Sensitivities of mRNA expression levels of *Kiss1* and its receptor, *Kiss1r*, to nutritional status are changed during the developmental period in female rats

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Abstract

Decreased activity of kisspeptin, the product of the hypothalamic *Kiss1* gene, is the major cause of the suppression of reproductive function in subnutritional conditions. The sensitivities of the endocrine and the hypothalamic neuronal systems to nutritional status develop during the neonatal period. We examined the developmental changes in the sensitivity of hypothalamic mRNA expression of *Kiss1* and its receptor, *Kiss1r*, to nutritional status in female rats. *Kiss1* mRNA expression was reduced by 24 h food deprivation (24 h FD) at postnatal day 25, but not at postnatal day 5 or 15. *Kiss1r* mRNA expression was reduced by the 12 or 24 h FD at postnatal days 5 and 25, but not at postnatal day 15. *Kiss1r* mRNA level was found to be correlated with the plasma leptin level, and the administration of leptin, which increased

the serum leptin concentration above the physiological range, restored the acute FD-induced suppression of *Kiss1r* mRNA expression. These data suggest that the hypothalamic *Kiss1* and *Kiss1r* mRNA expression is differentially affected by the nutritional condition at different age points. It is speculated that the sensitivity of *Kiss1* mRNA, which is expressed in kisspeptin neuron, to nutritional status develops during the neonatal period. On the other hand, it seems that the sensitivity of *Kiss1r* mRNA, which is expressed in GnRH neuron, to nutritional status has been already established during the early neonatal period. These data also show that hypoleptinemia plays a role in the reduction of hypothalamic *Kiss1r* mRNA expression under subnutritional conditions.

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Introduction

Reproductive function is highly sensitive to metabolic and nutritional status. Both pulsatile secretion of LH and the LH surge are inhibited by short-term food deprivation (FD) in ovariectomized estradiol (E₂) and/or progesterone-primed female rodents (McClure & Saunders 1985, Cagampang *et al.* 1991, Wade & Schneider 1992, Kohsaka *et al.* 2001). Similarly, the estrous cycle is disrupted (Knuth & Friesen 1983) and sexual maturation is delayed by chronic food restriction in gonadal intact female rats (Castellano *et al.* 2005). Recently, kisspeptin, the product of the *Kiss1* gene, and its receptor, *Kiss1r*, a product of the G protein-coupled receptor 54 (*GPR54*) gene, have emerged as essential gatekeepers of reproduction. GnRH and the gonadotrophin release are stimulated and sexual maturation is accelerated by the administration of kisspeptin in several species (Gottsch *et al.* 2004, Irwig *et al.* 2004, Navarro *et al.* 2004, 2005, Messenger *et al.* 2005, Shahab *et al.* 2005, Roseweir & Millar 2009). On the other hand, hypogonadotropic hypogonadism and delayed sexual maturation are induced

by loss-of-function point mutations and deletion of *GPR54* in humans and mice (de Roux *et al.* 2003, Seminara *et al.* 2003). Experimental evidence suggests that a decrease in the activity of kisspeptin is the main cause of the inhibition of reproductive function observed in subnutritional conditions. Hypothalamic *Kiss1* mRNA expression is reduced in acute food-deprived female rodents (Castellano *et al.* 2005, Luque *et al.* 2007, Brown *et al.* 2008) and streptozotocin-induced diabetic male rats (Castellano *et al.* 2006), and hypogonadotropic hypogonadism can be restored in these animals by the administration of kisspeptin (Castellano *et al.* 2005, 2006, Luque *et al.* 2007). On the other hand, the effects of FD on *Kiss1r* are disputed. Although hypothalamic *Kiss1r* mRNA expression is increased by 72 h FD in prepubertal rats (Castellano *et al.* 2005), its expression levels are reduced by 12–48 h FD in adult mice (Luque *et al.* 2007).

