Developmental changes in the responsiveness of hypothalamic ER alpha mRNA levels to food deprivation

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Abstract

OBJECTIVE: Estrogen plays an important role in feeding and energy balance, and the critical role of estrogen in the control of appetite and energy balance is mediated by hypothalamic estrogen receptor (ER) alpha. In undernourished rodents, hypothalamic ER alpha mRNA expression are decreased. Responses of some hypothalamic factors to negative energy balance develop during the early neonatal period.
DESIGN: In this study, we examined the developmental changes of fasting-induced alterations in hypothalamic ER alpha mRNA expression in female rats.
RESULTS: ER alpha mRNA expression was reduced after a 12-h or 24-h fast at postnatal days 15 and 25, but not at day 5. Serum estradiol levels in postnatal day 25 rats were not changed by fasting. Although serum leptin levels were suppressed by fasting at all ages, hypothalamic ER alpha mRNA expression at postnatal day 25 was not changed by leptin administration after a 24-h fast.
CONCLUSIONS: These data show that the sensitivity of hypothalamic ER alpha to negative energy balance may not be established in the early neonatal period, and that it develops by postnatal day 15. Decreased leptin levels might not be involved in the alterations of hypothalamic ER alpha mRNA expression in the undernourished condition.

ORIGINAL ARTICLE

INTRODUCTION

Estrogen plays an important role in appetite and energy control in many species. It suppresses appetite and increases energy expenditure, consequently reducing the amount of adipose tissue and preventing obesity. Estrogen deficiency induced by aging or ovariectomy results in hyperphagia, reduced energy expenditure, and increased adiposity in humans and rodents (Tchernof *et al.* 1998; Asarian & Geary 2006; Iwasa *et al.* 2011a). Because the absence of estrogen receptor (ER) function causes obesity and insulin resistance in humans and mice (Smith *et al.* 1994; Heine *et al.* 2000), the effects of estrogen on metabolic control may be mediated through ERs. Of the two main ER subtypes (alpha and beta), ER alpha plays an important role as a mediator of the effects of

estrogen on metabolic control. ER alpha knockout mice show decreased energy expenditure, increased adiposity, and insulin resistance in both sexes (Heine *et al.* 2000). ER alpha is widely distributed throughout the brain in female rodents, with greater concentrations in areas that regulate appetite and metabolism, such as the ventral medial hypothalamic nucleus (VMH) and arcuate nucleus (ARC) (Roemmich *et al.* 1997, Jones *et al.* 2002). Because the silencing of hypothalamic ER alpha leads to the characteristics of metabolic syndrome and obesity in female mice and rats (Musatov *et al.* 2007), ER alpha in hypothalamic areas may be important for the control of energy homeostasis.

Undernutrition induced by either food restriction or deprivation affects the hypothalamic ER levels in both male and female rodents. In both adult male and female rats, chronic food restriction and acute food deprivation suppress ER alpha immunoreactive cells and ER alpha mRNA expression in some hypothalamic areas, including the VMH and ARC (Lindblom *et al.* 2005; Jones *et al.* 2002). Similarly, the number of hypothalamic ER immunoreactive cells is decreased after food restriction in postweaning female rats (Roemmich *et al.* 1997). These alterations in ER alpha may be an adaptive response to conditions of undernourishment by which the anorectic action of estrogen in the hypothalamus is decreased, and, as a result, appetite is increased to promote survival during inadequate nutritional conditions. To the best of our knowledge, it is unknown if this response of ER alpha to undernutrition is innate or established after birth. In addition, the factor that regulates the suitable hypothalamic ER level to adapt to the level of nourishment has not been identified. Although it has been reported that estrogen levels and acyclicity have some effects on the regulation of hypothalamic ER alpha in adult female mice (Chakraborty *et al.* 2008), food deprivation did not alter the serum estradiol levels in prepubertal female rats in our previous study (Iwasa *et al.* 2010). On the other hand, some studies have shown that leptin affects the ER alpha levels in breast cancer cells and chondrogenic cells in vitro (Catalano *et al.* 2004; Wang *et al.* 2012), and that all ER-positive cells in the hypothalamus express leptin receptors (Diano *et al.* 1998). Leptin, which is mainly produced in adipose tissue, acts on the hypothalamus as an anorectic factor. The levels of ER alpha and leptin are decreased during undernourishment, and appetite subsequently increases via hypothalamic neural systems (Friedman 2009). To the best of our knowledge, the effect of endogenous or exogenous leptin on hypothalamic ER alpha has not been evaluated in any species.

