Food restriction during lactation suppresses *Kiss1* mRNA expression and kisspeptin-stimulated LH release in rats

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Abstract

Among the numerous physiological changes that accompany lactation is the suppression of the reproductive axis. The aim of this study was to investigate a possible role for the kisspeptin system in the restoration of the hypothalamic–pituitary–gonadal axis during late lactation in rats using a food restriction model that allows manipulation of the duration of lactational anovulation. *Kiss1* mRNA expression and kisspeptin-immunoreactive cell counts were examined in both food-restricted dams and *ad libitum* (AL)-fed dams across late lactation when LH concentrations begin to increase. In the arcuate nucleus, *Kiss1* mRNA expression and kisspeptin-positive cell counts were suppressed during late lactation. In the anteroventral periventricular (AVPV), day 15 food-restricted dams had significantly lower AVPV *Kiss1* mRNA expression and a decreased LH response to exogenous kisspeptin compared with the AL-fed dams. Following 5 days of *ad libitum* food intake, these values were restored to levels similar to those in dams that had been fed *ad libitum* throughout lactation. In conclusion, this study shows that delayed restoration of the reproductive axis due to food restriction is associated with a decrease in kisspeptin sensitivity and low AVPV *Kiss1* mRNA in late lactation.

Reproduction (2014) **147** 743–751

Introduction

In many species of animals, lactation is associated with a variable period of time during which ovulation does not occur. It has been shown previously in rats that this period of lactational anovulation is accompanied by a suppression of pulsatile release of luteinizing hormone (LH; Smith 1978, Walker *et al.* 1995) and a reduction in the ability of estrogen to stimulate a LH surge (Smith & Neill 1977, Smith 1978). Lactation is also associated with a huge metabolic demand due to the production of milk, and despite food consumption of ~300% of that of virgin rats, lactating dams enter a state of negative energy balance. Although it has long been known that food availability plays a key role in the maintenance of fertility, the negative energy balance experienced by lactating rats is not necessary for the maintenance of lactational anovulation (Woodside 1991, Woodside & Popeski 1999). However, food restriction in lactating rats for the first 2 weeks *postpartum* (pp), at a level of 50% of the *ad libitum* daily ration, prolongs the duration of lactational anovulation by ~7 days (Woodside 1991). In this model, food is returned *ad libitum* from day 15 of lactation onwards, yet the effects of this period of food restriction on the hypothalamic–pituitary–gonadal (HPG) axis persist after refeeding. In association with the prolonged period of lactational anovulation, pulsatile LH release remains

suppressed for longer than is observed in *ad libitum* (AL)-fed dams (Woodside 1991, Walker *et al.* 1995), and there is a delay in the restoration of estrogen's ability to induce a LH surge (Abizaid *et al.* 2003). Overall, the food-restricted dams experience a predictable and consistent delay in return to normal functioning of their HPG axis and the resumption of estrous cyclicity compared with the AL-fed dams, providing a useful model to investigate the mechanisms underlying the resumption of fertility after lactation. Furthermore, although the characteristics of lactational anovulation vary between different mammals, for example, *postpartum* ovulation is observed in rodents, the suppression of LH pulsatile release is thought to be a defining characteristic (McNeilly 1994). Given this similarity, along with the well-characterized changes in the HPG axis during lactation in rats (McNeilly 1994), this species provides a useful and relevant model to investigate the mechanisms underlying lactational anovulation.

Pituitary responsiveness to gonadotropin-releasing hormone (GNRH) administration and GNRH receptor levels are similar in AL-fed and food-restricted dams, suggesting that the effects of food restriction on lactational anovulation are mediated at the level of GNRH secretion. The central mechanisms underlying the inhibition of fertility during lactation, the further suppressive effects of food restriction, and the mechanisms involved in the normal restoration of the HPG axis in the *postpartum*

