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Involvement of the GHSR in the developmental programming and metabolic disturbances induced by maternal undernutrition

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Keywords: maternal undernutrition; high-fat diet; ghrelin receptor; mismatch model; development

ABSTRACT

The mismatch between maternal undernutrition and adequate nutrition after birth increases the risk of developing metabolic diseases. We aimed to investigate whether the hyperghrelinemia during maternal undernourishment rewires the hypothalamic development of the offspring and contributes to the conversion to an obese phenotype when fed a high-fat diet (HFD). Pregnant C57BL/6J, wild type (WT) and ghrelin receptor (GHSR)^{-/-} mice were assigned to either a normal nourished (NN) group, or an undernutrition (UN) (30% food restricted) group. All pups were fostered by NN Swiss mice. After weaning, pups were fed a normal diet, followed by a HFD from week 9. Plasma ghrelin levels peaked at postnatal day 15 (P15) in both C57BL/6J UN and NN pups. Hypothalamic *Ghsr* mRNA expression was upregulated at P15 in UN pups compared to NN pups and inhibited agouti-related peptide (AgRP) projections. Adequate lactation increased body weight of UN WT but not of GHSR^{-/-} pups compared to NN littermates. After weaning with a HFD, body weight and food intake was higher in WT UN pups but lower in GHSR^{-/-} UN pups than in NN controls. The GHSR prevented a decrease in ambulatory activity and oxygen consumption in UN offspring during *ad libitum* feeding. Maternal undernutrition triggers developmental changes in the hypothalamus *in utero* which were further affected by adequate feeding after birth during the postnatal period by affecting GHSR signaling. The GHSR contributes to the hyperphagia and the increase in body weight when maternal undernutrition is followed by an obesity prone life environment.

Keywords: maternal undernutrition; high-fat diet; ghrelin receptor; mismatch model; development

1. Introduction

Obesity is a multifactorial disease that affects millions of people throughout the world. Epidemiological and experimental studies in animals suggest that intrauterine undernutrition is closely associated with adulthood obesity [1]. Other studies also suggest that the nutritional status during gestation and lactation has long-term effects on the central and peripheral systems that regulate energy balance in the offspring [2]. Moreover, the association of impaired fetal development and obesity has been clearly demonstrated in the Dutch Famine study, where poor maternal nutrition resulted in increased rates of obesity in adult males [3]. To explain this developmental plasticity, one generally accepted hypothesis called the "Mismatch Hypothesis" has been proposed [2]. This hypothesis suggests that the offspring makes adaptations *in utero* or in the early postnatal developmental period, based on the predicted postnatal environment. The mismatch between maternal undernutrition and adequate nutrition after birth affects the developmental programming of the hypothalamus and increases the risk of developing metabolic diseases.

The hunger hormone ghrelin has been shown to play an important inhibitory role in the development of the neuronal circuits in the hypothalamus during the postnatal period. Ghrelin is a 28-amino acid gastrointestinal peptide, that undergoes octanoylation by the enzyme ghrelin O-acyl-transferase (GOAT) [4]. Octanoyl ghrelin binds to its endogenous receptor, the growth hormone secretagogue receptor (GHSR) which is expressed predominantly in the central nervous system (CNS) (arcuate nucleus, ventral tegmental area, hippocampus, pituitary etc.) with low levels of expression in the gut, pancreatic islets, adrenal gland etc [5]. Recently, liver-expressed antimicrobial peptide 2 (LEAP2) was discovered as an endogenous antagonist of GHSR in the small intestine that blocks constitutive and ghrelin-mediated GHSR activity [6, 7]. It blocks the major effects of ghrelin *in vivo*. Numerous studies have reported that peripheral administration of ghrelin stimulates food intake and adipogenesis [8-10]. Activation of the GHSR on the neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) increases the secretion of NPY and AgRP [9] to stimulate food intake. Studies in single and double mutant mice have shown that both NPY and AgRP are necessary for the effect of ghrelin on food intake [11]. Nevertheless, many studies using *ad libitum*, normal chow-fed conditions show no or only subtle body weight phenotypes for ghrelin^{-/-}, ghrelin receptor (GHSR)^{-/-} and GOAT^{-/-} mice [12-17]. GOAT^{-/-} subjected to severe caloric restriction failed to maintain their blood glucose sugar level and became moribund due to their failure to keep growth hormone levels elevated [18]. It has therefore been suggested that ghrelin is essential to promote survival during caloric restriction. Plasma ghrelin levels are dependent upon the nutritional status and are low in obesity and high during undernutrition and cachexia [19-22].

Nagata et al. [23] showed that rat pups from undernourished mothers, fostered by control mothers that were fed *ad libitum*, had higher plasma ghrelin levels than did controls and were hyperphagic from weaning time until adulthood.

We hypothesize that the hyperghrelinemia during maternal undernutrition will result in adaptations *in utero* to adapt to the restricted nutritional supply and will impair the normal development of the hypothalamic neurocircuitry controlling energy homeostasis during *ad libitum* feeding after birth. The first aim of this study was to investigate how maternal undernutrition affects the offspring's body weight, the timing and magnitude of the surge in postnatal plasma ghrelin levels and the development of AgRP fibers during adequate lactation. The second aim was to investigate the role of ghrelin during maternal undernutrition on lifelong metabolic changes using wild type (WT) and GHSR^{-/-} mice that are fed a high-fat diet (HFD) *ad libitum*.

2. Materials and Methods

2.1 Animals

Male and female C57BL/6J mice (11 weeks) were obtained from Janvier (France). Male and female Swiss (CD-1 (Hsd)) mice (8 weeks) were purchased from the animal facility KU Leuven. Male and female GHSR^{-/-} (129 Sv/Evbrd (LEX1) X C57BL/6 (albino)) mice and their WT littermates were bred in the animal facility KU Leuven and genotyped by PCR on total genomic DNA from the ear as previously described [24]. All mice were housed (20–22 °C) with free access to water under a 12-h: 12-h light-dark cycle. All experimental procedures were approved by the Ethical committee for Animal Experiments of the Catholic University of Leuven (P183/2016).

