, it is important to distinguish between its physiological role and the effect of the administration of exogenous AG on the GH axis. Administration of AG causes a marked increase in circulating GH in humans and mice [5,15]. In contrast, the role of endogenous AG on GH secretion has been more difficult to demonstrate. Several models have been used. Deletion of the preproghrelin gene does not affect GH secretion or growth in mice fed

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4 a standard diet [16,17]. However, because ghrelin production is completely abolished, this model
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6 cannot distinguish between the effects of AG and UAG. If present, anomalies of the
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8 preproghrelin gene do not seem to have major effects on growth in humans.
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11 Deletion of the GHS-R1a (through which AG exerts its effects) abolishes the GH
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13 response to exogenous AG without affecting the response to endogenous UAG. The modest but
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15 negative impact of GHS-R1a deletion on weight gain in these mice may be due to lower IGF-1
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17 production (likely reflecting impaired GH secretion) and decreased fat and muscle mass [18].
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19 The more pronounced effects of GHS-R1a deletion compared to preproghrelin gene deletion may
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21 reflect the fact that GHS-R1 has a high basal, constitutive activity (50% of its maximal activity)
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23 which is unaffected by preproghrelin gene deletion. In humans, variations in the preproghrelin
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25 and GHS-R1a receptor genes are not common causes of short stature [19]. However, point
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27 mutations in the GHS-R1a have been associated with shorter height, suggesting that the ghrelin-
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29 GHS-R1a pathway plays some role in the regulation of longitudinal growth [20,21].
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36 Finally, deletion of GOAT in animal models results in an absence of the acylation of
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38 ghrelin and undetectable circulating plasma AG concentrations. Production of UAG and the
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40 constitutive activation of the GHS-R1a remain present. Growth is not affected [22] and GH
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42 concentrations [23] in mice fed a normal diet remain normal. However, during prolonged
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44 starvation, Zhao *et al* (2010) observed that GOAT and AG were required for euglycemia
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46 maintenance, through their role in stimulating the secretion of GH, a counter regulatory hormone
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48 produced in response to hypoglycaemia. This concept has been recently challenged [24]. Taken
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50 together, these data suggest that there is redundancy in the mechanisms regulating GH secretion
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52 but that in specific circumstances, such as prolonged starvation, endogenous secretion of AG
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54 plays an important physiological role in boosting plasma GH and glucose concentrations.
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4 The cause of the marked increase in circulating GH observed during pregnancy in rodents
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6 remains unclear. The placenta is an unlikely source in rodents, in contrast to humans [25,26]. At
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8 the hypothalamo-pituitary level, a synergistic effect of GHRH and ghrelin could potentiate
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10 pituitary GH release [27-29]. Conversely, a decrease in somatostatin tone could also increase GH
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12 release by the pituitary. However, at least in rats, El Kasti, *et al.* (2008) [26] observed that
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14 hypothalamic GHRH gene expression was slightly decreased while somatostatin expression was
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16 markedly increased during pregnancy. Others reported that GHRH mRNA expression in the
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18 hypothalamus increased while GHRH-R mRNA expression decreased during the last week of rat
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20 pregnancy [30]. This was also associated with increased GHS-R1a expression in the pituitary.
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22 Taken together, these data do not support the concept that major changes in expression in the
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24 hypothalamo-pituitary axis could explain the changes in circulating GH. Recently, it was shown
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26 that the vagus nerve played a crucial role in modulating GH secretion in the rat [7]. Whether
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28 changes in afferent vagal tone could explain the increase in GH secretion by the pituitary in
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30 rodent pregnancy is unknown. Finally, while a decrease in peripheral GH degradation could also
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32 be invoked, we are not aware of any published data supporting this hypothesis.
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41 Our study specifically clarifies the role of ghrelin acylation in plasma GH concentrations
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43 during mice pregnancy. In NP mice, GH concentrations were similar in WT and KO animals
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45 despite the absence of circulating AG in the latter group, suggesting little role, if any, for AG in
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47 GH production under basal circumstances. As expected during pregnancy, we observed a marked
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49 increase in circulating GH in WT mice. Interestingly, this was not associated with a change in
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51 preproghrelin gene expression in the stomach or hypothalamus, but there was a 69% increase in
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53 pituitary preproghrelin mRNA. Consistent with the absence of effect of pregnancy on circulating
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55 AG, GOAT gene expression in the stomach or hypothalamus was not affected by pregnancy. We
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could not detect GOAT mRNA in the pituitary of WT animals, likely the low expression of the gene. Others have reported detectable amounts of GOAT in the mouse pituitary, although the results were lower than in the hypothalamus [32]. In KO animals, despite undetectable circulating AG concentrations, we observed a normal or slightly attenuated ($p=0.059$ compared to WT-P) surge in GH, in the presence of similar (stomach) or lower (pituitary and hypothalamus) preproghrelin gene expression. Taken together, these data suggest that the surge in GH during pregnancy is largely independent of AG concentrations or preproghrelin mRNA expression in the pituitary. The high constitutive activity of GHS-R1a may explain why the absence of AG does not have major effects on the GHS-R1a downstream mechanisms and on GH secretion. Another potential explanation is that, similar to rats, estrogens (known to be elevated during pregnancy) tend to decrease ghrelin secretion by the gastric mucosa in vivo and in vitro without affecting stomach preproghrelin gene expression [31].