period are not fully understood. The *Kiss1* gene codes for a family of neuropeptides called kisspeptins (Kotani *et al.* 2001), which are potent stimulators of GnRH neurons (Han *et al.* 2005). In rodents, there are two populations of kisspeptin neurons, one in the arcuate nucleus and the other in the anteroventral periventricular nucleus (AVPV). Estradiol stimulates *Kiss1* mRNA expression in the AVPV and suppresses *Kiss1* mRNA expression in the arcuate nucleus (Smith *et al.* 2005). Arcuate nucleus *Kiss1* mRNA expression has been shown to be suppressed during early and mid lactation (Yamada *et al.* 2007, Xu *et al.* 2009, True *et al.* 2011), while AVPV *Kiss1* mRNA is either unchanged (Yamada *et al.* 2007) or decreased (True *et al.* 2011). These data suggest that a decrease in kisspeptin expression might underlie the loss of fertility during lactation. The aim of the present study was to use the food restriction model to investigate *Kiss1* mRNA expression, kisspeptin-immunoreactive cell number, and responsiveness to kisspeptin during the later stages of lactation when the HPG axis is undergoing reactivation. We hypothesized that *Kiss1* mRNA would increase during the reactivation of the HPG axis and that this would be delayed in food-restricted dams experiencing a prolonged lactational anovulation. Furthermore, we predicted that the ability of kisspeptin to stimulate LH release would also vary as a function of the stage of lactation and/or food availability during early lactation.

Materials and methods

Animals

Virgin female Wistar rats, weighing 220–240 g at the start of the experiment, were obtained from Charles River Breeding Farms (St Constant, Quebec, Canada) and housed in groups of four or five per cage under a 12 h light:12 h darkness cycle with lights on at 0800 h. Temperature was maintained at 21 ± 1 °C, and all rats had free access to food and water, unless otherwise stated. A sexually experienced male rat was placed in each cage, and ~18 days later, pregnant female rats were individually caged. Virgin rats were housed individually in similar cages just before the onset of the study. This group of diestrous rats was included to identify any changes that were specific to lactation. The day on which the pregnant rats gave birth was assigned as day 0 pp, and on the following day, litter size was adjusted to eight pups per dam. Given the previously reported effects of ovariectomy on *Kiss1* mRNA expression in the AVPV and arcuate nucleus (Smith *et al.* 2005), a group of ovariectomized (OVX) rats was also included in this study to serve as a positive control. To generate the OVX group, female rats were anesthetized with isoflurane and ovaries were removed through bilateral dorsal incisions in the skin and body wall. Rats were then left to recover for ~1 week. Food intake, body weight, litter weight, where appropriate, and vaginal cytology were monitored throughout the experiment for all rats. For the lactating females, the percentage of cornified epithelial cells in the vaginal smear was rated by two independent judges. When 70% of the cells were assessed to be of the cornified type, the female was

considered to be in estrus and the anovulatory state to be terminated. The duration of the period of lactational diestrus was calculated as the number of days between the day of parturition and the first estrus and represents the length of time during lactation in which the animal presents a diestrous-like vaginal smear. All procedures were approved by the Concordia University Animal Research Ethics Committee under the guidelines of the Canadian Council on Animal Care.

Food restriction model

Lactating rats in the food-restricted group were fed 50% of a previously determined *ad libitum* amount of food for the first 2 weeks of lactation (days 1–14 pp inclusive) and were fed *ad libitum* from day 15 onwards. Thus, dams in the food-restricted group tested on day 20 pp had experienced 5 days of *ad libitum* access to food. Daily food rations were given to the food-restricted dams at ~0900 h. This food restriction paradigm predictably extends the period of lactational diestrus by ~1 week (Woodside 1991, Walker *et al.* 1995, Abizaid *et al.* 2003), allowing investigation of the HPG axis before and after reactivation at the end of lactation.