The present study was performed to determine the sensitivity of hypothalamic ER alpha to undernutrition is established during the neonatal to prepubertal period, and to determine whether decreased leptin levels in undernourished animals are involved in the changes in hypothalamic ER alpha.

MATERIALS & METHODS

Animals

Pregnant Sprague-Dawley rats were purchased (Charles River Japan Inc., Tokyo, Japan) and housed individually under controlled lighting (14 h light; 10 h darkness, lights on at 0800 and off at 2200) and temperature (24 °C). The day the litters were born was defined as postnatal day 1. Dams with fewer than 10 pups or more than 14 pups were excluded from the study. Only females were used because our previous study found that the sensitivity of hypothalamic factors to undernutrition developed during the neonatal to prepubertal period in female rats (Iwasa *et al.* 2010, 2011b). In addition, the hypothalamic ER in female mice is more sensitive to undernutrition than the hypothalamic ER in male mice (Roemmich 1997).

Female pups aged 5, 15 and 25 days were used in this study. To ensure that the litter size ranged from 10 to 12 pups per dam, pups were culled or moved to other dams and fostered until weaning. Pups used for day 25 experiments were weaned at postnatal day 21. All animal experiments were conducted in accordance with the ethical standards of the Animal Care and Use Committee of the University of Tokushima.

Effects of fasting on hypothalamic ER alpha mRNA and serum hormone levels

To determine the effects of fasting on the hypothalamic ER alpha mRNA levels at postnatal days 5, 15 and 25, female rats were randomly divided into the following three groups: fed; 12-h fast or 24-h fast (n=6–9 per group). Fasting was achieved by maternal separation for pups at postnatal days 5 and 15 and food deprivation for pups at postnatal day 25. The rats in all groups were weighed prior to fasting. At the end of the 12-h or 24-h fasting period, rats were weighed and sacrificed by decapitation. The rats in the fed group were weighed again after 24 h and then sacrificed by decapitation. The rats were sacrificed between 0900 h and 1100 h of the light cycle. Blood was collected, and serum was stored at −20 °C. Brains were collected and stored at −80 °C. Then, hypothalamic ER alpha mRNA, serum leptin and serum estradiol levels were measured. Serum estradiol levels were measured only in the postnatal day 25 rats because the amount of serum in rats at postnatal days 5 and 15 was insufficient for measurement.

Effects of leptin administration on hypothalamic ER alpha mRNA level after a 24-h fast

To determine whether the effects of the co-administration of leptin alter the ER alpha mRNA level under fasting conditions, female rats at postnatal day 25 of age were divided into two groups. All rats were subjected to 24-h food deprivation and then given intraperitoneal injections of leptin (3 mg/kg body weight) or saline at 12 h and 21 h after the initiation of food deprivation. This dose of leptin is sufficient to maintain the plasma leptin

levels above physiological levels for 13 h in neonatal rats (Proulx *et al.* 2002; Iwasa *et al.* 2010). At the end of the fasting period, the rats were sacrificed by decapitation and their brains were collected. Then, hypothalamic ER alpha mRNA levels were measured. Because it was possible that leptin administration itself affects the ER alpha regulation system, the effect of leptin administration on hypothalamic ER alpha mRNA levels was also evaluated in postnatal day 25 female rats. The rats fed ad libitum were given intraperitoneal (i.p.) injections of leptin or saline twice. The second injection was administered 9 h after the first injection. The brain was collected 3 h after the second injection.

Hormone assay

The serum leptin concentration was measured by using an I-125 radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, MO, USA). The sensitivity of the assay was 0.5 ng/ml. The inter- and intra-assay coefficients of variation were 4.8% and 2.4%, respectively. The serum E2 concentration was measured by using an I-123 RIA kit (Double Antibody E2 kit, Diagnostic Products Co., Los Angeles, CA, USA). The sensitivity of the assay was 1.4 pg/ml. The inter- and intra-assay coefficients of variation were 4.1% and 4.6%, respectively.