It is suggested that leptin transmits signals related to energy balance to kisspeptin neurons through its receptor, Ob-R (Smith *et al.* 2006, Castellano *et al.* 2009, Roa *et al.* 2010) and acts as a positive regulator of the hypothalamic kisspeptin system. *Kiss1* mRNA expression is lower in leptin-deficient

ob/ob mice than in wild-type mice (Smith *et al.* 2006, Castellano *et al.* 2009, Roa *et al.* 2010), and the reduced *Kiss1* mRNA expression seen in streptozotocin-induced diabetic male rats can be normalized by central leptin infusion (Castellano *et al.* 2006, 2009, Roa *et al.* 2010). In addition, the mRNA expression levels of *Kiss1* and *Kiss1r* in primary cultures of human fetal GnRH-secreting neuroblasts are increased by the addition of leptin (Morelli *et al.* 2008).

It has been reported that the neuronal pathways in the hypothalamus develop rapidly during the neonatal period (Bouret *et al.* 2004, Bouret 2010). The neuronal projections from the arcuate nucleus (ARC) to the periventricular nucleus (PeN) develop from postnatal day 8, and the mature patterns of innervation are achieved by postnatal day 16 (Bouret *et al.* 2004). Similarly, neuronal projection of kisspeptin also develops during the neonatal period (Clarkson & Herbison 2006). In addition, experimental data have suggested that the sensitivity of some endocrine and hypothalamic neuronal systems to metabolic and nutritional status develops simultaneously with their neuronal projection during the neonatal period. For example, the sensitivity of hypothalamic orexigenic and anorexigenic factors to leptin develops during the neonatal period, and the anorectic effects of leptin on appetite and feeding behaviors are acquired during the same period in mice and rats (Mistry *et al.* 1999, Carlo *et al.* 2007). Similarly, the adrenal response to FD is established during the neonatal period in mice (Ahima *et al.* 1998). It has also been reported that an abnormally high or low nutritional status during the fetal or neonatal period permanently affects hypothalamic function and induces metabolic diseases in later life in humans and experimental animals (Barker *et al.* 1993, Proulx *et al.* 2002, Yura *et al.* 2005, Bouret *et al.* 2008, Delahaye *et al.* 2008, Remmers *et al.* 2008, Breton *et al.* 2009, Kirk *et al.* 2009). Although the effects of undernutrition on kisspeptin action have been investigated in mice and rats at various reproductive ages, the developmental changes that occur in the sensitivity of the kisspeptin system to nutritional status have not been evaluated.

In this study, we examined the developmental change in the sensitivity of hypothalamic *Kiss1* and *Kiss1r* mRNA expression to nutritional status in female rats. In addition, we have examined whether the acquisition of leptin sensitivity by the kisspeptin system is involved in the developmental changes in its sensitivity to nutritional status because the leptin sensitivity of other hypothalamic factors is established during the neonatal period.

Materials and Methods

Animals

Sprague–Dawley rats (Charles River Japan Inc., Tokyo, Japan) were used. The day the litters were born was considered as postnatal day 1. The animals were housed in a room with controlled lighting (14 h light:10 h darkness cycle) and

temperature (24 °C) and 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 maternal and food deprivation on plasma hormone concentrations and hypothalamic neuropeptide (GnRH, Kiss1, and Kiss1r) mRNA levels at various developmental stages