Kiss1 mRNA expression during lactation

Kiss1 mRNA expression was determined in intact nonlactating (diestrous) rats, OVX rats, AL-fed dams (days 15 and 20 pp), and food-restricted dams (days 15 and 20 pp) by microdissection and quantitative RT-PCR. On the appropriate experimental day, the rats were decapitated and brains were rapidly removed and frozen on dry ice. Coronal brain sections (300 µm) were cut in a cryostat at –9 °C and then thaw-mounted on to glass slides. The AVPV and arcuate nucleus were dissected from these sections using a blunt-ended microdissection needle (Palkovits & Brownstein 1988). Dissected tissue was placed in 995 µl of TRIzol reagent (Invitrogen), along with 5 µl of glycogen (20 µg/µl), and briefly sonicated. Total cellular RNA was extracted from microdissected samples using TRIzol reagent according to the manufacturer's protocol. To denature any contaminating DNA, samples were treated with DNase I (amplification grade). For each sample, RNA concentration and quality were measured using a bioanalyzer (Agilent Technologies 2100 Bioanalyzer, Agilent Technologies Inc, Waldbronn, Germany). Only samples with intact 18S and 28S RNA peaks and RIN values >6.00 were included in the subsequent qPCR. Total RNA (100 ng) was reverse-transcribed using iScript Reverse Transcriptase (Bio-Rad) on a thermal cycler for 5 min at 25 °C and 30 min at 42 °C followed by 5 min at 85 °C. The resulting cDNA was analyzed by quantitative PCR for *Kiss1* mRNA (forward primer: 5'-ATGATCTCGCTGGCTTCTGG-3', reverse primer: 5'-GGTTCACCACAGGTGCCATTTT-3'; accession number: NM_181692) and β -actin mRNA (forward primer: 5'-AGATGACCCAGATCATGTTTGAGA-3', reverse primer: 5'-ACCAGAGGCATACAGGGACAA-3'; accession number: NM_031144) using SYBR Green on the Bio-Rad CFX96 Real-Time PCR Detection System. Final primer concentrations were 250 nM for β -actin and 500 nM for kisspeptin. Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was

amplified. Standard curves were used to confirm that the efficiency of amplification for each primer pair was 100%. No amplification was detected in the absence of template or in the no-RT control. Data were analyzed using the comparative C_q method, where the C_q value is defined as the cycle number in which the fluorescent signal for each reaction is first detected as significantly above the background fluorescent levels. There was no significant difference in average β -actin C_q levels between the groups (one-way ANOVA; $P < 0.05$), indicating that β -actin levels did not vary significantly across the experimental conditions. To normalize for any variation, the C_q value obtained for β -actin was subtracted from the C_q value obtained for kisspeptin to give a ΔC_q value. This value was then combined to form the average ΔC_q for each group. For relative quantitation, all the data points were compared with those of the diestrous control group, using the formula relative quantification = $2^{-\Delta\Delta C_q}$, where $\Delta\Delta C_q$ is the average diestrous ΔC_q , minus the average experimental group ΔC_q values. Data are presented as relative gene expression in comparison with the diestrous control group $\pm$ S.E.M.

Kisspeptin-immunoreactive cell number during lactation

The number of kisspeptin-immunoreactive cells in the arcuate nucleus was determined in the day 20 pp AL-fed and food-restricted dams as well as in the nonlactating group, all of which were in the diestrous phase of the estrous cycle. The AVPV population was not examined, as this population is very difficult to detect by immunohistochemistry in rats (Desrozières *et al.* 2010). On the designated day *postpartum* or of the estrous cycle, the rats were given an overdose of Euthanyl (sodium pentobarbital, 60 mg/rat) and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed, incubated at 4 °C in 4% paraformaldehyde containing 30% sucrose for 48 h and then stored at –80 °C. Frozen brains were sliced in a cryostat at –22 °C. Coronal sections (40 μ m) were collected through the arcuate nucleus of the hypothalamus and stored in a cryoprotectant at –20 °C. The sections were processed using single-label immunohistochemistry for kisspeptin. All washes consisted of three 10-min incubations in TBST (TBS/0.025% Triton X-100/130 mM NaCl) on an orbital shaker at room temperature. The sections were removed from storage, washed, and incubated for 10 min at room temperature in 0.3% H_2O_2 to block endogenous peroxide activity. The sections were then washed and incubated in a blocking buffer (wash buffer containing 2% normal goat serum and 0.25% BSA) for 1 h at room temperature. Following this, the sections were incubated at 4 °C in a blocking buffer containing a polyclonal rabbit anti-kisspeptin-10 antiserum (AB9754, Millipore, Billerica, MA, USA) at a concentration of 1:2000. This antibody was initially characterized and distributed by Dr Alan Caraty (Franceschini *et al.* 2006). Low levels of cross-reaction with RFRP1 have been suggested (True *et al.* 2011); however, similar to others, we did not detect any staining in the dorsomedial nucleus of the hypothalamus, suggesting that any cross-reaction is minimal (True *et al.* 2011). After ~48 h of incubation, the sections were washed and then incubated with a biotinylated goat anti-rabbit antibody for 1 h (1:1000 dilution in

