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The expression of orexigenic and anorexigenic factors in middle-aged female rats that had been subjected to prenatal undernutrition

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ABSTRACT

Fetal growth retardation, which affects short- and long-term fetal brain development, is associated with metabolic, hematological, and thermal disturbances, which can increase the risk of metabolic syndrome later in life. Orexigenic and anorexigenic factors regulate food intake and energy expenditure. We studied how the expression of these factors was affected by food deprivation (FD) in middle-aged female rats that had been subjected to prenatal undernutrition. Eight pregnant rats were divided into two groups, the normal nutrition (NN) ($n = 4$) group and the undernutrition (UN) ($n = 4$) group, which received 50% (approximately 11 g) of the daily food intake of the normal nutrition rats from day 13 of pregnancy to delivery. The pups from these dams were defined as the maternal NN (mNN) and maternal UN (mUN) groups, respectively. After weaning, all of the pups were housed and allowed ad libitum access to food and water. At the age of 6 months, both groups of pups were sub-divided into three groups. One group was allowed to consume normal amounts of food (Fed), and the other two groups were subjected to 24 h or 48 h FD ($n = 7$–8 per group). The rats' serum leptin levels and hypothalamic mRNA expression levels of various orexigenic or anorexigenic factors were measured. In both the mNN and mUN rats, the serum leptin levels of the 24 h and 48 h FD groups tended to be lower than those of the Fed group, and the serum leptin levels of the 24 h FD mUN rats and the Fed mUN rats differed significantly. The hypothalamic neuropeptide Y (NPY) mRNA expression levels of the 24 h and 48 h FD groups were significantly higher in the mUN rats than in the mNN rats. In addition, among the mUN rats the hypothalamic NPY mRNA expression levels of the 48 h FD group were significantly higher than those of the Fed group. In both the mNN and mUN rats, prepro-orexin mRNA expression was lower in the 48 h FD group than in the corresponding Fed group. Among the mUN rats, the 48 h FD group exhibited significantly lower hypothalamic proopiomelanocortin (POMC) mRNA expression than the Fed group, and a similar tendency was seen among the mNN rats. Among the mNN rats, the 24 h FD group displayed significantly higher hypothalamic leptin receptor (OBRb) mRNA levels than the Fed group. However, no such differences were seen among the mUN rats. As a result, the hypothalamic OBRb mRNA expression levels of the mUN rats in the 24 h and 48 h FD groups were lower than those of the corresponding mNN rat groups. These findings indicate that rats that are subjected to prenatal undernutrition exhibit upregulated expression of orexigenic factors and are more sensitive to FD in middle age, which might increase their risk of developing metabolic disorders in later life.

1. Introduction

Maternal protein restriction induces fetal growth retardation (FGR), which influences short- and long-term fetal brain development and can result in metabolic, hematological, and thermal disturbances that promote the development of metabolic syndrome in later life (Barker et al., 2002; Wang et al., 2015). In animal models, low birth weight offspring display reduced circulating leptin levels and downregulated hypothalamic leptin signaling, which alter orexigenic and anorexigenic regulatory mechanisms. Furthermore, they tend to exhibit hyperleptinemia and leptin resistance, which is associated with obesity in adulthood (Desai et al.,

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2005). Orexigenic and anorexigenic factors in the hypothalamus and peripheral tissues play important roles in the regulation of food intake. Neuropeptide Y (NPY), which is a major orexigenic peptide, is mostly synthesized in the hypothalamic arcuate nucleus (ARC). Food deprivation (FD) increases hypothalamic NPY mRNA expression, which stimulates appetite (Allen et al., 1983; Stanley and Leibowitz, 1985; White and Kershaw, 1990). As the ability of the hypothalamus to synthetize NPY falls with age, NPY release and hypothalamic prepro-NPY (ppNPY) mRNA expression decrease in an age-dependent manner (Gruenewald et al., 1994). Orexins are hypothalamic neuropeptides that localize in the lateral hypothalamus. Orexin-immunoreactive nerve fibers are also present in the ARC, paraventricular nucleus, dorsomedial hypothalamus, and other brain regions including the cerebral cortex, medial thalamic nuclei, circumventricular organs, limbic system, and brain stem. A previous study found that hypothalamic prepro-orexin (ppORX) mRNA expression was upregulated in rats that were subjected to FD for 48 h and then centrally administered orexin A and orexin B. This suggests that orexin also plays a role in the central regulation of feeding behavior (Sakurai et al., 1998; Nambu et al., 1999). Leptin is an anorexigenic factor and is derived from adipose tissue. The OB gene encodes leptin, which reduces food intake, increases energy expenditure, and modulates glucose and fat metabolism through its receptor OBRb in the hypothalamic ARC (Zhang et al., 1994; Friedman and Halaas, 1998). A previous study found that functional hypothalamic leptin receptor (OBRb) expression was increased in 1-day-old FGR rats, and this pattern was reversed in adult FGR rats (Khorram et al., 2015). Proopiomelanocortin (POMC) is another anorexigenic factor. It is predominantly found in the lateral ARC, the neurons of which are activated by leptin (Cowley et al., 2001). This study was conducted to examine the effects of FD on the expression of orexigenic and anorexigenic factors in the hypothalamus in middle-aged female rats that were subjected to prenatal undernutrition.