2.2 Experimental design

2.2.1 C57BL/6J offspring

Swiss mice were mated on the same day as the C57BL/6J mice. Pregnant C57BL/6J mice were assigned to one of two nutritional groups: 1) a normal nourished (NN) group, fed a normal diet *ad libitum* throughout gestation, 2) an undernutrition (UN) group, fed 70% of the food consumed by the NN group from 10 days postcoitum to the day of delivery. The evening before parturition the mice received 2.5 g extra chow to prevent that they will eat their pups. Pups were fostered by Swiss mice whose maternal behavior is not affected by interstrain cross-fostering [25] and who accepted pups from other dams much more easily than C57BL/6J mice. Pregnant Swiss mice were fed a normal diet *ad libitum* throughout gestation and lactation. After delivery, litters from either NN or UN C57BL/6J mothers containing 7-10 pups were selected and fostered by Swiss mothers who gave birth to pups at the same day. Litter size was adjusted to 9 pups per litter by keeping 1-2 Swiss pups or by removing 1 C57BL/6J pup based on C57BL/6J litter size.

After weaning, male offspring from either NN or UN group were fed a normal diet *ad libitum* until week 8, followed by a HFD (protein: 20% kcal, fat: 60% kcal, carbohydrate: 20% kcal, energy density: 5.21 kcal/g, D 12492, Research Diets, Inc., USA) until week 17. Body weight and body length were monitored during the postnatal period (P0-P21) and once a week after weaning.

Pups at P10, P15, P21 and week 17 were anaesthetised through intraperitoneal (i.p.) injection with Anesketin (10 mg/ml; 200 µl) and Xyl-m (1%, 200 µl) after which blood was taken by cardiac puncture or decapitation. Blood samples were supplemented with EDTA (1 mg/ml) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (4 mM), centrifuged and plasma was acidified (10%) with 1 N HCl and frozen in liquid nitrogen. The hypothalamus was dissected using a neonatal brain matrix (Zivic Instruments, USA) and stored in RNALater. Stomach and duodenum were harvested and divided along their longitudinal axis into 2 parts: the right part was kept in RNALater; the left part was kept in liquid nitrogen for tissue extraction.

Fig. 1. Experimental design

2.2.2 WT and GHSR^{-/-} offspring

The same breeding procedure was performed on WT and GHSR^{-/-} mice. After weaning, male WT and GHSR^{-/-} offspring from either NN or UN group were fed a normal diet *ad libitum* until week 8, followed by a HFD until week 19. Body weight and body length were monitored during the postnatal period and once a week after weaning.

2.2.3 Immunostaining

Male C57BL/6J pups at P15 and male WT and GHSR^{-/-} mice at week 19 were anesthetized and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in PBS. The whole brains were collected, post-fixed overnight, then cryoprotected in 30% sucrose (4 °C) for 2 days. The brains were coated with Tissue-Tek® OCT and 20 µm thick coronal sections were cut on a cryostat (Thermo CryoStar NX70, Germany). Sections were stored at -20 °C until use.

Brain sections underwent antigen retrieval (30 min) in sodium citrate buffer (10mM pH=6) at 80 °C, and were blocked with 10% (v/v) donkey serum in 0.1 M PBS-0.3% (v/v) Triton X-100 for 2 h. Afterwards, sections were incubated with primary antibody rabbit anti-AgRP (1:500; Phoenix Pharmaceuticals, USA) 2h at 37 °C and subsequently incubated with secondary antibody Alexa 488 donkey anti rabbit (1:1000; Life technologies, USA) for 90 min at room temperature. Finally, brain sections were mounted with Citifluor AF1. For the negative control, primary antibody was replaced by normal rabbit serum (1:500).

2.2.4 Quantitative analysis of fiber density

The paraventricular nucleus (PVN) region was identified by the morphological landmarks defined in mouse brain atlas (The Mouse Brain in Stereotaxic Coordinates, 2001). Sections through the PVN (Bregma -0.82-0.94 mm) were recorded using a Zeiss LSM780 confocal microscope (25 x, H2O immersion lens, Cell and Tissue Imaging Cluster, KU Leuven, Belgium) assisted with ZEN software (Zeiss). Confocal images were captured using the same settings from 2 to 6 sections (2 views/section) per mouse (n=5-8 mice per group) and image stacks were collected through the Z-axis (5 planes in 10 µm). The density of AgRP immunoreactive fibers of each region of interest (ROI) (200 µmx200 µm) was analyzed using ImageJ/Fiji. All images were adjusted with the same threshold to eliminate background and the integrated intensity of the ROIs was calculated.

2.2.5 Preparation of tissue extracts for ghrelin measurement

Tissues collected from stomach and duodenum were boiled in water for 10 minutes, dried, weighed and 3 volumes of protease inhibitor cocktail (5 mg/ml EDTA; 1.45 µM pepstatin A; 2.1 µM leupeptin; 1.67 mM AEBSF) and 9 volumes of 6% acetic acid were added, followed by homogenization. After centrifugation, the supernatant was boiled for 10 minutes and

centrifuged (45 min) at 4 °C. The supernatant was collected and subjected to radioimmunoassay. Protein levels were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

2.2.6 Ghrelin Radioimmunoassay

Plasma samples were extracted on a Sep-Pak C18 cartridge (Waters Corporation), vacuum-dried. Tissue extracts of stomach and duodenum and plasma samples were subjected to an in house-developed radioimmunoassay which discriminates between octanoyl and total ghrelin [26].

2.2.7 Real-time PCR for *Ghrelin*, *Ghsr*, *Goat*, *Agrp*, *Proopiomelanocortin (Pomc)* and *Leap2*

Total RNA of mouse hypothalamus, stomach and duodenum was isolated using the RNeasy kit (Qiagen) and treated with Turbo DNA-free™ kit (Thermo Fisher Scientific, USA). The RNA was reverse transcribed to cDNA using the high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA). The qRT-PCR reaction was performed using the Lightcycler 480 (Roche Diagnostics, Switzerland) with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics, Switzerland). A calibrator was used to correct for inter-run variability between plates [27]. Results were expressed relative to the geometric mean of the normalized expression [28] of the three most stable housekeeping genes ($2^{-\Delta\Delta C_t}$) (hypothalamus: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), ribosomal protein L13a (*Rpl13a*), cyclophilin (*Cycloph*); stomach: β -actin, *Rpl13a*, hydroxymethylbilane synthase (*Hmbs*); duodenum: *Cycloph*, β -actin, TATA box binding protein (*Tbp*)). Primer sequences are shown in Table 1.