TBST containing 2% normal goat serum). Next, the sections were washed and treated with an avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) for 1 h. After washing, staining was developed using 3,3'-diaminobenzidine (DAB, nickel-intensified substrate kit from Vector Laboratories). This reaction was stopped by three 10-min incubations in a wash buffer. The sections were mounted on gelatin-coated slides and, when dry, the slides were dehydrated through an alcohol series ending with two 20-min xylene incubations. The slides were coverslipped with Permount and left to air-dry.

The sections were viewed using a Leica DMR-HC microscope mounted with a Hitachi 3CCD camera (Model #HV-C20), and images were captured on a G4 Macintosh computer using Scion Image Software 1.63. All the sections were analyzed by an observer blind to the experimental group. The number of kisspeptin-positive cells in the arcuate nucleus was determined (–2.00 to –3.25 mm posterior to bregma (Paxinos & Watson 1986)), and at least four sections per brain were examined. The mean number of stained cells per section was calculated for each rat.

LH response to exogenous kisspeptin during lactation

The LH response to centrally administered exogenous kisspeptin was examined on day 15 and day 20 pp in the AL-fed and food-restricted dams and diestrous rats. Separate groups of lactating rats were used on day 15 and day 20 of lactation. Lactating dams on days 1–3 pp and nonlactating rats on metestrus were anesthetized (6 mg ketamine/1.1 mg xylazine per 100 g of body weight) and placed in a stereotaxic apparatus. A 23-gauge stainless-steel guide cannula was implanted in the lateral ventricle with the following coordinates: anteroposterior 0:00; lateral 0:16; and ventral 0:50 (Paxinos & Watson 1986). The cannula was secured to the skull with jeweler's screws and dental cement. Following blood sampling, the animals were transcardially perfused with saline followed by 4% paraformaldehyde, and brains were collected, processed, and sectioned as described above. The location of the tip of the cannula was determined in the brain slices and animals in which the cannulas were found to be misplaced were removed from all the subsequent analyses.

Two days before blood sampling, the animals were anesthetized with isoflurane, and an indwelling silastic cannula (outer diameter = 0.047 inches and inner diameter = 0.025 inches; Dow Corning, Midland, MI, USA) was inserted into the right atrium via the right jugular vein. The end of the cannula was exteriorized through the skin between the scapulae. For the nonlactating rats, this surgery took place on estrus after at least two complete estrous cycles following the surgery for the placement of lateral ventricle cannula. For the lactating rats, this surgery took place on day 13 pp for the dams that were tested on day 15 pp or day 18 pp for the dams that were tested on day 20 pp. On the day of blood sampling, between 0800 and 1000 h, the first blood sample (400 μ l) was collected from rats directly before the rats were administered a 2 μ l injection of 1 nmol kisspeptin-10 (kisspeptin (110–119) Amide, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) in saline or vehicle (saline) into the left lateral ventricle using a Hamilton syringe connected to a stainless-steel injection

cannula. Injections were carried out in conscious animals that had previously been habituated to this procedure for 5 days prior to the injection. The solution was injected over a 30-s period, and the injection cannula was then left in position for an additional 30 s before being removed. The guide cannula was then sealed to prevent possible leakage. Subsequent blood samples were collected 15, 60, and 180 min after lateral ventricle injection. After the collection of each sample, blood was centrifuged and plasma samples were stored at -20°C until further processing. Plasma LH concentrations were measured in all samples by RIA (conducted by the University of Virginia Core Ligand and Assay Laboratory). Leptin concentrations were measured in the first blood sample using an ELISA kit from Linco Research, Inc. (St Charles, MO, USA). Progesterone concentrations were measured in the second blood sample (collected 15 min after i.c.v. injection) using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). For each hormone measurement, all the samples were measured in one assay, and the intra-assay coefficients of variation were 5.6% for LH, 7.8% for leptin, and 10.3% for progesterone.