2. Materials and methods

2.1. Animals

Pregnant Sprague-Dawley rats (gestational age: 13 days, 280–360 g) were purchased from Charles River Japan, Inc. (Tokyo, Japan) and housed individually. The animal rooms were maintained under controlled lighting (14 h light, 10 h dark cycle) and temperature (24 °C) conditions. All animal experiments were conducted in accordance with the ethical standards of the Animal Care and Use Committee of Tokushima University. In total, 8 pregnant rats and their offspring were used in this study. The pregnant rats were divided into two groups. In the normal nutrition (NN) ($n = 4$) group, the dams were allowed ad libitum access to water and food during the gestation and lactation periods. In the undernutrition (UN) ($n = 4$) group, the dams received 50% (approximately 11 g) of the daily food intake of the NN rats from day 13 of pregnancy to delivery. Thereafter, they were allowed ad libitum access to water and food during lactation period. After birth, the pups from the NN and UN dams were defined as the maternal NN (mNN) and maternal UN (mUN) groups, respectively. Both mNN and mUN pups were weighed and randomly assigned to each dam (10–12 per dam) to avoid any confounding litter size effect. The pups were culled and fostered to other dams until weaning. The day the litters were born was defined as postnatal day (PND) 1. The pups were weaned at PND 21. After weaning, the pups in both groups were housed 3–4 animals per cage and allowed ad libitum access to food and water. Only female pups were used for this experiment. At the age of 6 months, the mNN and mUN rats were sub-divided into three groups. One group was allowed to consume normal amounts of food (Fed), and the other two groups were subjected to 24 h or 48 h FD ($n = 7$–8 per group). The body weights of rats were measured at before and after 24 h, 48 h of FD.

2.2. Serum and tissue collection

After 24 h or 48 h fasting, all of the rats were killed by decapitation between 0900 and 1100 h of the light cycle. Their blood and whole brains were collected. The serum samples were stored at −20 °C, and the brain tissues were stored at −80 °C.

2.3. Hormone assay

Serum leptin levels were measured using an I-125 radioimmunoassay (RIA) kit (rat leptin RIA kit, Linco Research Inc., St. Charles, MO, USA). The sensitivity of the assay was 0.5 ng/ml and its inter-and intra-assay coefficients of variation were 4.8% and 2.4%, respectively.