Real-time reverse-transcription PCR analysis

Hypothalamic explants were dissected from frozen brains as described previously (Iwasa *et al.* 2011b). These hypothalamic blocks included the medial preoptic area, ARC, VMH and paraventricular nucleus, where ER alpha mRNA is expressed. Total RNA was isolated from the hypothalamus by 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 by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Co.). Real-time polymerase chain reaction (PCR) analysis was performed by using the StepOnePlus™ Real-time PCR System (PE Applied Biosystems, Foster City, CA, USA) and SYBR® green. Standard curves, generated from at least 4 dilution series of an abundant sample, were used for the relative quantification of ER alpha and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels of ER alpha were normalized by dividing by GAPDH mRNA expression levels. Dissociation curve analysis was also performed for each gene at the end of PCR. Each amplicon generated a single peak. The following forward and reverse primers were used: ER alpha: F: 5'-CAT CGA TAA GAA CCG GAG GA-3', R: 5'-TCT GAC GCT TGT GCT TCA AC-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: initial denaturation and enzyme activation at 95 °C for 20 sec; followed by 45 cycles of denaturation at 95 °C for 3 sec, 62 °C for 30 sec (ER alpha), or 64 °C for 30 sec (GAPDH).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with post-hoc Dunnett's test or unpaired Student's *t*-test. All results are presented as means±S.E.M. Differences were considered to be statistically significant at $p<0.05$.

RESULTS

Effects of fasting on hypothalamic ER alpha mRNA and serum hormone levels

The body weights of rats at postnatal days 5, 15, and 25 were 12.5±0.2 g (mean±SEM), 37.1±0.5 g, and 63.0±0.7 g, respectively. No significant differences in the mean body weights of the rats were noted among the fed, 12-h fast and 24-h fast groups at any age.

In the postnatal day 5 rats, body weights (one-way ANOVA; $p<0.001$, $F(3,55)=18.47$) and the serum leptin levels (one-way ANOVA; $p=0.048$, $F(3,68)=3.74$) were significantly lower in the 12-h and 24-h fast groups than in the fed group (Figure 1). However, no significant differences were found in the hypothalamic ER alpha mRNA levels (one-way ANOVA; $p=0.41$, $F(5,93)=0.93$) among these three groups.

In the postnatal day 15 and 25 rats, body weights (one-way ANOVA; $p<0.001$, $F(3,68)=85.45$ (day 15), $p<0.001$, $F(3,63)=110.19$ (day 25)) and serum leptin levels (one-way ANOVA; $p<0.001$, $F(3,68)=65.00$ (day 15), $p<0.001$, $F(3,55)=21.57$ (day 25)) were significantly lower in the 12-h and 24-h fasting groups than in the fed group (Figure 1). In addition, the hypothalamic ER alpha mRNA levels (one-way ANOVA; $p=0.007$, $F(3,59)=6.85$ (day 15), $p=0.022$, $F(3,47)=4.55$ (day 25)) in the 12-h and/or 24-h fasting groups were significantly lower than in the fed group. In the postnatal day 25 rats, serum E2 levels were not different among the fed, 12-h fast and 24-h fasting groups (5.5±0.9, 7.4±0.5, 6.5±0.6 pg/ml, respectively).

Effects of leptin administration on hypothalamic ER alpha mRNA level after a 24-h fast

Hypothalamic ER alpha mRNA levels were not significantly different between the 24-h fasting rats with leptin injection and those with saline injection (Figure 2). Serum leptin levels at the point of tissue harvest were significantly higher in the leptin-treated rats compared with the rats that received saline (269.4±6.4, 1.1±0.0 ng/ml respectively). Similarly, although exogenous leptin administration induced supraphysiological leptin levels under the fed condition (245.8±7.3 ng/ml in the rats that received leptin, and 1.7±0.2 ng/ml in the rats that received saline), exogenous leptin administration did not affect the hypothalamic ER alpha mRNA levels.