To determine the effects of maternal deprivation (MD) and/or FD on hypothalamic neuropeptide mRNA levels at various developmental stages, female rats at postnatal days 5, 15, and 25 of age were divided into three groups: the control, the 12 h MD or FD, and the 24 h MD or FD groups ($n=7-8$ per group). The rats in all the groups were weighed, and the rats in the 12 and 24 h deprivation groups were separated from their mothers (postnatal day 5 or 15) or were deprived of food (postnatal day 25). At the end of the MD or FD, the animals were weighed and killed by decapitation between 0900 and 1000 h of the light cycle, and their blood and whole brain were collected. The rats in the control group were neither separated from their mothers nor deprived of food and were weighed again after 24 h and then killed by decapitation. The serum was separated by centrifugation and stored at -20°C , and the whole brain was snap frozen and stored at -70°C . Before the RNA analysis, hypothalamic explants, including the median preoptic area, the anteroventral PeN (AVPV), and the ARC, were dissected out from the frozen brains according to the following methods, as described elsewhere (Castellano *et al.* 2006). A brain section was dissected out through an anterior coronal cut at 1 mm (postnatal days 5 and 10) or 2 mm (postnatal day 25) anterior from the optic chiasm and a posterior coronal cut at the posterior border of the mammillary bodies. Then, these tissue blocks were cut through two parasagittal cuts along the hypothalamic fissures and dorsally cut at 2.0 mm (postnatal days 5 and 10) or 2.5 mm (postnatal day 25) from the ventral surface.

The plasma leptin and LH (postnatal days 15 and 25) concentrations and the hypothalamic *Kiss1*, *Kiss1r*, and *GnRH* mRNA levels were then measured. As the sample volume for the postnatal day 5 rats was insufficient, the plasma LH concentration could not be measured. In the postnatal day 25 rats, the serum E_2 concentration, the ovarian weight, and the uterine weight were also measured because a change in the E_2 concentration might have affected the hypothalamic *Kiss1* mRNA level.

Effects of the co-administration of leptin on FD-induced alterations in plasma hormone concentrations and hypothalamic neuropeptide (Kiss1 and Kiss1r) mRNA levels

To determine the effects of the co-administration of leptin on FD-induced alterations in plasma hormone concentrations and hypothalamic neuropeptide mRNA levels, the postnatal day 25 female rats were divided into three groups: the control,

the 24 h FD, and the 24 h FD with leptin administration (24 h FD + lep) groups ($n=7-8$ per group). The rats in the 24 h FD + lep group were given i.p. injections of leptin (3 mg/kg body weight) at 12 and 21 h after the initiation of FD. This dose of leptin is sufficient to maintain the plasma leptin concentration above physiological levels for 13 h in neonatal rats (Proulx *et al.* 2002). We measured the plasma leptin concentration following exogenous leptin administration at the dose used in this study as a preliminary experiment. The rats in the control and 24 h FD groups were administered i.p. injections of saline at the same time points. At the end of the FD, the animals were killed by decapitation. The blood and whole brain were collected, and hypothalamic explants were dissected out as mentioned above. Then, plasma LH concentrations and hypothalamic *GnRH*, *Kiss1*, and *Kiss1r* mRNA levels were measured.

Hormone assays

The plasma leptin concentration was measured using an I-125 RIA kit (Rat leptin 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 (CV) were 4.8 and 2.4% respectively. The plasma LH concentration was measured using an I-125 RIA kit (Rat LH (I-125) RIA kit, Institute of Isotopes Co., Ltd, Tokyo, Japan). The sensitivity of the assay was 0.2 ng/ml. The inter- and intra-assay CV values were 6.6 and 6.5% respectively. The plasma E_2 concentration was measured using an I-123 RIA kit (Double Antibody E_2 kit, Diagnostic Products Co., Los Angeles, CA, USA). The sensitivity of the assay was 1.4 pg/ml. The inter- and intra-assay CV values were 4.1 and 4.6% respectively.

Quantitative real-time PCR

Total RNA was isolated from the hypothalamus using a TRIzol reagent kit (Invitrogen Co.) and an RNeasy Mini kit (Qiagen GmbH). cDNA was synthesized with oligo (deoxythymidine) primers at 50 °C using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Co.). Real-time PCR analysis was performed using the PCR System 7500 (PE Applied Biosystems, Foster City, CA, USA) with SYBR green. Sequence-specific primers were generated on the basis of the published rat sequences using the Primer3 software (Whitehead Institute/MIT Center for Genome Research; <http://www.genome.wi.mit.edu>). The forward and reverse primers used were as follows: *Kiss1*: F: 5'-AGC TGC TGC TGC TTC TCC TCT GT-3', R: 5'-AGG CTT GCT CTC TGC ATA CC-3'; *Kiss1r*: F: 5'-GCA GAC CGT CAC CAA TTT CT-3', R: 5'-GGG AAC ACA GTC ACG TAC CA-3'; *GnRH*: F: 5'-GCA GAA CCC CAG AAC TTG GA-3', R: 5'-TGC CCA GCT TCC TCT TCA AT-3'; and β -actin: F: 5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3', R: 5'-CTT AGA AGC ATT TGC GGT GCA CG-3'. The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95 °C for