Table 1. Primer sequences

Genes	Forward primer	Reversed primer
<i>Ghrelin</i>	CCAgAggACAgAggACAAgC	ACATCgAAgggAgCATTgAA
<i>Ghsr</i>	TCAgggACCAgAACCACAAA	CCAgCAgAggATgAAAgCAA
<i>Goat</i>	ATTTgTgAAgggAAgTggAg	CAGgAgAgCAgggAAAAgAg
<i>Agrp</i>	gCggAggTgCTAgATCCA	AggACTCgTgCAgCCTTA
<i>Pomc</i>	ACCTCACCACggAgAgCA	gCgAgAggTCgAgTTTgC
<i>Leap2</i>	CTGCTGGGTCAGGTCAATAGT	ACACTTAGGGAACAGCGTCT
<i>Gapdh</i>	CCCCAATgTgTCCgTCgTg	gCCTgCTTCACCACCTTCT
<i>Rpl13a</i>	CACTCTggAggAgAAACggAAgg	gCAGgCATgAggCAAACAgTC
<i>Cycloph</i>	ggAgATggCACAggAggAAA	CCCgTAgTgCTTCAgCTTgAA
β -actin	gATCTggCACACACCTTCTAC	TggATggCTACgTACATggCTg
<i>Hmbs</i>	CTgAAggATgTgCCTACCATAC	AAGgTTTCCAgggTCTTTCC

Tbp

AggATgCTCTAgggAAgAT

TgAATAggCTgTggAgTAAgT

2.2.8 Blood glucose levels and oral glucose tolerance test (OGTT)

Blood glucose levels were measured using a glucometer (Glucomen LX Plus⁺, A. Menarini Diagnostics, Belgium) at the time of sacrifice (P10, P15, P21 and week 17) between 9-12 A.M.. C57BL/6J mice (week 13) and WT and GHSR^{-/-} mice (week 17) were fasted 6 hours with free access to water from 7 A.M. to 1 P.M. before gavage with 2g D-glucose/kg. Blood glucose levels were measured with a glucometer from the tail vein at 0, 15, 30, 60 and 120 minutes after glucose administration.

2.2.9 Metabolic changes

After 6 weeks on a HFD, mice were housed individually in automated calocages for indirect calorimetry (Phenomaster Calocages TSE systems, Germany) on a 12-h: 12-h light-dark cycle at 20–22 °C with *ad libitum* access to HFD and water. Prior to actual measurements, mice were acclimatized to single housing and specific drinking bottles for 1 week. Food intake, O₂ & CO₂ consumption, heat production and ambulatory activity were continuously recorded. Respiratory exchange ratio and energy expenditure were calculated. To exclude new cage environment bias, measurement of 72 hours of which the last 48 hours were used.

2.2.10 Echo MRI measurement

At week 18, total fat and lean mass of WT and GHSR^{-/-} mice were measured using Echo MRI-100H system (EchoMRI LLC, TX, USA).

2.3 Statistics

Results are presented as mean ± SEM. Differences in body weight, body length, food intake, body composition and density of AgRP fibers were analyzed using a mixed model (SAS/Stat 14.1, SAS Institute Inc., Cary, USA). Data of ghrelin levels and mRNA expression were analyzed using two-way ANOVA (factors: age and nutritional status), followed by planned comparisons (Statistica 13, StatSoft) and corrected by Holm-bonferroni correction. For oral glucose tolerance tests and metabolic changes, Repeated Measures ANOVA was used (Statistica 13, StatSoft). Significance was accepted at the 5% level.

3. Results

3.1 Effect of maternal undernutrition on developmental changes in body weight, ghrelin parameters and hypothalamic neuropeptide expression during lactation

3.1.1 Effect on body weight and length

At birth, body weight was lower ($P<0.05$) and body length was shorter ($P<0.001$) of maternal undernourished (UN) compared to normal nourished (NN) C57BL/6J pups. After fostering *ad libitum* by NN Swiss mothers, body weight gain (age x group: $P<0.01$) and body length gain (age x group: $P<0.001$) was higher in UN pups than in NN pups after the 3 weeks of lactation (Fig. 2 A, B). Post-hoc analysis showed that catch-up growth already started at P2. Body weight of NN pups was higher when fostered by NN Swiss mothers compared to NN C57BL/6J mothers, indicating that pups were overfed during lactation (Fig. S1).

Fig. 2. Changes in body weight/length and octanoyl ghrelin levels in NN and UN pups during lactation (A, B) Percent increase in body weight and body length in NN pups and UN pups during lactation (P0: n=95-135, P2: n=79-85, P5: n=77-85, P10: n=74-78, P15: n=60-68, P21: n=35) (a, b) body weight gain and body length gain at P21 (C) Age-dependent changes in plasma ghrelin levels of NN and UN pups during lactation (P10: n=12-14; P15: n=16-20; P21: n=30) (D, E) Postnatal changes in stomach and duodenal octanoyl ghrelin tissue content in NN and UN pups during lactation (P10: n=16-26; P15: n=17-24; P21: n=31) (F) Correlations between plasma octanoyl ghrelin levels and ghrelin tissue content in stomach and duodenum (Stomach: Black dots; Duodenum: Grey dots). Data are presented as mean $\pm$ SEM. **: $P < 0.01$, ***: $P < 0.001$ vs. P10 in NN and UN; ££: $P < 0.01$ vs. P15 in NN and UN; ##: $P < 0.01$, ###: $P < 0.001$ UN vs. NN, &: $P < 0.05$, &&: $P < 0.01$, &&&: $P < 0.01$ vs. NN.