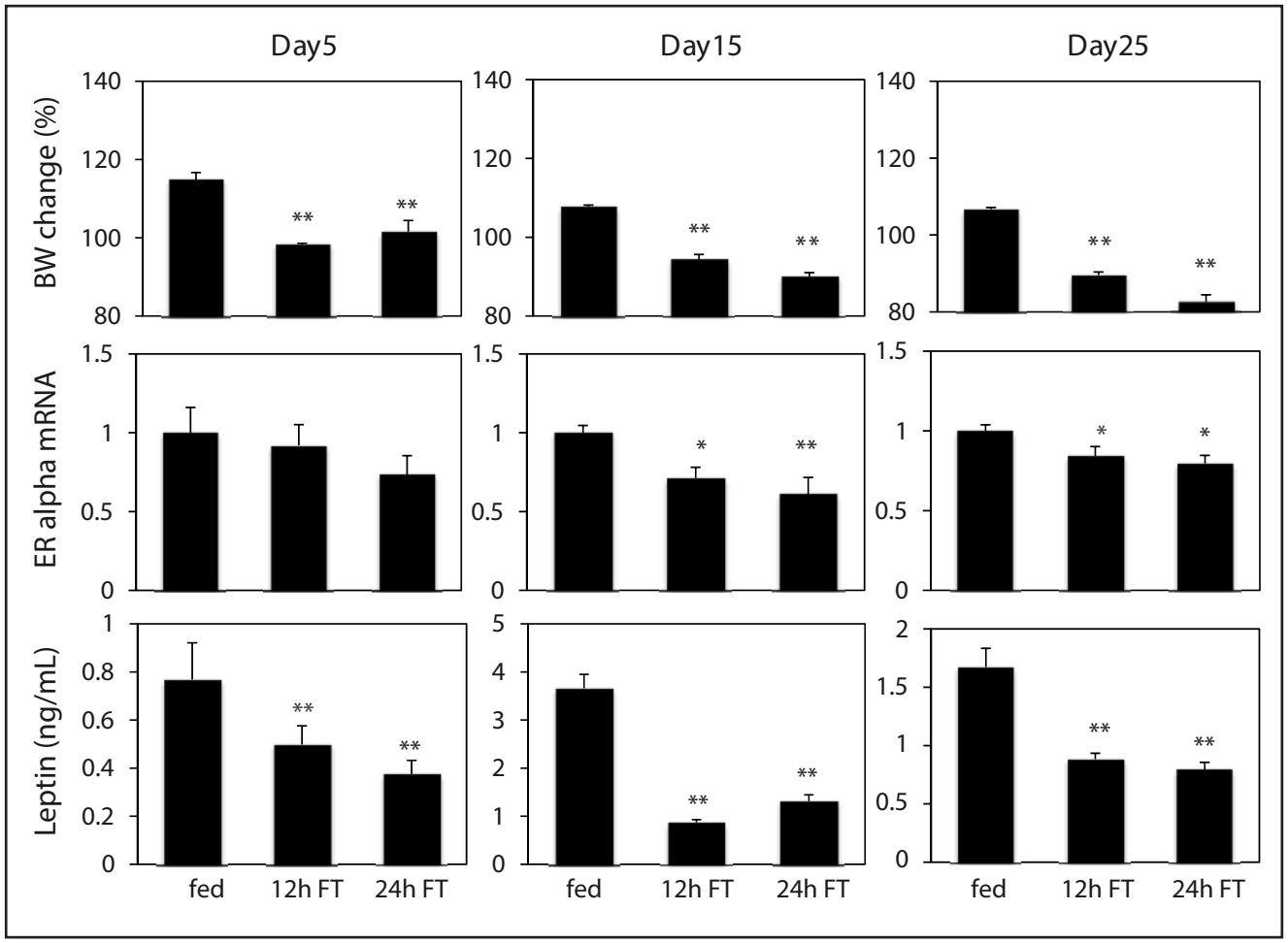

Fig. 1. Effects of maternal separation or food deprivation on body weight (BW), hypothalamic estrogen receptor (ER) alpha mRNA expression, serum estradiol and serum leptin levels in 5-, 15- and 25-day-old female rats. Animals were fed ad libitum (fed group), subjected to 12 h of maternal separation or food deprivation (12 h FT group), or subjected to 24 h of maternal separation or food deprivation (24 h FT group). Values are expressed as the mean with SEM above. n = 6–9 per group. The relative expression levels of ER alpha mRNA were calculated by dividing their GAPDH mRNA expression levels, and expressed as the ratio of the fed group values. *p<0.05; **p<0.01 vs. fed groups by one-way ANOVA with post-hoc Dunnett's test.