10 min, followed by 45 cycles of denaturation at 95 °C for 15 s; annealing at 63 °C for 30 s (*Kiss1*, *Kiss1r*), 58 °C for 30 s (*GnRH*), or 65 °C for 30 s (β -actin); and extension at 72 °C for 1 min. The copy numbers of the transcripts were normalized against those of β -actin transcripts for each sample.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Fisher's protected least significant difference test. All results are presented as means $\pm$ S.E.M. Differences were considered to be statistically significant at $P<0.05$. Correlation analyses were performed using Spearman's correlation as appropriate.

Results

Effects of MD and FD on the plasma leptin concentration and hypothalamic neuropeptide (*GnRH*, *Kiss1*, and *Kiss1r*) mRNA levels at various developmental stages

In postnatal day 5 rats, body weight (one-way ANOVA; $P<0.001$, $F(3,47)=22.38$) and plasma leptin concentration (one-way ANOVA; $P=0.006$, $F(3,52)=6.89$) were significantly lower in the 12 h MD and 24 h MD groups than in the control group (Fig. 1). Similarly, the *Kiss1r* mRNA level was significantly lower in the 24 h MD group (one-way ANOVA; $P=0.002$, $F(3,49)=5.01$) than in the control group. However, the mRNA levels of *GnRH* (one-way ANOVA; $P=0.306$, $F(3,47)=1.26$) and *Kiss1* (one-way ANOVA; $P=0.751$, $F(3,47)=0.29$) were not altered by MD.

Figure 1 Effects of maternal food deprivation (MD) on body weight (A), plasma leptin concentration (B), and hypothalamic neuropeptide (*GnRH*, *Kiss1*, and *Kiss1r*) mRNA levels (C–E) in postnatal day 5 female rats. Values are expressed as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$.

In the postnatal day 15 rats, body weight (one-way ANOVA; $P < 0.001$, $F(3,59) = 102.86$) and the plasma leptin concentration (one-way ANOVA; $P < 0.001$, $F(3,63) = 34.08$) were significantly lower in the 12 h MD and 24 h MD groups than in the control group (Fig. 2). However, the *GnRH* (one-way ANOVA; $P = 0.310$, $F(3,59) = 1.25$), the *Kiss1* (one-way ANOVA; $P = 0.683$, $F(3,59) = 0.39$), and the *Kiss1r* (one-way ANOVA; $P = 0.624$, $F(3,59) = 0.48$) mRNA levels were not altered by MD. Similarly, the plasma LH concentration was not altered by MD (one-way ANOVA; $P = 0.404$, $F(3,89) = 0.98$).

In the postnatal day 25 rats, body weight (one-way ANOVA; $P < 0.001$, $F(3,47) = 127.30$) and plasma leptin concentration (one-way ANOVA; $P < 0.001$, $F(3,47) = 22.95$) were significantly lower in the 12 h FD and 24 h FD groups than in the control group (Fig. 3). Similarly, the *Kiss1* (one-way ANOVA; $P = 0.040$, $F(3,47) = 3.73$) and *Kiss1r* (one-way ANOVA; $P < 0.001$, $F(3,47) = 13.54$) mRNA levels and the plasma LH concentration (one-way ANOVA; $P = 0.008$, $F(3,49) = 6.23$) were significantly lower in the 24 h FD group than in the control group. However, the *GnRH* (one-way ANOVA; $P = 0.446$, $F(3,47) = 0.84$) mRNA levels were not altered by FD. In addition, the serum E_2 concentration (one-way ANOVA; $P = 0.249$, $F(3,52) = 1.50$), the ovarian weight (one-way ANOVA; $P = 0.423$, $F(3,47) = 0.90$), and the uterine weight (one-way ANOVA; $P = 0.530$, $F(3,52) = 0.66$) were not altered, showing that E_2 was not associated with changes in the *Kiss1* or *Kiss1r* mRNA level.