3.1.2 Effect on plasma ghrelin levels and ghrelin tissue content

A steep increase in plasma octanoyl ghrelin levels was observed between P10 and P15 in both NN (age effect: $P<0.001$) and UN (age effect: $P<0.001$) groups (Fig. 2 C). Between P15 and P21 octanoyl ghrelin levels significantly decreased in both groups but remained significantly higher compared to P10. Two-way ANOVA analysis did not reveal an effect of maternal undernourishment on the age-dependent changes in plasma ghrelin levels during lactation. Gender did not affect age-dependent changes in plasma ghrelin levels in NN and UN pups (Fig. S2).

The octanoyl ghrelin content in stomach tissue extracts gradually increased from P10 to P21 and was not affected by maternal undernourishment nor by gender (Fig. 2 D, S3). However, no age-dependent changes in ghrelin content were observed in the duodenum (Fig. 2 E). Plasma octanoyl ghrelin levels correlated moderately with stomach ($R=0.2212$; $P=0.014$) but not with duodenal ($R=0.0036$; $P=0.969$) ghrelin tissue content (Fig. 2 F).

3.1.3 Effect on *Ghrelin*, *Goat* and *Leap2* mRNA expression

The relative mRNA expression levels of *Ghrelin* ($P<0.001$) and *Goat* ($P<0.05$) in the stomach were significantly increased at P15 and P21 compared to P10 in both NN and UN groups and were not affected by maternal undernutrition (Fig. 3 A, B). Moreover, a decrease ($P<0.001$) (Fig. 3 B) was observed in *Goat* expression between P15 and P21 in UN pups.

In contrast to the stomach, duodenal mRNA expression levels of *Ghrelin* ($P<0.001$) were significantly decreased at P15 and P21 compared to P10 in both NN and UN groups (Fig. 3 C). Duodenal *Goat* mRNA expression differed between NN and UN pups (age x group: $P<0.001$). Compared to NN pups, UN pups had lower duodenal *Goat* ($P<0.001$) mRNA expression at P15 but higher *Goat* ($P<0.05$) mRNA expression at P21 (Fig. 3 D). The relative mRNA expression of the GHSR antagonist *Leap2* showed an age-dependent decrease ($P<0.05$) in both groups but did not differ between NN and UN pups (Fig. 3 E). The relative mRNA expression of *Ghrelin* in the stomach showed a trend ($R=-0.328$; $p=0.058$) towards a negative correlation with duodenal *Leap2* mRNA levels (Fig. S4).

Fig. 3. Relative mRNA expression of *Ghrelin*, *Goat* and *Leap2* in NN and UN male pups during lactation (A, B) Relative mRNA expression of *Ghrelin* and *Goat* in the stomach (C-E) Relative mRNA expression of *Ghrelin*, *Goat* and *Leap2* in the duodenum from NN and UN groups during lactation (P10: n=5-8; P15: n=6-8; P21: n=7-8). Data are presented as mean $\pm$ SEM. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ vs. P10 in NN and UN; £££: $P<0.001$ vs. P15 in NN and UN; #: $P<0.05$, ####: $P<0.001$ UN vs. NN.

3.1.4 Effect on hypothalamic *Ghsr*, *Agrp* and *Pomc* mRNA expression

In contrast to the UN pups, the relative mRNA expression levels of *Ghsr* in NN pups were not age-dependent resulting in a significant interaction effect (age $\times$ group: $P<0.05$). *Ghsr* mRNA expression was significantly ($P<0.05$) upregulated in UN pups at P15 compared to NN pups (Fig. 4 A). Age-dependent changes were observed in the mRNA expression of *Agrp* (age $\times$ group: $P<0.05$) and *Pomc* (age $\times$ group: $P<0.01$) in both NN and UN groups. Notably, both *Agrp* ($P<0.05$) and *Pomc* ($P<0.001$) mRNA expression showed a significant increase at P15 in UN pups compared to NN pups (Fig. 4 B, C).

Ghrelin has been shown to inhibit the density of the neural projections from the ARC to the PVN during the preweaning period in pups from mice that were fed ad libitum during pregnancy [29]. The upregulation of the GHSR in UN pups prompted us to investigate its role on the density of AgRP fibers which can be used as a selective marker for ARC projections to the PVN [30]. The density of AgRP-immunoreactivity (IR) fibers innervating the PVN was downregulated ($P<0.05$) in UN pups compared to NN pups at P15 (Fig. 4 D, E). This was in agreement with the increase in hypothalamic *Ghsr* mRNA expression at P15 in NN compared to UN pups and the known inhibitory effect of ghrelin on AgRP fiber density.

Fig. 4. Relative mRNA expression of *Ghsr*, *AgRP* and *Pomc* in the hypothalamus and AgRP-IR fiber density in the PVN of NN and UN male pups during lactation (A-C) Relative mRNA expression of *Ghsr*, *AgRP* and *Pomc* in the hypothalamus of NN and UN groups during lactation (P10: n=7; P15: n=6-8; P21: n=7-8) (D, E) Effects of maternal undernutrition on AgRP-IR fiber density in the PVN of NN and UN pups at P15. Representative confocal image including an insert with a higher magnification to depict AgRP fibers (D) and quantification with Image J (E). The square indicates the region of interest (ROI) in the PVN used for quantification, Scale bars: 50 μ m. NN (n=6), UN (n=7). Data are presented as mean $\pm$ SEM. *: $P < 0.05$, ***: $P < 0.001$ vs. P10 in NN and UN; ££: $P < 0.01$ vs. P15 in NN and UN; #: $P < 0.05$, ###: $P < 0.001$ UN vs. NN.

3.2 Effect of maternal undernutrition on changes in body weight, glucose tolerance, ghrelin parameters and hypothalamic neuropeptide expression after feeding a HFD *ad libitum*

3.2.1 Effect on changes in body weight

After weaning with a normal diet, no difference in body weight was observed between both groups until week 8. After feeding a HFD from week 9 to week 17, UN offspring gained more weight (time $\times$ group: $P < 0.001$) than NN offspring (Fig. 5 A). A significant increase in body weight was observed at week 17 in UN offspring compared to NN offspring (Fig. 5 C). No difference in body length was observed between the two groups after feeding with either a normal diet or a HFD (Fig. 5 B).