DISCUSSION

Our results show that hypothalamic ER alpha mRNA expression can respond to fasting at postnatal days 15 and 25, but not at postnatal day 5. Because leptin supplementation did not restore ER alpha mRNA expression during fasting, decreased leptin may not be involved in the regulation of hypothalamic ER alpha, at least in this model. To the best of our knowledge, this is the first report that examines the developmental change of the ER alpha response to undernutrition and the relationship between leptin and hypothalamic ER alpha mRNA expression.

Physiological functions that are involved in the response to stress and undernourishment develop during the neonatal to prepubertal period. Previously, we reported that the response of hypothalamic Kiss-1 mRNA, which is a positive regulator of gonadotropin-releasing hormone (GnRH), to undernutrition was established at postnatal day 25, but not at days 5 and 15, and that the responses of hypothalamic inflammatory cytokines to immune stress were dramatically increased at postnatal day 15 as compared with postnatal day 5 (Iwasa *et al.* 2010, 2011c). Hypothalamic neuropeptide Y (NPY) mRNA, which is an orexigenic factor, can respond to undernutrition around postnatal day 10–15, but not at postnatal day 5 (Kowalski *et al.* 1998; Iwasa *et al.* 2010). Estradiol directly or indirectly suppresses NPY mRNA expression in the hypothalamus of adult female mice and inhibits NPY secretion from murine immortalized hypothalamic neurons (Olofsson *et al.* 2009; Dhillon *et al.* 2010). In addition, these actions of estradiol are mainly mediated through membrane-bound ER alpha, but not ER beta (Dhillon *et al.* 2010). Therefore, we speculate that changes in ER alpha responses to undernutrition are involved, at least in part, in the development of the NPY mRNA response during the neonatal to prepubertal period because the

responses of ER alpha and NPY to fasting are established during the same period. However, further studies are needed to clarify this hypothesis.

It has been reported that ER alpha in the hindbrain, as well as in the hypothalamus, also plays an important role in the effect of estradiol on appetite, and that the hindbrain is important for appetite control in the early postnatal phase. Administration of estradiol to the dorsal raphe nucleus (DRN) reduces food intake in ovariectomized rats (Santollo *et al.* 2011). Similarly, hindbrain administration of estradiol activates ER alpha in the nucleus tractus solitarius (NTS) and inhibits feeding in ovariectomized rats (Thammacharoen *et al.* 2007). In addition, in breast-fed early neonatal rats, after 12 h of isolation from their mothers, the number of fos-immunoreactive neurons in the NTS becomes increased. This indicates that the NTS is involved in feeding (eating) behavior during the early neonatal period (Hironaka *et al.* 2000). Consequently, it can be supposed that the activity of ER alpha in the hindbrain is also changed according to feeding status, and that such alterations would affect eating behavior even in the neonatal period. To confirm this hypothesis, ER alpha mRNA levels in the hindbrain, for example in the NTS and DRN, should be measured and compared between normally fed and fasting animals in future experiments.

Estrogen is one of the candidates for the ER alpha regulation factor. In adult mice, ovariectomy was observed to increase the number of hypothalamic ER alpha immunoreactive cells (Chakraborty *et al.* 2008). Similarly, estrogen suppressed ER alpha mRNA expression in specific clonal hypothalamic neurons in vitro (Titolo *et al.* 2006). Thus, it can be assumed that estrogen is also involved in fasting-induced changes of hypothalamic ER alpha mRNA during the prepubertal period. However, the estradiol level in postnatal day 25 rats was not changed by fasting, indicating that estradiol might not be involved in the suppression of ER alpha in this experimental model. One possibility is that changes in another type of estrogen, for example, estrone, affect ER alpha. Another possibility is that the levels of other factors are changed by undernutrition and that these factors regulate ER alpha under fasting conditions. As noted above, some in vitro experiments have revealed that leptin affects ER alpha mRNA expression levels; therefore, we hypothesized that decreased leptin levels during fasting play a role in the regulation of hypothalamic ER alpha. However, we could not detect any effects of leptin administration on ER alpha mRNA expression in 24-h fasted rats, although the leptin levels were increased to supraphysiological levels. We used the acute food or maternal deprivation model to induce undernourishment. It is possible that the administered leptin could not overcome the pivotal effect of acute food deprivation on hypothalamic energy control systems. To address this hypothesis, chronic food restriction, but not deprivation, should be used to confirm the leptin action on hypothalamic factors, including