In the postnatal day 25 rats, the *Kiss1* mRNA expression level was not correlated with the plasma leptin

concentration (Fig. 4). On the other hand, the *Kiss1r* mRNA expression level was positively correlated with the plasma leptin concentration.

Effects of leptin administration on FD-induced alterations in the plasma LH concentration and hypothalamic neuropeptide (*Kiss1* and *Kiss1r*) mRNA levels

In the postnatal day 25 rats, the plasma leptin concentration following exogenous leptin administration at the dose used in this study (3 mg/kg body weight) was significantly increased at each time point (4.5, 9, and 12 h) compared with that of the saline-administered rats (Fig. 5). Co-administration of leptin restored the 24 h FD-induced reduction of *Kiss1r* mRNA expression (*Kiss1r* mRNA expression was significantly higher in the 24 h FD+lep group than in the 24 h FD group; Fig. 6). On the other hand, it did not restore the 24 h FD-induced reduction of *Kiss1* mRNA expression or the plasma LH concentration.

Discussion

In this study, we have shown that the sensitivity of the hypothalamic kisspeptin system to nutritional status in female rats changes during the developmental period. Hypothalamic *Kiss1* mRNA expression was reduced by FD at postnatal day 25, but not in the early developmental period (postnatal days 5 and 15). These developmental changes in the sensitivity of *Kiss1* mRNA expression to nutritional status resemble their neuronal projection developmental patterns. It has been reported that no kisspeptin neurons were detected in the AVPV or PeN of female mice at postnatal day 15, after which they increased in number from postnatal day 25 until they had reached adult levels at the onset of puberty (Clarkson & Herbison 2006). In addition, the close apposition between kisspeptin fibers and GnRH cell bodies becomes apparent at postnatal day 25 (Clarkson & Herbison 2006). Thus, it is assumed that the sensitivity of the *Kiss1* mRNA expression to metabolic and nutritional status develops simultaneously with its neuronal projections during the neonatal period. These developmental patterns of the kisspeptin system resemble those of other endocrine and neuronal systems, i.e. the hypothalamus–pituitary–adrenal axis and the hypothalamic appetite regulatory system (Ahima *et al.* 1998, Mistry *et al.* 1999, Carlo *et al.* 2007). Many epidemiological and experimental studies have indicated that an abnormally high or low nutritional status or putative stress during the plastic period induces long-term alterations in physiological systems (Barker *et al.* 1993, Proulx *et al.* 2002, Yura *et al.* 2005, Bouret *et al.* 2008, Delahaye *et al.* 2008, Remmers *et al.* 2008, Breton *et al.* 2009, Kirk *et al.* 2009). Although these alterations are adaptations to environmental conditions, they often increase the risks of certain diseases in later life (Barker *et al.* 1993). Similarly, we have shown that prenatal undernutrition reduces hypothalamic *Kiss1* mRNA expression throughout

Figure 2 Effects of maternal food deprivation (MD) on body weight (A), plasma leptin concentration (B), plasma LH concentration (C), and hypothalamic neuropeptide (*GnRH*, *Kiss1*, and *Kiss1r*) mRNA levels (D–F) in postnatal day 15 female rats. Values are expressed as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