Fig. 5. Changes in some metabolic parameters in NN and UN male offspring after feeding with normal diet (week 3-8) and a HFD (week 9-17) (A) Body weight of NN and UN offspring (NN: n=5, UN: n=5) (B) Body length of NN and UN offspring (NN: n=5, UN: n=5) (C) Body weight of NN and UN offspring at week 17 (NN: n=5, UN: n=5) (D) Plasma octanoyl ghrelin levels at week 17 (NN: n=5, UN: n=5) (E-G) Relative mRNA expression of *Ghhr*, *AgRP* and *Pomc* in the hypothalamus of NN and UN groups at week 17 (NN: n=5, UN: n=5). Data are presented as mean $\pm$ SEM. &&: $P < 0.01$ vs. NN; ###: $P < 0.001$ UN vs. NN on a HFD.

3.2.2 Effect on changes in plasma ghrelin levels, hypothalamic *Ghhr*, *AgRP* and *Pomc* mRNA expression and glucose tolerance

There was no difference in plasma octanoyl ghrelin levels and in the relative mRNA expression of *Ghhr*, *AgRP* and *Pomc* between the two groups at postnatal week 17 (Fig. 5 E-G).

An oral glucose tolerance test was performed 5 weeks after starting a HFD (postnatal week 13). Offspring were glucose intolerant but no difference between NN and UN offspring was observed (Fig. S5 A, B). However, UN offspring had higher ($P < 0.05$) non-fasted plasma

glucose levels compared to NN offspring at the time of sacrifice (postnatal week 17) (Fig. S5 C).

3.3 Role of the GHSR in the metabolic changes induced by maternal undernutrition after feeding a HFD *ad libitum*

3.3.1 Effect on body weight

The role of the GHSR in the increase in body weight after feeding a HFD after maternal undernutrition was investigated in GHSR^{-/-} mice. After fostering *ad libitum* by Swiss mice, body weight of WT UN pups was significantly (age x group: $P < 0.001$) higher compared to WT NN pups from P10 onwards until the end of the lactation period (Fig. 6 A). However, maternal undernourishment did not result in changes in body weight in GHSR^{-/-} UN pups compared to GHSR^{-/-} NN pups (Fig. 6 B).

After weaning with a normal diet (week 4-8), no difference in body weight was observed between NN and UN groups in WT offspring (Fig. 6 C). However, body weight of GHSR^{-/-} UN offspring was lower (age x group: $P < 0.001$) compared to NN offspring (Fig. 6 D). After feeding a HFD from week 9 to week 19, WT UN offspring gained more weight (age x group: $P < 0.01$) while GHSR^{-/-} UN offspring gained less weight (age x group: $P < 0.001$) compared to NN offspring (Fig. 6 C, D). The time-dependent changes after weaning in body weight between both maternal nutritional conditions were genotype-dependent (age x group x genotype: $P < 0.05$). At week 18, body weight, fat and lean mass, as measured with Echo-MRI, did not differ anymore in WT NN and UN offspring. However, GHSR^{-/-} UN offspring showed lower ($P < 0.05$) body weight and lean mass compared to NN offspring (Fig. 6 E, F).

Fig. 6. Changes in body weight and body composition of WT and GHSR^{-/-} NN and UN offspring
 (A, B) Changes in body weight of WT and GHSR^{-/-} pups in NN and UN groups during lactation (P0: n=24-35, P2: n=21-33, P5: n=21-32, P10: n=21-33, P15: n=21-33, P21: n=21-33) (a, b) body weight at P21 in WT and GHSR^{-/-} pups (C, D) Changes in body weight of WT and GHSR^{-/-} NN and UN male offspring (NN: n=8, UN: n=11-14) after feeding with a normal diet (week 4-8) and a HFD (week 9-19) (E, F) Body weight, fat and lean mass in NN and UN male offspring at week 18 after feeding a HFD from week 9 (NN: n=8, UN: n=11-12). Data are presented as mean $\pm$ SEM. &&&: P<0.01 vs. NN; #: P<0.05, ##: P<0.01, ###: P<0.001 UN vs. NN.

3.3.2 Effect on metabolic parameters

Compared to NN offspring, maternal undernutrition decreased oxygen consumption (Day: $P<0.01$, Night: $P<0.001$) and ambulatory activity (Day: $P<0.05$, Night: $P<0.001$) in $GHSR^{-/-}$ but not in WT offspring at week 15 (Fig. 7 A, C). These changes were genotype-dependent (oxygen consumption, Day: $P<0.05$, Night: $P<0.01$; ambulatory activity, Day and Night: $P<0.01$). A decreased ($P<0.05$) heat production was observed in both WT and $GHSR^{-/-}$ UN offspring compared to NN offspring during the night (Fig. 7 B). Neither maternal undernutrition nor genotype affected the mean respiratory exchange rate of the offspring (Fig. 7 D).

Fig. 7. Metabolic changes in WT and $GHSR^{-/-}$ NN and UN male offspring after feeding a HFD (week 9-15) (A) Oxygen consumption, (B) Heat production, (C) Ambulatory activity and (D) Mean respiratory exchange rate of NN and UN offspring at week 15 after feeding a HFD (NN: $n=8$, UN: $n=11-14$). Data are presented as mean $\pm$ SEM. *: $P<0.05$, **: $P<0.01$ WT vs. $GHSR^{-/-}$; #: $P<0.05$, ##: $P<0.01$; ###: $P<0.001$ UN vs. NN.

3.3.3 Effect on food intake, glucose tolerance and hypothalamic density of AgRP projections

After weaning, food intake of WT UN offspring was higher than of NN offspring after feeding with either a normal diet (age x group: $P<0.01$) or a HFD (age x group: $P<0.05$) (Fig. 8 A). $GHSR^{-/-}$ UN offspring ate less ($P<0.05$) food only after feeding with a HFD from week 9 to week 19 compared to NN offspring (Fig. 8 B). The time-dependent changes after a HFD fed in food intake between both maternal nutritional conditions were genotype-dependent (age x group x genotype: $P<0.01$).