2.4. Quantitative real-time PCR analysis

Before the RNA analysis, hypothalamic explants including the ARC were dissected out from the rats' frozen brains. The brain sections were dissected via a coronal cut at 2 mm anterior to the optic chiasm and a posterior cut at the posterior border of the mammillary bodies. These tissue blocks were cut 2.5 mm from the bottom of the hypothalamus and then trimmed at 2.5 mm lateral from the midline of each side. Total RNA was extracted from the anterior and posterior blocks using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® mini kit (Qiagen Gmbh, Hilden, Germany). cDNA was synthesized with oligo (deoxythymidine) primers at 50 °C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (RT-PCR; Invitrogen Co.). RT-PCR analysis was performed using the StepOnePlus™ RT-PCR system (PE Applied Biosystems, Foster City, CA, USA) and FAST SYBR® green. Hypothalamic tissue expression levels were normalized to the mRNA expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is the most stable housekeeping gene in the brain (Vandesompele J et al., 2002). The following forward and reverse primers were used: NPY: F: 5′-GGG GCT GTG TGG ACT GAC CCT-3′, R: 5′-GAT GTA GTG TCG CAG AGC GGAG-3′; ppORX: F: 5′-GCC GTC TCT ACG AAC TGT TG-3′, R: 5′-CGA GGA GAG GGG AAA GTT AG-3′; POMC: F: 5′-CCC GAG AAA CAG CAG CAC TC-3′, R: 5′-AGG GGG CCT TGG ACT GAG AA-3′; OBRb: F: 5′-GCA GCT ATG GTC TCA CTT CTT TTG-3′, R: 5′-GGT TCC CTG GGT GCT CTGA-3'; GAPDH: F: 5′ -ATG GCA CAG TCA AGG CTG AGA-3′, R: 5′ - CGC TCC TGG AAG ATG GTG AT-3′. The PCR conditions were as follows: the initial denaturation and enzyme activation were performed at 95 °C for 20 s, followed by 45 cycles of denaturation at 95 °C for 3 s and annealing at 65 °C for 30 s (NPY), 60 °C for 30 s (ppORX), 65 °C for 30 s (POMC), 63 °C for 30 s (OBRb), or 64 °C for 30 s (GAPDH), and a final extension step of 72 °C for 1 min. The copy numbers of the transcripts were normalized against those the GAPDH transcript for NPY, ppORX, POMC, and OBRb (Iwasa et al., 2011a,b, 2015). Shi et al., reported that mRNA expression and peptide expression of NPY are strongly correlated (Shi et al., 2009). Therefore, we did not estimated peptide expression by Western blot analysis in this experiment.

2.5. Statistical analysis

Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA), and *post hoc* comparisons were carried out using Dunnett's test. All data are presented as mean ± SE values. *P*-values of <0.05 were considered to be statistically significant.

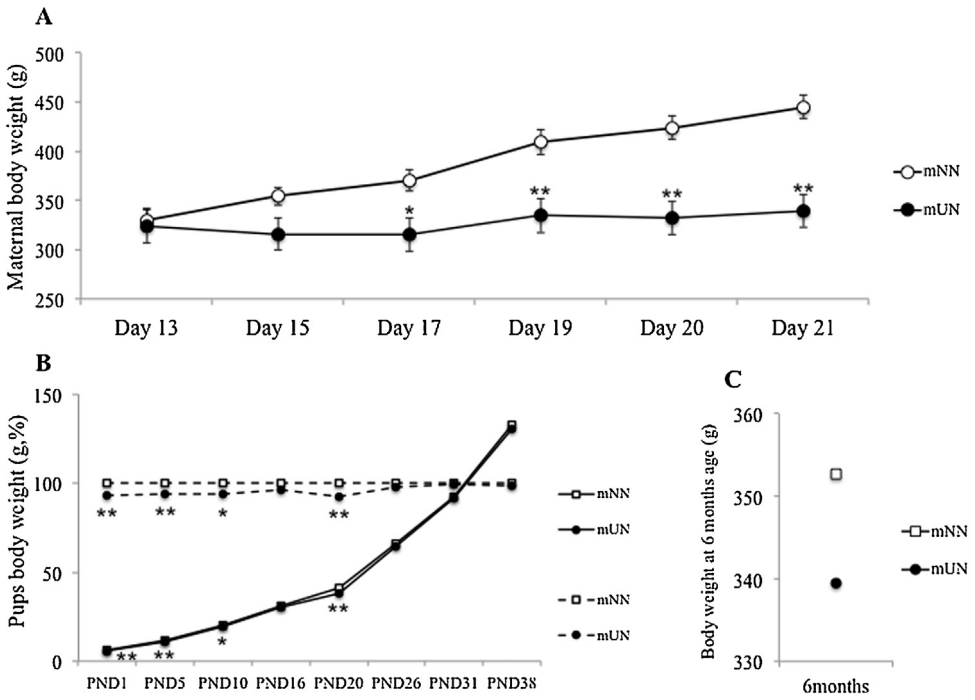

Fig. 1. Body weight of the dams and their offspring.
(A): The body weight of the NN dams increased during pregnancy. On the other hand, the body weight of the UN dams did not increase and was significantly lighter than that of the NN dams during pregnancy (from day 17–21). (B): The mUN pups were significantly lighter than the mNN pups at PND1 to PND26. (C): The body weight was not differ between mNN and mUN at 6 months age. Data are expressed as mean ± SE values. $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Effects of 24 h and 48 h FD on the body weight and serum leptin levels of the mNN- and mUN rats

