Impact of changes in postnatal nutrition on puberty onset and the expression of hypothalamic GnRH and ghrelin

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Abstract. – OBJECTIVES: Ghrelin is an octanoylated peptide hormone with multiple and diverse physiologic functions including an important role in energy homeostasis and reproduction. In this study, the adjustment effects of different postnatal nutritional status on puberty onset and the expression of hypothalamic ghrelin and gonadotrophin-releasing hormone (GnRH) were examined in 1 day-old female Sprague-Dawley rats.

MATERIALS AND METHODS: Animals were randomly assigned into four groups: overnutrition group (Group O), normal group (Group N, control group), and undernutrition groups (Group U and U2). Western blot analysis and immunohistochemistry were used to analyze the expression of hypothalamic ghrelin and GnRH.

RESULTS: With a low level expression of hypothalamic ghrelin, the appearance of puberty onset and secretion peak of GnRH in Group O was earlier than the other groups.

CONCLUSIONS: Undernutrition delayed puberty onset and the GnRH peak, at the same time, promoted the expression of hypothalamic ghrelin. While, the expression of hypothalamic ghrelin was suppressed at puberty onset.

Key Words: Ghrelin, GnRH, Nutrition, Puberty onset.

Introduction

The mechanisms for the connection between energy balance and reproduction have been the subject of considerable attention. However, our understanding of the neurobiological basis for this phenomenon is still incomplete. Identification of ghrelin in late 1999, opened up a new era in our understanding of neuroendocrine systems. The 28 amino acid peptide ghrelin as the endogenous bioactive ligand for growth hormone secretagogue (GHS) receptor, this was considered the end-point of the search for the endogenous counterpart of GHS. The peptide was named ghrelin, from “ghre-”, the Proto-Indo-European root for “growth”; “GH” and “relin” are also the abbreviations for “growth-hormone-release”, the characteristic effect of ghrelin1. Ghrelin is highly conserved across several species and widely expressed in different tissues indicating its physiological importance2. Ghrelin mRNA is widely expressed in the body, with the highest levels found in the X-like neuroendocrine cells of the stomach, from which the majority of circulating ghrelin is derived, whilst lower levels are expressed in the small intestine, pancreas, lymphocytes, placenta, kidney, lung, pituitary, brain and gonads2. Ghrelin potently stimulates GH release and food intake and exhibits diverse effects, including ones on glucose metabolism and on secretion and motility of the gastrointestinal tract. Besides these effects on food intake and energy homeostasis, ghrelin is also involved in controlling reproductive functions3, but the role of ghrelin in sexual development remains largely unexplored to date. In the present study, we investigated the adjustment effects of different postnatal nutrition on puberty onset. Furthermore, we examined the expression of hypothalamic ghrelin and GnRH by establishing different nutrition models of female rats.

Materials and Methods

Animals and Laboratory Chow

One hundred and sixty newborn female Sprague–Dawley rats (1 day old, approximately 5 g body weight) were selected from twenty litters, and randomly assigned to 4 experimental groups (n = 40). Female pups from the same litter were cross-fostered and grouped into different litter sizes: the overnutrition group (Group O; two pups per litter), the normal group (Group N; ten pups per litter), and the undernutrition group
(Group U; twenty pups per litter), and the undernourishment group 2 (Group U2; twenty pups per litter). The animals were weaned on postnatal day (PND) 21. After weaning, Group O received a high-fat diet (HFD), Group N received a normal diet (NFD), while, Group U and U2 received a low-fat diet (LFD). Animals were maintained in 12-h light/dark conditions, with lighting beginning at 7:00 A.M. and the temperature maintained at approximately 21°C in concordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All animals had access to water ad libitum. All procedures used for all experiments were approved by the Institutional Animal Care and Use Committee of Shanghai, China. Standard laboratory chow (NFD) contained 4 g fat, 21 g protein, and 1193 kJ/100 g. The HFD contained 25.71 g fat, 19.54 g protein, and 1987 kJ/100 g; the HFD was prepared by mixing lard (20%, w/w), sugar (4%, w/w), whole-milk powder (2%, w/w), and cholesterol (1%, w/w) into standard laboratory chow. The LFD contained 1.7 g fat, 13.7 g protein, and 761 kJ/100 g; the LFD was prepared by bean pulp (15%, w/w), Indian corn (15%, w/w), wheat (23%, w/w), cellulose (35%, w/w), and yeast (1.6% w/w) into standard laboratory chow. The experimental diets were freshly prepared once a month and stored at –20°C to avoid rancidity.