All mice were glucose intolerant but no difference in glucose levels was observed between NN and UN offspring of both genotypes at week 17 during an OGTT (Fig. 8 C, D).

At week 19, no differences in the density of AgRP-IR fibers were observed between NN and UN offspring of both WT and $GHSR^{-/-}$ genotypes (Fig. 8 E, F).

Fig. 8. Changes in food intake, glucose tolerance and density of AgRP projections in the PVN in male offspring (A, B) Changes in food intake of WT and GHSR^{-/-} NN and UN offspring after feeding with normal diet (week 4-8) and a HFD (week 9-19) (NN: n=8, UN: n=11-14) (a, b) food intake at week 19 in WT and GHSR^{-/-} pups (C, D) Glucose levels and area under curve during an OGTT (postnatal week 17) in WT and GHSR^{-/-} NN and UN offspring at week 17 after feeding a HFD from week 9 (NN: n=8, UN: n=11-14) (E, F) Effects of maternal undernutrition on AgRP-IR fiber density in the PVN of WT and GHSR^{-/-} offspring after feeding a HFD (week 9-19). Representative confocal image (E) and quantification with Image J (F). (WT: NN: n=8, UN: n=6; GHSR^{-/-}: NN: n=7, UN: n=5) Square indicates the ROI in the PVN used for quantification, Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM. #: P<0.05, ##: P<0.01 UN vs. NN.

4. Discussion

In the current study, a maternal undernourished mouse model was used to investigate how the mismatch between undernutrition during pregnancy and adequate nutrition after birth affects the developmental programming of the hypothalamus and increases the risk of developing metabolic diseases. Hormones and nutrients are considered as important mediators that transmit signals from the mother to program the metabolic phenotype of the developing offspring. It has been shown that there is an early perinatal window during which developmental programming can be reset if adequate nutrition is available after birth [31]. The hunger hormone ghrelin is known to exert an organizational effect on neural projections from the hypothalamus, the key brain center involved in the control of food intake [29].

Our data showed that body weight and length of maternal undernourishment pups was lower than of normal nourished pups. After fostering by Swiss mice with adequate nutrition, a catch-up growth was observed in UN pups compared to NN pups as reported in previous studies [32]. Also children that are born small for gestational age show catch-up growth during the first 2 years of life [33]. This growth acceleration in UN pups during the postnatal period may result from fetal programming under maternal undernourishment or altered development after birth. Indeed, environmental factors during gestation can influence the epigenetic state of the genome and this has been shown convincingly for diet [34]. For example, maternal undernutrition has been shown to induce epigenetic changes in sheep fetal and hypothalamic proopiomelanocortin and glucocorticoid receptor genes [35]. There is evidence that several hormones such as corticosterone, insulin and leptin can cross the placental-fetal barrier to potentially affect fetal development and thus it is likely that fetuses have hormone levels that mirror those of their dams [36-38]. Apart from these hormones also ghrelin is a prime candidate for perinatal metabolic programming [39]. Ghrelin is present in the rat fetal amniotic fluid [40] and ghrelin has been shown to stimulate proliferation from the spinal cord and hypothalamus in the neonatal period in rats [41].

Ghrelin levels fluctuate in accordance with the nutritional status and increase during diet-induced weight loss [42]. We hypothesized that the increase in plasma ghrelin levels due to undernourishment in the pregnant mice would be transmitted to the fetuses and maintained in UN pups after birth. Ghrelin levels could not be measured immediately after birth but did not differ between NN and UN pups at postnatal day 10. This was probably due to the fact that both groups were fostered by *ad libitum* fed dams during this period. It has been shown that postnatal overfeeding attenuates ghrelin levels [43].

In contrast to humans, development of hypothalamic neurocircuits in rodents is not completed at birth but continues to develop until the third week of life [44-46]. Thus, with respect to neurobiological development, the lactation period in mice is comparable with the third trimester of human pregnancy. Previous studies have shown that ghrelin and leptin play an important role in the development of hypothalamic neuronal connections during this period. Plasma leptin level show a surge between P4-16 and promote the development of projections from the ARC to other hypothalamic nuclei, including the dorsomedial hypothalamus (P6), PVN (P8-P10), and lateral hypothalamus (P12) [44]. This is followed by an increase in plasma ghrelin levels at the end of the second week which limit leptin-stimulated growth of hypothalamic connections [29]. Our results confirm the surge in plasma ghrelin levels at P15 in both NN and UN groups during the postnatal period. Furthermore, the increase in plasma ghrelin levels was mirrored by an increase in gastric ghrelin tissue content and gastric *Ghrelin* and *Goat* mRNA production in both NN and UN groups. In contrast, duodenal ghrelin tissue content did not change during the postnatal period and duodenal *Ghrelin* and *Goat* mRNA production even decreased. These findings indicate the stomach was the source of the increase in plasma ghrelin levels at P15. Recently it was shown that plasma levels of LEAP2, an endogenous GHSR antagonist present in the small intestine fluctuate inversely with plasma octanoyl ghrelin levels under different metabolic conditions [47]. Although we did not measure plasma LEAP2 levels, our results showed a trends towards an inverse correlation between gastric *Ghrelin* and duodenal *Leap2* mRNA

expression suggesting that LEAP2 may facilitate the action of ghrelin in the postnatal period. These parameters were not influenced by maternal undernutrition.

Ghrelin stimulates food intake by acting on the GHSR in the ARC to induce the release of NPY and AgRP. In contrast to plasma ghrelin levels, no time-dependent changes were observed in hypothalamic *Ghsr* mRNA expression during the postnatal period in NN pups but in UN pups an increase in *Ghsr* mRNA expression was observed at P15. Epigenetic adaptations or other effects during maternal undernourishment may contribute to this effect. Of note, *Ghsr* mRNA expression did not differ between both feeding groups at P10 suggesting that fetal programming *in utero* is not the only reason for the developmental changes. These data are also in agreement with the 'mismatch' hypothesis that the mismatch interferes with the postnatal development. An altered milieu during the early postnatal period can not only predispose to metabolic disorders but may override prenatal factors to develop obesity [48].