Statistical analysis

Data for relative gene expression, immunohistochemistry cell counts, and hormone concentrations were analyzed using one-way ANOVA. Data for LH concentrations were analyzed using a three-way ANOVA with two between-subjects variables: group (virgin, *ad libitum* lactating and food restricted lactating) and treatment (vehicle vs kisspeptin) and one within-subjects variable: time (-2 , 15, 60, and 180 min after kisspeptin injection). Each time point during lactation was analyzed separately, and the diestrous group was also included in the analysis to assess for any possible effect of lactation. The area under the curve for the kisspeptin-induced LH response over the 180-min time period was calculated using the trapezium rule formula, taking into account the nonuniform time points on the x-axis. For this parameter, data from day 15 to day 20 of lactation were analyzed separately using a two-way group (*ad libitum* vs food restriction) $\times$ treatment (vehicle vs kisspeptin) between-subjects ANOVA, while in the diestrous groups, for this parameter, the effects of vehicle and kisspeptin were compared using a one-way ANOVA. Where appropriate, overall ANOVAs were followed by analyses of simple main effects and/or pairwise *post hoc* tests.

Results

Circulating concentrations of LH in the food-restricted and AL-fed dams prior to i.c.v. injection of kisspeptin or vehicle are shown in Fig. 1A. With the caveat that LH secretion is pulsatile, single measurements of LH concentrations indicated that on day 15 pp lactating dams had significantly lower basal LH concentrations than the diestrous rats. By day 20 pp, concentrations were increased, and the concentrations in the AL-fed dams were similar to those in the diestrous group. These rising concentrations of LH are associated with the resumption of estrous cycles, as all the day 20 pp AL-fed dams

Figure 1 (A) LH concentrations in nonlactating (D, virgin control rats) and lactating rats (AL, FR and pp). Groups with different letters are significantly different ($P < 0.05$). Group sizes are as follows: D $n = 14$, day 15 pp AL $n = 11$, day 15 pp FR $n = 10$, day 20 pp AL $n = 10$, and day 20 pp FR $n = 10$. (B and C) *Kiss1* mRNA levels in the AVPV and arcuate nucleus in nonlactating and lactating rats. D, diestrous rats; OVX, ovariectomized rats; AL, *ad libitum*-fed dams; FR, food-restricted dams; pp, *postpartum*. Bars represent mean ($\pm$ S.E.M.). Day 20 food-restricted dams were food restricted from days 1 to 14 pp, after which food access was *ad libitum*. All values are expressed relative to the levels of mRNA measured in the diestrous group. *Significant with respect to the diestrous group ($P < 0.05$). For the AVPV, group sizes are as follows: D $n = 8$, OVX $n = 5$, day 15 pp AL $n = 9$, day 15 pp FR $n = 8$, day 20 pp AL $n = 7$, and day 20 pp FR $n = 11$. For the Arc, group sizes are as follows: D $n = 7$, OVX $n = 5$, day 15 pp AL $n = 8$, day 15 pp FR $n = 8$, day 20 pp AL $n = 8$, and day 20 pp FR $n = 9$.

Figure 2 (A) Kisspeptin-positive cell counts in the arcuate nucleus on day 20 pp in the *ad libitum*-fed (AL, $n=5$) and food-restricted (FR, $n=5$) dams and nonlactating rats on the day of diestrus (D, $n=7$). Bars represent means ($\pm$ S.E.M.). (B) Representative images of immunohistochemical staining for kisspeptin in the arcuate nucleus on diestrus and day 20 of lactation (*ad libitum* fed). Day 20 FR dams were food restricted for the first 15 days of lactation, after which food access was *ad libitum*. *Significant with respect to the diestrous group ($P<0.05$). Scale bar: 200 μ m.

displayed a first estrus. By day 20 pp, none of the food-restricted dams displayed a first estrus. LH concentrations in the food-restricted dams were significantly reduced compared with those in the AL-fed controls, but still exhibited a significant increase between day 15 and day 20 pp. Nevertheless, basal LH concentrations were lower in the food-restricted dams than in the AL-fed dams and diestrous rats on both day 15 pp and day 20 pp (Fig. 1A).