**Tissue Preparation**

Twenty animals (10 animals for immunohistochemistry [IHC] and 10 animals for western blot analysis) in Group O, Group N and Group U were randomly sacrificed when Group O and Group N showed complete vaginal opening (VO) (postnatal days (PND) 35 and 42, respectively). The animals were sacrificed with an overdose of sodium nitrate and each rat was perfused transcardially with saline containing 2.5% sodium nitrite and then with 100 ml of a mixture of 4% buffered paraformaldehyde (PFA), pH 6.8 (Sigma, St. Louis, MO, USA) and 2.5% acrolein (EM grade; Polysciences, Warrington, PA, USA). After perfusion, the brain was removed and postfixed in 4% PFA for 6 h, first submersed in 20% aqueous sucrose solution at 4°C for 72 h, then submersed in 30% aqueous sucrose solution, and stored at 4°C till each brain was serially sectioned (25-µm thickness) in the coronal plane at the bregma level, ranging from –1.30 mm to –5.30 mm on a freezing microtome. The sections were transferred to a cryoprotectant antifreeze solution and stored at –20°C, until they were processed for IHC. For western blotting, the brains were quickly removed and frozen on dry ice. The hypothalami were separated and stored at –80°C until use. The animals were observed the complete vaginal opening time without sacrificed in Group U2.

**Immunohistochemistry**

For GnRH IHC staining, sections were incubated 2 hours with mouse anti-GnRH (luteinizing hormone-releasing hormone) monoclonal antibody (1:500 dilution, cat. no. MAB5456; Chemicon International, Inc., Temecula, CA, USA). After the unbound primary antibodies were removed, sections were incubated with a corresponding biotinylated secondary antibody (1:200 dilution) for 45 min at 37°C, followed by incubation with avidin-biotin peroxidase for 45 min at 37°C (cat. no. PK-6102; Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA). Immunoreactivity was visualized with 0.05% diaminobenzidine in Tris-HCl buffer (0.1 M, pH 7.6) containing 0.03% H2O2. Negative control sections received identical treatment except for the incubation with primary antibody and showed no positive signal. Every fourth section through the arcuate nucleus (ARC) (11 sections, from 0.30 to 4.30 mm posterior to the bregma) from each rat was stained. Typical sections of the ARC (from 2.56 mm posterior to the bregma) were used for quantification. Analysis of the results of GnRH neurons was undertaken by determining the total number of cell bodies in every positive region bilaterally. Slides were evaluated and imaged using a ×20 objective microscope (DMRA2; Leica, Wetzlar, Germany) for analysis of the GnRH neurons. For better visualization images, brightness and contrast were adjusted using exact parameters for all samples. Scale bars indicate 50 µm. Identical images were captured using bright field, and images were merged using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA). All adjustments for brightness and contrast were kept identical to ensure comparability. Data were analyzed for the total number of GnRH neurons in ARC by IMS (Image Movement Signal) image analysis system (Shanghai Shenteng Information Technology co., Ltd). The ARC area was outlined on the grayscale image and processed for density measurement. Nonspecific background density points were eliminated using the same threshold for each rat.
Western Blot Analysis

Western blot analysis was performed for protein analysis of ghrelin in the hypothalamus. Briefly, cellular protein lysates were obtained, and 20 µg of total protein was separated on 10% Sodium Dodecyl Sulphate (SDS)/polyacrylamide gels and transferred to nitrocellulose membrane. The blots were incubated overnight with a primary antibody, goat anti-ghrelin polyclonal antibody (1:1000, cat. no. sc-10368; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in the blocking buffer (1% bovine serum albumin: BSA, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20). The membranes were washed and incubated with their appropriate rabbit anti-goat secondary antibody (1:2000). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000), and immunoreactivity was visualized by ECL (electrochemiluminescence).

Statistical Analysis

The values are the means±SEM for each group. One-way ANOVA followed by the least significance difference (LSD) test was performed to assess the variation among experimental groups. Statistical significance was set at $p < 0.05$. All analyses were performed with GraphPad Prism 5 software (San Diego, CA, USA) for analysis.

Results

Impact of Changes in Postnatal Nutrition on Body Weight Gain

The difference of body weight among Group O, Group N and Group U was significant since PND7 (21.8±2.0 g vs. 15.7±3.0 g vs. 10.5±2.1 g, $p < 0.05$) (ANOVA F value = 107.55), and resulted in wide divergence. As shown in Figure 1, the body weight of female rats in Group O (136.5±13.3 g) was significantly increased compared with Group N (113.8±12.1 g) and Group U (84.8±8.9 g) at PND35 ($p < 0.05$) (ANOVA F value = 99.94). The difference among Group O, Group N and Group U was still significantly increased at PND42 (164.7±5.5 g vs. 145.7±11.3 g vs. 115.1±8.6 g, $p < 0.05$) (ANOVA F value = 80.69).

Impact of Changes in Postnatal Nutrition on the Timing of Puberty

All rats in Group U were sacrificed when Group N showed complete VO at PND42, even though not all of them showed complete VO at that time. To research the effect of undernutrition on puberty onset, we established Group U2 as same as Group U to observed the exact complete VO time. As shown in Figure 2, Group O showed complete VO earlier than the other groups, at a mean age of 29.20 ± 1.96d compared with 39.05 ± 1.93d in Group N, and 42.70 ± 1.69d in Group U2 ($p < 0.01$).