Figure 3 Effects of food deprivation (FD) on body weight (A), plasma leptin concentration (B), plasma LH concentration (C), ovarian weight (D), uterine weight (E), plasma estradiol concentration (F), and hypothalamic neuropeptide (*GnRH*, *Kiss1*, and *Kiss1r*) mRNA levels (G–I) in postnatal day 25 female rats. Values are expressed as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

the developmental period and that this alteration retards sexual maturation in female rats (Iwasa *et al.* 2010). Furthermore, another group demonstrated that neonatal immune stress reduces hypothalamic *Kiss1* mRNA levels during the prepubertal period in female rats (Knox *et al.* 2009). Therefore, it can be assumed that a negative energy balance in the early developmental period induces alterations in the hypothalamic kisspeptin system and negatively affects sexual maturation and reproductive function in later life. Further epidemiological and experimental studies are needed to clarify this hypothesis.

It has been reported that the sensitivities of some hypothalamic factors to leptin are established in the early developmental period. Therefore, we assumed that the acquisition of leptin sensitivity was involved in the establishment of the sensitivity of *Kiss1* mRNA expression to acute FD. However, contrary to our expectations, hypothalamic *Kiss1* mRNA expression was not correlated with the plasma leptin level, and the administration of leptin did not restore the reduced *Kiss1* mRNA and plasma LH levels induced by acute FD at postnatal day 25. As mentioned above, the plasma leptin concentration observed following exogenous leptin administration at the dose used in this study was significantly

increased compared with that of the saline-administered rats. It is disputed whether hypoleptinemia plays a role in the suppression of reproductive hormones under acute food-restricted conditions. For example, the suppression of plasma LH and testosterone levels in acute food-restricted male monkeys is not restored by short-term leptin administration (Lado-Abeal *et al.* 1999). Our present data also support the hypothesis that hypoleptinemia is not involved in the suppression of reproductive function induced by acute food restriction. As whole hypothalamic blocks containing both the AVPV and ARC were used for the gene expression analyses, the effects of FD and leptin on the *Kiss1* mRNA

Figure 4 Correlations between the *Kiss1* (A) or *Kiss1r* (B) mRNA level and the plasma leptin concentration in postnatal day 25 female rats.

Figure 5 Time course of the changes in plasma leptin concentrations after saline or exogenous leptin (3 mg/kg body weight) administration in postnatal day 25 female rats. Values are expressed as the mean $\pm$ S.E.M. ** $P < 0.01$ compared with the saline group.

expression in individual hypothalamic nuclei could not be examined. Therefore, it remains possible that subtle changes in gene expression in each nucleus might have escaped detection due to the use of whole hypothalamic blocks. In addition, it is possible that leptin regulates *Kiss1* mRNA expression in an opposing manner in the AVPV and ARC, similar to estrogen, which stimulates *Kiss1* mRNA expression in the AVPV but inhibits it in the ARC (Oakley *et al.* 2009, Uenoyama *et al.* 2009). If this is true, the opposing regulatory effects of leptin might have canceled each other out, resulting in no change in the *Kiss1* mRNA expression level of the whole hypothalamic block. Further precise examinations, i.e. through *in situ* hybridization, are needed to clarify the effects of metabolic status and leptin on *Kiss1* mRNA expression in each nucleus.

We have shown that hypothalamic *Kiss1r* mRNA expression was reduced by acute FD at postnatal days 5 and 25 but not at day 15. These findings suggest that the hypothalamic *Kiss1r* is sensitive to metabolic status from the early neonatal period onward. These developmental changes in the sensitivity of *Kiss1r* mRNA expression to metabolic status were different from those of *Kiss1* mRNA expression. As *Kiss1* mRNA is expressed in kisspeptin neurons, it is reasonable that the developmental changes observed in the sensitivity of *Kiss1* mRNA expression to metabolic status fit well with the pattern of kisspeptin neuron development during the neonatal period. On the contrary, *Kiss1r* mRNA is expressed in another neuronal population, i.e. GnRH neurons in the preoptic area. Thus, the developmental changes observed in the sensitivity of *Kiss1r* mRNA expression to metabolic status do not fit with the developmental pattern of kisspeptin neurons.