The OVX rats had significantly lower expression of *Kiss1* mRNA in the AVPV than the diestrous rats (Fig. 1B), as previously reported. On both day 15 and day 20 pp, the AL-fed dams had *Kiss1* mRNA levels similar to those in the diestrous rats. By contrast, on day 15 pp, *Kiss1* mRNA levels in the food-restricted dams were similar to those in the OVX rats and significantly lower than those in both diestrous rats and AL-fed dams. By day 20 pp, following 5 days of refeeding, *Kiss1* mRNA levels in the food-restricted dams increased and were similar to those in the AL-fed dams and diestrous rats.

Ovariectomy significantly increased *Kiss1* mRNA expression in the arcuate nucleus as described previously (Smith *et al.* 2005). Lactating rats exhibited significantly lower *Kiss1* mRNA expression than both the OVX rats and diestrous rats (Fig. 1C) in this area, and there was no significant difference in *Kiss1* mRNA expression between any of the lactating groups. Results of the immunohistochemical analysis were similar to those of qPCR: while kisspeptin-immunoreactive neurons could be readily detected in the arcuate nucleus in diestrous rats, lactating rats on day 20 pp, whether food-restricted or AL-fed, had significantly fewer kisspeptin-immunoreactive cells than the diestrous rats (Fig. 2).

A robust increase in circulating LH concentrations was induced in the diestrous rats by i.c.v. kisspeptin-10 administration (Fig. 3A and D), and on both day 15 and day 20, the AL-fed dams exhibited a LH response similar to that observed in the diestrous group (Fig. 3B and C).

The food-restricted dams exhibited a suppressed response to kisspeptin-10 administration on day 15 pp compared with the AL-fed dams and diestrous rats. However, on day 20 pp, after 5 days of *ad libitum* food intake, the LH response to kisspeptin in the food-restricted dams was similar to that of the day 20 pp AL-fed dams and the diestrous group (Fig. 3B and C). Analysis of the area under the curve of the LH response to the vehicle and kisspeptin supported these results (Fig. 3D, E, and F), indicating a significantly reduced effect of kisspeptin in the food-restricted dams than in the AL-fed dams only on day 15 pp.

Plasma leptin concentrations were significantly lower during all stages of lactation compared with those in the nonlactating rats (Fig. 4A). On day 15, the food-restricted dams had significantly lower leptin concentrations than the AL-fed dams. On day 20 pp, after 5 days of refeeding, leptin concentrations were similar in the food-restricted and AL-fed dams. Progesterone concentrations were significantly increased in the food-restricted dams than in the AL-fed dams and nonlactating, diestrous rats on both day 15 and day 20 pp (Fig. 4B).

The AL-fed and food-restricted dams and their litters exhibited different patterns of weight gain across lactation. Overall, from day 1 of lactation to day 20 of lactation, the AL-fed dams gained significantly more weight than the food-restricted dams (53.0 ± 8.1 g vs 18.2 ± 10.2 g). During the period of food restriction, the food-restricted dams lost, on average, 64.0 ± 6.7 g of weight, whereas during the same period, the AL-fed dams gained, on average, 43.8 ± 12.3 g of weight. During the 5 days of refeeding, the food-restricted dams gained, on average, 82.2 ± 5.6 g of weight, which was significantly greater than the weight gain in the AL-fed dams over the same time period (9.2 ± 7.7 g). From day 1 to day 20 of lactation, litters from the AL-fed dams gained significantly more weight than those from

Figure 3 LH response to i.c.v. kisspeptin-10 administration in diestrus rats (A and D) and day 15 pp (B and E) and day 20 pp (C and F) *ad libitum*-fed (AL) and food-restricted (FR) dams. (A, B, and C) Plasma LH concentrations following i.c.v. kisspeptin-10 (1 nmol) administration. (D, E, and F): Integrated LH secretion after i.c.v. kisspeptin-10 administration during the 180-min study period. Day 20 FR dams were food restricted for the first 15 days of lactation, after which food access was *ad libitum*. *Significant with respect to the vehicle-treated group of the same physiological state ($P < 0.05$). †Significant with respect to the AL-fed group of the same day *postpartum* ($P < 0.05$).

the food-restricted dams (384.3 ± 16 g vs 271.1 ± 14.4 g). During the period of food restriction, litters from the food-restricted dams gained significantly less weight than those from the AL-fed dams (144.4 ± 9.8 g vs 253.3 ± 7.6 g). However, during the period of refeeding, litters from the AL-fed dams and those from the food-restricted dams gained similar amounts of weight (131.1 ± 9.1 g vs 126.7 ± 4.9 g). Dams and litters from the kisspeptin-induced LH secretion experiment were not included in these comparisons because these dams underwent extra manipulations during lactation. However, changes in body weight and litter weight that were similar to those in the other groups were observed in these dams.