The UN dams were significantly lighter than the NN dams from gestational day 17 to delivery (gestational day 21) (Fig. 1A) . Similarly, the mUN pups were significantly lighter (5.7 ± 0.1 g, mean ± SE) than the mNN pups at delivery (PND1) (6.2 ± 0.1 g); however, this significant difference disappear at PND 16, mUN rats showed that significantly lower body weight at PND20. Therefore, mUN rats showed catch-up growth at PND 26 (Fig. 1B). At the age of 6 months, the body weight of the mUN rats (339.5 ± 8.2 g) did not differ from that of the mNN rats (352.7 ± 8.6 g) (Fig. 1C). In addition, the body weight changes observed after 24 h and 48 h FD did not differ between the mNN and mUN rats (Table 1). Specifically, the body weight of the mNN and mUN rats decreased by 5.2% and 5.5% after 24 h FD and by 7.7% and 7.9% after 48 h FD, respectively. There was no significant difference between the body weights of the mNN and mUN before or after FD.

In both the mNN and mUN rats, the serum leptin levels of the 24 h and 48 h FD groups were lower than those of the Fed groups (two-way ANOVA; $F(1,44) = 2.58$, $p < 0.11$). The mUN rats tended to have higher serum leptin levels than the mNN rats, and the difference was significant in the 24 h FD groups ($p < 0.05$) (Fig. 2).

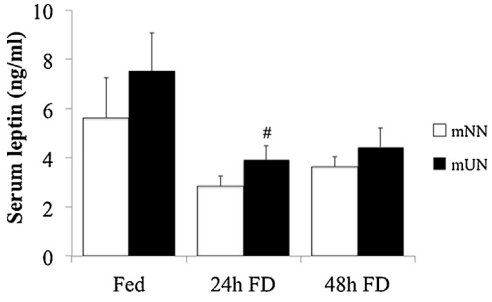

Fig. 2. Serum leptin levels of the Fed and 24 h or 48 h FD groups.
In both the mNN and mUN rats, the 24 h and 48 h FD groups exhibited lower serum leptin levels than the Fed group. Data are expressed as mean + SE values. $^{\#}p < 0.05$ vs. Fed mUN rats.

3.2. Effects of 24 h and 48 h FD on the hypothalamic NPY, ppORX, POMC, and OBRb mRNA expression levels of the mNN and mUN rats

The hypothalamic mRNA expression level of the orexigenic peptide NPY was significantly higher in the 24 h and 48 h FD mUN rats than in the 24 h and 48 h FD mNN rats, respectively ($p < 0.01$). Among the mUN rats, the hypothalamic NPY mRNA expression level of the 24 h FD group tended to be higher and that of the 48 h FD group was significantly higher than that seen in the Fed group, ($p < 0.05$), whereas the mNN rats exhibited similar hypothalamic NPY mRNA levels regardless of whether or not they were subjected to FD (Fig. 3A) . The hypothalamic mRNA expression level of ppORX, another orexigenic peptide, was lower in the 48 h FD group than in the corresponding Fed group in the both mNN and mUN rats ($p < 0.01$, $p < 0.05$) (Fig. 3B). Among the mUN rats, the hypothalamic POMC mRNA expression level of the 48 h group was lower than that of the Fed group, and a similar tendency was seen in the mNN rats (Fig. 4A) . Among the mNN rats, hypothalamic OBRb mRNA expression was significantly higher in the 24 h FD group than in the Fed group ($p < 0.05$). However, the mUN rats did not display any such differences. As a result, the hypothalamic OBRb mRNA expression levels of the 24 h and 48 h FD mUN rats were lower than those of the corresponding mNN rats ($p < 0.01$, $p < 0.05$) (Fig. 4B).

4. Discussion

In this study, we found that 24 h or 48 h FD in adulthood increased the hypothalamic NPY mRNA expression level of mUN

Table 1
Body weight before and after fasting in maternal normal or undernutrition rats at 6 months after birth.

Body weight (g)	mNN			mUN		
	Fed ($n=7$)	24 h FD ($n=8$)	48 h FD ($n=8$)	Fed ($n=7$)	24 h FD ($n=7$)	48 h FD ($n=8$)
before fasting	355.7 ± 14.2	342.6 ± 14.8	360.3 ± 16.8	336.7 ± 12.1	331.2 ± 12.1	349.1 ± 17.9
after 24 h fasting		324.8 ± 14.6	341.5 ± 15.4		312.7 ± 11.2	328.7 ± 18.0
after 48 h fasting			332.2 ± 15.4			321.3 ± 17.3

Body weight decreased 24 h and 48 h food deprivation in both mNN and mUN groups. There was no significance in body weight between mNN and mUN groups. mNN: maternal normal nutrition, mUN: maternal undernutrition, Fed: consume normal amounts of food, FD: food deprivation.