GnRH Expression in Hypothalamus After Postnatal Nutritional Challenges

Group O showed a biggest number of hypothalamic GnRH-positive neurons compared with...
the other groups ($p < 0.05$) at both PND35 and PND42, and Group U showed smallest number of GnRH-positive neurons ($p < 0.05$). While, between PND35 and PND42, there was no significant difference in expression of GnRH in Group O ($p > 0.05$). The expression of GnRH in Group U increased significantly at PND42 compared with PND35 ($p < 0.05$), as same as Group N ($p < 0.05$) (Figures 3 and 4).

**Ghrelin Expression in Hypothalamus After Postnatal Nutritional Challenges**

At PND35, the expression of hypothalamic ghrelin in Group U was significantly higher than the other groups ($p < 0.05$), and the expression in Group O was significantly depressed ($p < 0.05$). As puberty onset drew near, the expression of hypothalamic ghrelin in Group U was significantly depressed at PND42 ($p < 0.05$), such as Group N.

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**Figure 3.** Panels **A1** and **a1**, the expression of GnRH-positive neurons of Group O at PND35; **Panels B1** and **b1**, the expression of Group N at PND35; **Panels C1** and **c1**, the expression of Group U at PND35; **Panels A2** and **a2**, the expression of Group O at PND42; **Panels B2** and **b2**, the expression of Group N at PND42; **Panels C2** and **c2**, the expression of Group U at PND42; III, third ventricle. Scale bars, 50 µm (panels A1-C1 and A2-C2), 20 µm (panels a1-c1 and a2-c2).
However, the expression in Group O increased significantly at PND42 compared with PND35 (p < 0.05), although significantly lower than the expression in Group U at PND35 (p < 0.05) (Figures 5, 6, 7).

## Discussion

A mystery that still puzzles researchers is what initiates reproduction. However, the impact of postnatal metabolic challenges on the puberty onset has been only fragmentarily addressed. In recent years, the influence of nutrition on puberty and reproductive physiology has been recognized. Over the last decades a secular trend towards earlier puberty has occurred in association with improvements in nutrition and the increasing number of obese patients. Several cross-sectional studies have shown an association between increased BMI during childhood and early pubertal development in girls. Terry et al have reported that size at birth and infant weight gain from 4 months to 1 year of life are associated with earlier age at menarche in girls. In the present study, we observed that postnatal overnutrition induced a persistent increase in BW gain and advanced complete VO, also advanced hypothalamic GnRH secretion peak. These data demonstrate that persistent overfeeding during early development might contribute to precocious pubertal activation.

Most of the rodent studies addressing the impact of early nutritional programming of puberty have focused on the consequences of undernutrition during pregnancy or lactation. However, these studies remain scarce and have yielded contradictory findings. There was so far limited information regarding the impact of underfeeding during early critical periods on the developmental programming of puberty. Sánchez-Garrido et al and Castellano et al reported that peripubertal subnutrition female rats displayed a marked delay in body weight gain and puberty onset, overtly de-
layed VO has not been detected in all studies involving postnatal malnutrition due to large litter size\textsuperscript{13,14}. Our findings in persistent underfeeding rats were in keeping with the predominant low body weight and document an unambiguous delay of the age of VO, meanwhile, delayed the GnRH secretion peak in hypothalamus.

Taking into account the fact that the reproductive axis is highly dependent on the body energy status, ghrelin, could be one of the signal mechanisms linking nutritional balance to the hypothalamic-pituitary-gonadal (HPG) axis\textsuperscript{18}. Several scientists have established Ghrelin inhibits GnRH\textsuperscript{19} and LH secretions\textsuperscript{20} in the pre-pubertal period. PND35, the expression of hypothalamic ghrelin in Group U was unambiguously higher than the other groups, with delayed GnRH secretion peak and VO. That consistmented with the role of ghrelin in modulating gonadotropin secretion and delaying pubertal onset\textsuperscript{21,22}. Previous studies reports were plausible that higher ghrelin levels among the girls before puberty reflect a normal response to the demands of growth at that age. Reasons for higher ghrelin and greater hunger in peri-pubertal female are not clear, but may be due in part to their lower estradiol\textsuperscript{23}. In our research, the expression of hypothalamic ghrelin in Group N and Group U remarkably increased at PND35, when was the pre-pubertal period. The body weight of the two groups also increased, of course, to prepare for puberty onset. On the other hand, at the complete VO time, it was significantly supressed that the expression of hypothalamic ghrelin in Group O at PND35 and in Group N and U at PND42. We assumed that GnRH secretion peak was negative feedback to the expression of ghrelin in hypothalamus at puberty onset.

**Conclusions**

Ghrelin acted as a signal of energy insufficiency to the HPG axis. Undernutrition exacerbates ghrelin’s inhibition of hypothalamic GnRH secretion and puberty onset. Most importantly, down-regulation of ghrelin expression in the hypothalamus at puberty onset, provided generous response from energy metabolism to reproduction.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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