Neonatal ghrelin specifically inhibits the development of ARC projections [29]. AgRP immunolabeling can be used as a marker of ARC projections because AgRP expression is restricted to the ARC in the adult mouse brain [49]. The PVN acts as the 'second order' nucleus responding indirectly to AgRP and NPY released from ghrelin-activated ARC neurons. Immunostaining showed that the density of AgRP-IR fibers innervating the PVN was downregulated in UN pups compared with NN pups at P15. This downregulation was consistent with the upregulation of the hypothalamic mRNA expression of *Ghsr* where ghrelin binds to and with the known inhibitory effect of ghrelin on axonal projections from the ARC. In addition, both *Agrp* and *Pomc* mRNA expression showed a significant increase at P15 in UN pups compared to NN pups which may be the result of an increase in AgRP and POMC neurons due to fetal programming during undernutrition. However, since UN pups showed less AgRP fibers in PVN, the elevated *Agrp* mRNA expression may represent a feedback signal as well. Our findings indicate that maternal undernutrition does not only remodel AgRP related pathways but also other hypothalamic peptidergic pathways like the *Pomc* related pathway.

After weaning with a normal diet, no differences in body weight were observed between both groups. However after feeding a HFD, UN offspring gained more weight than NN offspring. OGTT could not indicate a difference in glucose tolerance between both groups but non-fasted glucose levels were higher at week 17 in the undernourished offspring. It cannot be excluded that changes in insulin sensitivity occur in UN offspring. Our findings indicate that maternal undernutrition predisposes the offspring for metabolic diseases.

To study the role of the GHSR in our mismatch model that predisposes to body weight gain, maternal undernourished WT and GHSR^{-/-} mice were fostered by *ad libitum* fed dams and after weaning, subjected to 4 weeks of a normal diet followed by 11 weeks of a HFD. Unlike in WT mice, maternal undernourishment did not result in catch-up growth in GHSR^{-/-} UN pups during lactation. Moreover, whereas WT mice were hyperphagic and gained more weight during the HFD, GHSR^{-/-} UN offspring ate less food and gained less weight. Our findings indicate that the GHSR is involved in the hyperphagia and body weight gain in the mismatch model.

It was previously shown that pups from normal nourished mothers that were injected with an anti-ghrelin compound during lactation developed higher body weights [29]. This phenotype is opposite to the decrease in body weight gain observed in GHSR^{-/-} UN and shows that the GHSR plays an important role in fetal development during maternal undernutrition and not only in the postnatal window. The decrease in the density of AgRP projections observed during the lactation period was lost in adult UN versus NN mice of both genotypes which point towards the development of compensatory mechanisms.

Of note, while the increase in body weight after HFD feeding in C57BL/6J UN offspring compared to NN offspring was maintained until the end of the study, in WT UN mice it was lost at week 18. As mentioned in the methods, WT littermates were derived from a mixed background (C57BL/6, 129Sv). It has been shown that mouse strains respond with a different sensitivity to a HFD exposure which could affect the severity of metabolic outcomes including diet-induced obesity, glucose intolerance and insulin resistance [50]. For example for GHSR,

it has been shown that the magnitude of the body weight difference between WT and GHSR^{-/-} mice fed a HFD was dependent on the background of the mice [16, 51]. In addition, the susceptibility to diet-induced obesity of different mouse strains is highly dependent on the feeding duration, diet composition and housing conditions.

Surprisingly, although ghrelin is known to stimulate adipogenesis, the decrease in body weight in GHSR^{-/-} UN was not due to a decrease in fat mass but to a decrease in lean mass. It has been shown that both ghrelin and a GHSR agonist can improve food intake, weight gain and lean body mass retention in a rat model of cachexia via an increase in the expression of hypothalamic orexigenic neuropeptides and a decrease in IL-1 receptor expression [52]. Maternal malnutrition is known to alter the offspring's exposure to immune-related factors [53]. In GHSR^{-/-} UN mice, loss of the GHSR may promote inflammatory changes and affect lean mass.

Compared to NN offspring, maternal undernutrition decreased oxygen consumption and ambulatory activity in GHSR^{-/-} but not in WT offspring. These findings indicate a protective role of the GHSR in these metabolic changes. Ghrelin is known to activate the reward system and to promote food seeking behaviour [54]. The decreased locomotor activity in GHSR^{-/-} UN mice may be a protective mechanism to prevent food seeking behaviour and hence an increase in body weight as observed in WT UN mice. Decreased heat production was observed in both WT and GHSR^{-/-} UN offspring compared to NN offspring during the night. Thus, thermogenesis of UN offspring was disturbed by maternal undernourishment but was not affected by the GHSR.

In conclusion, our study shows that the GHSR plays a role in the regulatory system of the energy balance that has been programmed for a thrifty postnatal lifestyle by restricted feeding in utero but that has been challenged by adequate nutrition after birth.

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Declarations of interest

None

Author Contributions

Shu Sun, Bart Van Der Schueren and Inge Depoortere conceived and designed the experiments. Shu Sun, Katrien Corbeels, Louis Desmet, Anneleen Segers and Qiaoling Wang performed the experiments. Shu Sun and Inge Depoortere analysed the results and wrote the manuscript. All authors reviewed the manuscript.

Ethics approval

Ethical Committee for Animal Experimentation of the KU Leuven.