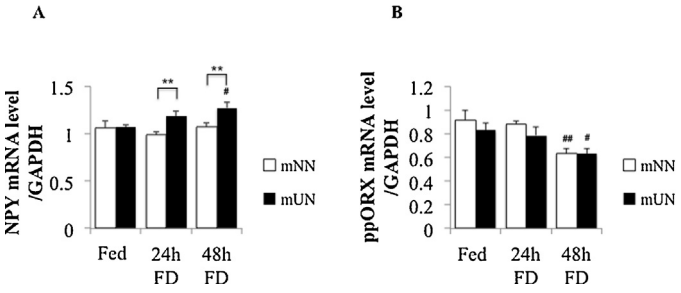

Fig. 3. NPY and ppORX mRNA expression levels of mNN and mUN female rats at 6 months of age.
The 24 h and 48 h FD mUN rats exhibited significantly higher NPY mRNA levels than the 24 h and 48 h FD mNN rats. In both the mNN and mUN rats, the 48 h FD displayed lower ppORX mRNA expression levels than the corresponding Fed group. Relative expression levels were calculated by using the expression level of GAPDH mRNA as an internal control. Data are presented as mean + SE values. (A) and (B) ** $p < 0.01$ vs. each other; # $p < 0.05$ vs. the corresponding Fed group.

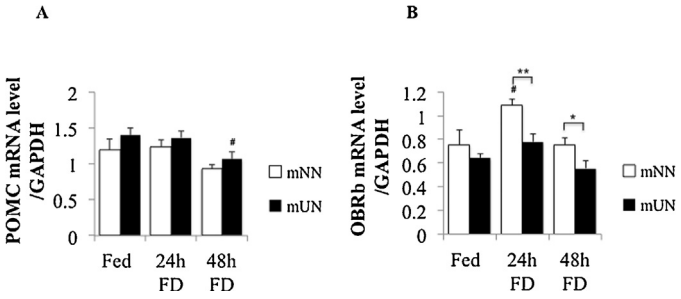

Fig. 4. POMC and OBRb mRNA expression levels of mNN and mUN female rats at 6 months of age.
Among the mUN rats, POMC mRNA expression was lower in the 48 h FD group than in the Fed group. Among the mNN rats, OBRb mRNA expression was significantly higher in the 24 h FD group than in the Fed group. Relative expression levels were calculated by using the mRNA expression level of GAPDH as an internal control. Data are presented as mean + SE values. (A) and (B) * $p < 0.05$, * $p < 0.01$ vs. each other; # $p < 0.05$ vs. the corresponding Fed group.

female rats, whereas no such difference was seen in mNN rats. As far as we know, this is the first study to show that female rats that had been subjected to undernutrition in the neonatal period possessed orexigenic hypothalami in middle age.

Early life environmental conditions, especially nutritional conditions, can influence fetal neuronal development and metabolic regulatory systems, and adverse environmental conditions can increase the risk of various conditions in adulthood, including cardiovascular disease, hypertension (Barker et al., 2005), obesity, type 2 diabetes, and insulin resistance (Eriksson et al., 2002). In humans, such developmental programming is closely associated with FGR; i.e., undernutrition in the fetal period. In a rat model, the offspring of undernourished dams exhibited insulin resistance and leptin resistance, which can lead to hyperinsulinism, hyperleptinemia, hypertension, and hyperphagia-induced obesity in later life (Vickers et al., 2000). The brain integrates information about changes in the blood levels of energy substrates such as glucose, fatty acids, and ketone bodies through specific sensory systems (Shuichi and Koji, 2015). In addition, our previous studies demonstrated that FGR rats undergo rapid catch-up growth in the developmental period, which can result in the delayed onset of puberty and lower hypothalamic kisspeptin mRNA expression levels due to accelerated leptin resistance in the prepubertal period (Iwasa et al., 2010; Gereltsetseg et al., 2012).