Discussion

During lactation, there is a reduction in basal LH secretion and in the ability of estrogen to induce a LH surge. As weaning approaches, basal LH concentrations begin to increase (Walker *et al.* 1995) and the ability of estrogen to invoke a LH surge gradually returns

(Abizaid *et al.* 2003). In the present study, we used a model in which food restriction for the first 2 weeks of lactation delays the resumption of LH secretion and the estrous cycle, allowing a comparison of changes in *Kiss1* mRNA at the same stage during lactation, but either before or after the resumption of cycles. As has been reported previously, food-restricted dams in the present study had increased progesterone concentrations and decreased leptin concentrations and had not resumed estrous cyclicity by day 20 pp, demonstrating the consistency of this model (Woodside 1991, Walker *et al.* 1995, Abizaid *et al.* 2004). The results of the present study demonstrate that prolonged lactational diestrus induced by food restriction for the first 2 weeks of lactation is associated with a reduction in AVPV *Kiss1* mRNA expression and a decrease in kisspeptin-induced secretion of LH on day 15 pp compared with that in AL-fed lactating dams. By day 20 of lactation, after dams have had access to *ad libitum* food for 5 days, these changes in the kisspeptin-GRP54 network were restored to levels similar to those observed in dams that have been fed *ad libitum* for the duration of lactation, but the

Figure 4 Plasma leptin (A) and progesterone (B) concentrations in nonlactating and lactating dams. D, diestrus rats; AL, *ad libitum*-fed dams; FR, food-restricted dams; pp, *postpartum*. Bars represent means ($\pm$ S.E.M.). Day 20 FR dams were food restricted for the first 15 days of lactation, after which food access was *ad libitum*. Groups with different letters are significantly different ($P < 0.05$). Group sizes are as follows: D $n = 8$, day 15 pp AL $n = 7$, day 15 pp FR $n = 7$, day 20 pp AL $n = 6$, and day 20 pp FR $n = 6$.

food-restricted dams had not yet displayed a resumption of estrous cyclicity. Furthermore, during the last week of lactation, *Kiss1* mRNA in the arcuate nucleus was low in the AL-fed lactating rats and food-restricted dams, despite increases in overall LH concentrations.

Increases in AVPV kisspeptin expression across pubertal development in rodents (Han *et al.* 2005, Clarkson & Herbison 2006, Takase *et al.* 2009, Clarkson *et al.* 2010) and increases in POA kisspeptin expression across the photoperiodic transition from long days to short days in ewes (Chalivoix *et al.* 2010) have been reported to be associated with the activation or reactivation respectively of the HPG axis. By contrast, in the AL-fed lactating rats, *Kiss1* mRNA levels in the AVPV remained stable, between day 15 pp and day 20 pp, and were similar to those in the nonlactating controls, even though basal LH concentrations increased over this period. Although not measured in the present study, decreased AVPV *Kiss1* mRNA expression has been observed earlier in lactation (day 10/11 pp) in intact dams using *in situ* hybridization (True *et al.* 2011). This suggests that during early lactation a reduction in AVPV *Kiss1* mRNA expression may be involved in the mechanisms suppressing the HPG axis, and as weaning approaches, and the HPG axis is slowly reactivated, AVPV *Kiss1* mRNA expression increases back to control levels. This possibility is supported by the current data, which demonstrated that delaying the resumption of cycles by food restriction in early lactation results in lower *Kiss1* mRNA levels, perhaps maintaining the suppression into the latter part of lactation. Thus, it is possible that restoration of AVPV *Kiss1* mRNA expression is a necessary step in the early cascade of events that ultimately lead to the full restoration of reproductive function as lactation wanes. That this event occurs later when rats are food restricted for the first 2 weeks *postpartum* is consistent with the fact that other indexes of reproductive function, including sensitivity to estrogen positive feedback and restoration of cyclic hormone release, are also delayed by this manipulation (Woodside 1991, Walker *et al.* 1995, Abizaid *et al.* 2003).