We have also shown that the hypothalamic *Kiss1r* mRNA level was correlated with the plasma leptin level and that the administration of leptin restored the reduced *Kiss1r* mRNA expression level induced by acute FD at postnatal day 25, suggesting that hypoleptinemia plays a role in the reduction of *Kiss1r* mRNA expression induced by acute FD. On the other hand, the administration of a dose of leptin above the physiological range in normal fed rats did not increase

the hypothalamic *Kiss1r* mRNA levels in our preliminary evaluation (1.50 ± 0.19 (control) versus 1.24 ± 0.13 (leptin administration), mean $\pm$ S.E.M.). Therefore, it is assumed that *Kiss1r* mRNA expression remains stable when the serum leptin levels are within or above the normal range and that its expression decreases when the plasma leptin level falls below the lower threshold of the normal range. In rodents, the plasma leptin level increases around postnatal days 8–16, which is called the leptin surge (Yura *et al.* 2005). In this study, the plasma leptin level of the control group at postnatal day 15 (4.04 ± 0.45 ng/ml, mean $\pm$ S.E.M.) was significantly higher than that at day 5 (0.88 ± 0.14 ng/ml) and day 25 (1.62 ± 0.15 ng/ml). In addition, the plasma leptin level of the 24 h FD group at postnatal day 15 (1.31 ± 0.13 ng/ml, mean $\pm$ S.E.M.) was also significantly higher than that at day 5 (0.40 ± 0.05 ng/ml) and day 25 (0.78 ± 0.06 ng/ml). We suppose that the plasma leptin level had not fallen below the level required to maintain the hypothalamic *Kiss1r* mRNA level by postnatal day 15, even after FD. If the MD had been continued for longer than 24 h in the postnatal day 15 rats, we assume that the plasma leptin level would have fallen below the lower threshold of the normal range, causing hypothalamic *Kiss1r* mRNA expression to decrease. We also speculate that the lower threshold of the plasma leptin level required to maintain *Kiss1r* mRNA expression is raised during development and that higher plasma leptin levels are required to maintain *Kiss1r* mRNA expression in postnatal day 25 rats compared with postnatal day 15 rats. However, it is unclear whether alterations in hypothalamic *Kiss1r* mRNA expression at postnatal day 5 or 25 play a role in fasting-induced suppression of reproductive function because the restoration of the *Kiss1r* mRNA level by leptin administration did not affect the plasma LH level in this study. Further examinations are needed to clarify the physiological roles of these alterations.

Figure 6 Effects of leptin administration on 24 h food deprivation (FD)-induced alterations in body weight (A), plasma LH concentration (B), and neuropeptide (*Kiss1* and *Kiss1r*) mRNA levels (C and D) in postnatal day 25 female rats. Values are expressed as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

In this study, we have focused on the acute changes that occur following MD or FD and leptin administration. However, leptin is regarded as a key signal for the long-term control of energy balance. It has been reported that the chronic negative energy balance caused by diabetes mellitus reduces *Kiss1* mRNA expression and that chronic leptin infusion is sufficient to normalize the *Kiss1* mRNA level (Castellano *et al.* 2006). Thus, it remains possible that longer treatment with leptin can rescue *Kiss1* mRNA expression under chronic food restriction.

In summary, we have shown that the sensitivity of the hypothalamic mRNA expression of *Kiss1* and *Kiss1r* mRNA to nutritional status is altered during the development in female rats. The sensitivity of hypothalamic *Kiss1* mRNA expression to acute FD was not established during the early neonatal period; however, it had become apparent by postnatal day 25. It is speculated that these alterations in the sensitivity of kisspeptin neurons to nutritional status develop simultaneously with their neuronal projections during the neonatal period. On the other hand, the sensitivity of hypothalamic *Kiss1r* mRNA expression to acute FD may be established in the early neonatal period. In addition, the reduced *Kiss1r* mRNA expression observed in undernourished conditions may be caused by decreases in serum leptin levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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