In conclusion, we show that acylation of ghrelin by GOAT is not required for the physiological surge in plasma GH concentrations observed during pregnancy in mice. Our study did not address whether ghrelin plays a role in the fine tuning of GH release outside of pregnancy (e.g. by modulating amplitude or timing [1]), but our results further emphasize the difficulty in separating the pharmacological actions from the physiological role of ghrelin in the regulation of GH metabolism.

CONFLICT OF INTEREST

Dr. Mark Heiman (3rd author) is currently an employee at MicroBiome Therapeutics. All other authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Median (interquartile) concentrations for plasma acylated ghrelin (AG), unacylated ghrelin (UAG) and growth hormone (GH) in wild type (WT) and GOAT knock out (KO), non-pregnant (NP) and pregnant (P) mice.

Kruskal-Wallis: $P < 0.0001$ for GH. AG was statistically NS. N=6-14/gr

Mann-Whitney: *: $P < 0.0001$ vs. corresponding group as indicated in the figure.

ND: not detected

Figure 2. Median (interquartile) gene expression for stomach, pituitary and hypothalamus preproghrelin (A) and for stomach and hypothalamus GOAT (B) in wild-type (WT) and GOAT Knock-Out (KO), non-pregnant (NP) and pregnant (P) mice.

Kruskal-Wallis: $P < 0.05$ for pituitary and hypothalamus preproghrelin. All other GOAT and preproghrelin expression was statistically NS. N=6-11/gr (N=4 only for pituitary preproghrelin KO-P group).

Mann-Whitney: *: $P < 0.05$; §: $0.05 < P < 0.1$ vs. corresponding group as indicated in the figure.

TABLE AND TABLE LEGEND

Table 1. Body weights for wild type (WT) and GOAT knock out (KO) non-pregnant (NP) and pregnant (P) mice at Day 0 and 18.

Data are presented as Median [interquartile]

N=10-15/gr for WT and N=6-16/gr for KO mice.

Kruskal-Wallis: $P < 0.001$ for Day 18

Mann-Whitney: * : $P < 0.001$ vs. NP mice counterparts

	Day 0 (grams)	Day 18 (grams)
WT-NP	20.4 [19.2-22.4]	21.9 [20.8-22.9]
WT-P	19.3 [18.6-20.9]	33.6 [32.3-35.4]*
KO-NP	23.6 [21.5-25.18]	23.2 [21.8-24.4]
KO-P	21.2 [20.03-21.9]	34.1 [33.2-35.6]*

Figure 1 (revised)

Figure 1

Figure 2 (revised)

Figure 2

Table 1 (revised)

Table 1

	Day 0 (grams)	Day 18 (grams)
WT-NP	20.4 [19.2-22.4]	21.9 [20.8-22.9]
WT-P	19.3 [18.6-20.9]	33.6 [32.3-35.4]*
KO-NP	23.6 [21.5-25.18]	23.2 [21.8-24.4]
KO-P	21.2 [20.03-21.9]	34.1 [33.2-35.6]*