Yura et al. reported that undernutrition-associated hypothalamic programming that occurs during the prenatal or postnatal periods can have long-lasting effects on energy and appetite regulatory systems (Yura et al., 2005). The hypothalamus, especially the ARC, plays a key role in the systems regulating energy balance and appetite (Sainsbury et al., 2002), which are heavily influenced by prenatal nutritional conditions. A previous study reported that perinatally undernourished rats demonstrated higher hypothalamic levels of NPY, a major orexigenic peptide, than normally nourished rats. On the other hand, the perinatally undernourished rats exhibited decreased hypothalamic levels of POMC, an anorexigenic peptide (Delahaye et al., 2008). Our findings showed that hypothalamic NPY mRNA expression was increased by 24 h or 48 h FD in middle-aged female rats that had been subjected to undernutrition in the neonatal period. However, the hypothalamic NPY mRNA levels of the mNN rats did not increase much in response to 24 h or 48 h FD. Gruenewald et al. reported that ppNPY mRNA hybridization was markedly increased in the ARC in middle-aged and old rats after fasting, but another group found that such fasting-induced increases become less marked with age (Li et al., 1998). Furthermore, after fasting the old rats consumed less food and found it more difficult to regain body weight than the young rats (Gruenewald et al., 1994). In this study, the hypothalamic NPY mRNA level was not increased by fasting in middle-aged female mNN rats, which might have been due to the effects of aging. On the contrary, the hypothalamic NPY mRNA levels of the mUN rats continued to increase after fasting even in middle age, which might have been due to programming induced by the fasting they experienced in the prenatal period. Breton et al., evaluated peripheral nutritional regulatory parameters and hypothalamic appetite regulatory factors in nonfasted and 48 h fasted adult offspring by using model of maternal 70% food restricted diet. After fasting, NPY mRNA expression was increased and POMC mRNA expression was decreased in fasted control rats. These mRNA expressions showed quite small difference compared to those of the experimental group, however, such small differences were associated with actual phenotype differences, i.e. higher food intake in re-feeding period for one hour (Breton et al., 2009). The detailed mechanisms responsible for these phenomena should be clarified in a future study.

Leptin, which is produced in adipose tissue, acts as a feedback signal that is transmitted from the energy stores in peripheral adipose tissue to the hypothalamus. Serum leptin levels are usually closely related to the amount of body fat an animal possesses. In fasting conditions, serum leptin levels fall, and leptin activity in the hypothalamus also decreases, resulting in increased expression of orexigenic factors such as NPY or orexin. In this study, the serum leptin levels of the mNN and mUN rats did not differ significantly;

however, the mUN rats tended to have higher serum leptin levels in both the fed and fasted conditions. Desai et al., reported that mUN rats increased their food intake and exhibited hyperphagia after the weaning period, which tended to persist through to adulthood. They also demonstrated rapid catch-up growth and increased ghrelin levels, which led to hyperleptinemia, leptin resistance and obesity in later life (Desai et al., 2005).

After 24 h fasting, the mNN rats exhibited significantly higher hypothalamic OBRb mRNA levels than the Fed mNN rats, but no such difference was detected in the mUN rats. Matsuzaki et al. reported that hypothalamic OBRb mRNA expression is upregulated by fasting during the neonatal-prepubertal period in male rats (Matsuzaki et al., 2015). Additionally, moderate caloric restriction can also increase OBRb mRNA expression in maternal calorie-restricted male pups during lactation, and female rats showed that similar tendency (Palou et al., 2011). These findings indicate that such rats are highly sensitive to leptin; therefore, they become resistant to obesity. This increased hypothalamic OBRb mRNA expressions might be one of the protective mechanism against negative energy balance. On the contrary, the mUN rats did not exhibit upregulated hypothalamic OBRb mRNA expression after fasting. In fact, they displayed significantly lower hypothalamic OBRb mRNA expression than the mNN rats after 24 h and 48 h FD. The mUN rats seemed to be able to adaptive and compensated to fasting; i.e., they remained in an orexigenic state, such changes increase the risk of metabolic disturbances occurring later in life. In addition, mUN male and female offspring developed obesity, hyperinsulinemia and leptin resistance in adult life, particularly under high fat diet (Vickers et al., 2000). These metabolic disturbances could be reversed with leptin treatment during postnatal age 3–13 days (Vickers et al., 2008).

In summary, hypothalamic orexigenic factors dominated the responses to FD seen in middle-aged female rats that had been subjected to prenatal undernutrition. This study found that prenatally undernourished rats have orexigenic hypothalami and are more sensitive to FD in middle age, which might make them more susceptible to metabolic disorders in later life. These findings suggest that avoiding and/or correcting negative energy balances during pregnancy might help to ensure the normal development of the hypothalamic appetite regulatory system.

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