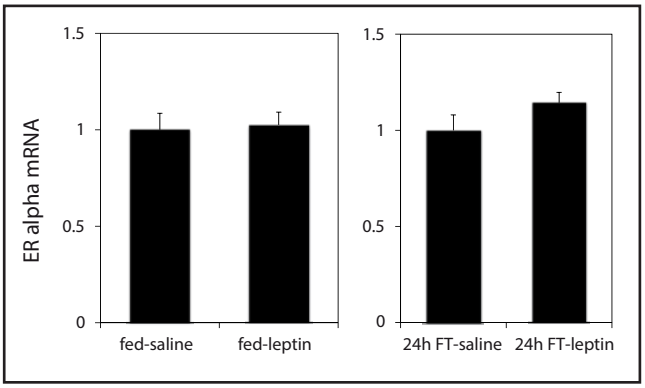

Fig. 2. Effects of leptin administration on hypothalamic estrogen receptor (ER) alpha mRNA levels in 25-day-old female rats under fed or fasting conditions. In the experiment under fed conditions, rats that were fed ad libitum were given intraperitoneal (i.p.) injection of leptin or saline twice. (The second injection was administered 9 h after the first injection. The hypothalamus was harvested 3 h after the second injection). In the experiment under fasting conditions, rats were subjected to 24 h of food deprivation (FD) and given intraperitoneal injections of leptin or saline at 12 h and 21 h after the initiation of FD. Values are expressed as the mean with SEM above. n=8 per group. The relative expression levels of ER alpha mRNA were calculated by dividing their GAPDH mRNA expression levels, and expressed as the ratio of the saline-received group values in the corresponding feeding condition. No significant difference was observed between the leptin- and saline-received groups with the unpaired Student's *t*-test under fed or fasting conditions.

ER alpha. In addition, we cannot exclude the possibility that supraphysiological levels of leptin, as found in this experimental model, exert other effects that prevent the restoration of the ER alpha mRNA expression level under the undernourished condition. Therefore, in future studies, another leptin dose should be selected that increases the blood leptin level to the level in the fed state for fasted rats. Also, experiments that evaluate the effects of food deprivation on hypothalamic ER alpha in mice lacking the leptin receptor (db/db mice) should be performed to accurately clarify the relationship between leptin and ER alpha.

This study had several limitations: 1) The entire hypothalamic block was used, and ER alpha mRNA expression could not be clarified in each hypothalamic nucleus. ER alpha mRNA expression levels were detected, but not the corresponding protein levels. ER alpha is abundant in two hypothalamic nuclei, the VMH and the ARC. In some cases, ER alpha in these areas changes differently during undernourishment. While ER alpha in the VMH, but not in the ARC, is decreased by chronic food restriction in prepubertal female mice and adult male rats (Roemmich *et al.* 1997; Lindblom *et al.* 2005), ER alpha in both areas is decreased by acute food deprivation in adult female rats (Jones *et al.* 2002). If ER alpha in these two areas were to change conversely (i.e., one is upregulated and another is downregulated), under the present experimental condi-

tions, the changes in each area would be overlooked. In addition, it is unclear whether mRNA levels of ER alpha can reflect the actual function of ER alpha in basal and undernourished conditions, although another research group has shown that changing the pattern of ER alpha mRNA level and protein level almost corresponds to estrogen-exposed clonal hypothalamic neurons (Titolo *et al.* 2006). Other research techniques, for example in situ hybridization and immunohistochemistry, should be used in future studies to improve spatial information. 2) The second limitation was that this study did not examine alterations of brain estradiol levels. It has been shown that steroidogenesis occurs in some brain areas – hippocampus, cortex, and hypothalamus – until the perinatal period, and that aromatase activity in these areas is related to the regional estradiol content (Konkle & McCarthy 2011). If central aromatase activity and estradiol levels are altered by food deprivation, these changes might induce ER alpha mRNA expression in some brain areas.

Taken together, these findings indicate that the sensitivity of hypothalamic ER alpha mRNA to negative energy balances may be established by postnatal day 15. Decreased leptin levels under these conditions are not involved in the changes of hypothalamic ER alpha mRNA. These changes might be adaptive responses to undernutrition that decrease the anorectic function of estrogen and increase appetite under inadequate nutritional conditions.

Conflict of interest

The authors have no conflicts of interest to declare that would prejudice the impartiality of this scientific work.

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