Figure Captions

Fig. 2. Changes in body weight/length and octanoyl ghrelin levels in NN and UN pups during lactation (A, B) Percent increase in body weight and body length in NN pups and UN pups during lactation (P0: n=95-135, P2: n=79-85, P5: n=77-85, P10: n=74-78, P15: n=60-68, P21: n=35) (a, b) body weight gain and body length gain at P21 (C) Age-dependent changes in plasma ghrelin levels of NN and UN pups during lactation (P10: n=12-14; P15: n=16-20; P21: n=30) (D, E) Postnatal changes in stomach and duodenal octanoyl ghrelin tissue content in NN and UN pups during lactation (P10: n=16-26; P15: n=17-24; P21: n=31) (F) Correlations between plasma octanoyl ghrelin levels and ghrelin tissue content in stomach and duodenum (Stomach: Black dots; Duodenum: Grey dots). Data are presented as mean $\pm$ SEM. *: $P<0.01$, ***: $P<0.001$ vs. P10 in NN and UN; ££: $P<0.01$ vs. P15 in NN and UN; ###: $P<0.01$, ####: $P<0.001$ UN vs. NN, &: $P<0.05$, &&: $P<0.01$, &&&: $P<0.01$ vs. NN.

Fig. 3. Relative mRNA expression of *Ghrelin*, *Goat* and *Leap2* in NN and UN male pups during lactation (A, B) Relative mRNA expression of *Ghrelin* and *Goat* in the stomach (C-E) Relative mRNA expression of *Ghrelin*, *Goat* and *Leap2* in the duodenum from NN and UN groups during lactation (P10: n=5-8; P15: n=6-8; P21: n=7-8). Data are presented as mean $\pm$ SEM. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ vs. P10 in NN and UN; £££: $P<0.001$ vs. P15 in NN and UN; #: $P<0.05$, ####: $P<0.001$ UN vs. NN.

Fig. 4. Relative mRNA expression of *Ghsr*, *Agrp* and *Pomc* in the hypothalamus and AgRP-IR fiber density in the PVN of NN and UN male pups during lactation (A-C) Relative mRNA expression of *Ghsr*, *Agrp* and *Pomc* in the hypothalamus of NN and UN groups during lactation (P10: n=7; P15: n=6-8; P21: n=7-8) (D, E) Effects of maternal undernutrition on AgRP-IR fiber density in the PVN of NN and UN pups at P15. Representative confocal image including an insert with a higher magnification to depict AgRP fibers (D) and quantification with Image J (E). The square indicates the region of interest (ROI) in the PVN used for quantification, Scale bars: 50 μ m. NN (n=6), UN (n=7). Data are presented as mean $\pm$ SEM. *: $P<0.05$, ***: $P<0.001$ vs. P10 in NN and UN; ££: $P<0.01$ vs. P15 in NN and UN; #: $P<0.05$, ####: $P<0.001$ UN vs. NN.

Fig. 5. Changes in some metabolic parameters in NN and UN male offspring after feeding with normal diet (week 3-8) and a HFD (week 9-17) (A) Body weight of NN and UN offspring (NN: n=5, UN: n=5) (B) Body length of NN and UN offspring (NN: n=5, UN: n=5) (C) Body weight of NN and UN offspring at week 17 (NN: n=5, UN: n=5) (D) Plasma octanoyl ghrelin levels at week 17 (NN: n=5, UN: n=5) (E-G) Relative mRNA expression of *Ghsr*, *Agrp* and *Pomc* in the hypothalamus of NN and UN groups at week 17 (NN: n=5, UN: n=5). Data are presented as mean $\pm$ SEM. &&: $P<0.01$ vs. NN; ####: $P<0.001$ UN vs. NN on a HFD.

Fig. 6. Changes in body weight and body composition of WT and *GHSR*^{-/-} NN and UN offspring (A, B) Changes in body weight of WT and *GHSR*^{-/-} pups in NN and UN groups during lactation (P0: n=24-35, P2: n=21-33, P5: n=21-32, P10: n=21-33, P15: n=21-33, P21: n=21-33) (a, b) body weight at P21 in WT and *GHSR*^{-/-} pups (C, D) Changes in body weight of WT and *GHSR*^{-/-} NN and UN male offspring (NN: n=8, UN: n=11-14) after feeding with a normal diet (week 4-8) and a HFD (week 9-19) (E, F) Body weight, fat and lean mass in NN and UN male offspring at week 18 after feeding a HFD from week 9 (NN: n=8, UN: n=11-12). Data are presented as mean $\pm$ SEM. &&&: $P<0.01$ vs. NN; #: $P<0.05$, ###: $P<0.01$, ####: $P<0.001$ UN vs. NN.

Fig. 7. Metabolic changes in WT and *GHSR*^{-/-} NN and UN male offspring after feeding a HFD (week 9-15) (A) Oxygen consumption, (B) Heat production, (C) Ambulatory activity and (D) Mean respiratory exchange rate of NN and UN offspring at week 15 after feeding a HFD (NN: n=8, UN: n=11-14). Data are presented as mean $\pm$ SEM. *: $P<0.05$, **: $P<0.01$ WT vs. *GHSR*^{-/-}; #: $P<0.05$, ##: $P<0.01$, ####: $P<0.001$ UN vs. NN.

Fig. 8. Changes in food intake, glucose tolerance and density of AgRP projections in the PVN in male offspring (A, B) Changes in food intake of WT and *GHSR*^{-/-} NN and UN offspring after feeding with normal diet (week 4-8) and a HFD (week 9-19) (NN: n=8, UN: n=11-14) (a, b) food intake at week 19 in WT and *GHSR*^{-/-} pups (C, D) Glucose levels and area under curve during an OGTT (postnatal week 17) in WT and *GHSR*^{-/-} NN and UN offspring at week 17 after feeding a HFD from week 9 (NN: n=8, UN: n=11-14) (E, F) Effects of maternal undernutrition on AgRP-IR fiber density in

the PVN of WT and GHSR^{-/-} offspring after feeding a HFD (week 9-19). Representative confocal image (E) and quantification with Image J (F). (WT: NN: n=8, UN: n=6; GHSR^{-/-}: NN: n=7, UN: n=5) Square indicates the ROI in the PVN used for quantification, Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM. #: P<0.05, ##: P<0.01 UN vs. NN.

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Highlights

- Maternal undernourishment triggers developmental changes in the hypothalamus
- Postnatal overfeeding enhances ghrelin signalling and inhibits hypothalamic fibers
- The mismatch with the predicted thrifty postnatal life style promotes obesity
- The ghrelin receptor is a key factor in the hyperphagia in this mismatch model

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