Arcuate nucleus *Kiss1* mRNA levels were profoundly suppressed on days 15 and 20 pp in both the AL-fed and food-restricted dams compared with those in the nonlactating controls. On day 20 of lactation, basal LH concentrations in the AL-fed dams were similar to those in the nonlactating controls, although the lactating group had both lower levels of arcuate nucleus *Kiss1* mRNA and a decreased number of kisspeptin-immunoreactive cells at this time. Although LH concentrations were measured only in a single blood sample in this study, the results were similar to those of our previous study in which LH concentrations were determined in a number of serial blood samples (Walker *et al.* 1995). These data indicate that LH concentrations increase, despite constant low arcuate nucleus *Kiss1* mRNA expression during late lactation. However, pulsatile LH release was attenuated

during lactation in both the AL-fed and food-restricted dams, and this suppression of pulsatility was still observed on days 20–22 pp despite an increase in basal LH concentrations (Walker *et al.* 1995). It has been demonstrated that the administration of a kisspeptin antagonist directly into the arcuate nucleus results in a decrease in LH pulse frequency without affecting pulse amplitude in rats, suggesting a role for arcuate nucleus kisspeptin in the regulation of LH pulse frequency (Li *et al.* 2009). Therefore, it is tempting to speculate that low arcuate nucleus *Kiss1* mRNA may contribute to the reduction in the pulsatile release of LH during lactation. Indeed, recent work showing a correlation between suppression of both arcuate nucleus *Kiss1* mRNA and LH pulsatility in estrogen-treated day 16 pp OVX dams (Yamada *et al.* 2012) also supports the hypothesis that suppression of kisspeptin expression in the arcuate nucleus may contribute to the attenuated pulsatility of LH at this time. The rising concentrations of LH at the end of lactation may, however, be independent of this system.

In addition to hypothalamic *Kiss1* gene expression, the ability of exogenous kisspeptin administration to stimulate LH release in late lactation was also investigated. In the present study, LH secretion in response to exogenous kisspeptin administration in AL-fed dams tested on either day 15 or day 20 pp was similar to that observed in rats in the diestrous phase of the estrous cycle. By contrast, the day 15 pp food-restricted dams had an attenuated LH response to exogenous kisspeptin administration, suggesting that prolonged lactational diestrus during lactation is associated with decreased sensitivity to kisspeptin in terms of LH release. Previous studies using this food restriction model have demonstrated that the LH response to a GnRH challenge is similar in the AL-fed and food-restricted dams on day 15 of lactation (Walker *et al.* 1995), indicating that the decrease in LH release after kisspeptin administration is unlikely to be due to an insensitivity of the pituitary to GnRH. Therefore, it could be hypothesized that the response of GnRH neurons to kisspeptin may be attenuated in the food-restricted lactating dams at this late stage of lactation and thus may contribute to the prolongation of lactational diestrus in this model.

Previous studies in lactating rats have shown that on day 5 pp the sensitivity to kisspeptin-induced LH release is decreased in intact Wistar rats (Roa *et al.* 2006), although others have shown in OVX day 7 and intact day 16 Wistar-Imamichi dams that kisspeptin-induced LH release is not altered during lactation (Yamada *et al.* 2007, 2012). Whether these inconsistencies are due to strain differences, stage of lactation, or steroid hormone status is yet to be determined. Nevertheless, together with those of the study carried out by Yamada *et al.* (2012), the results of the present study indicate that kisspeptin sensitivity is unaltered during late lactation in AL-fed rats. Given the conflicting results in early lactation (Roa *et al.* 2006, Yamada *et al.* 2007), however,

it remains to be established whether decreased sensitivity to kisspeptin contributes to lactational anovulation in early lactation. Thus, whether this decreased sensitivity in the food-restricted dams on day 15 pp is a prolonged effect of a suppressive mechanism that is normally present during the early part of lactation is, at this point, unknown. Furthermore, the mechanism underlying this decreased sensitivity in the day 15 pp food-restricted dams remains elusive. Although leptin concentrations were further reduced on day 15 pp in the food-restricted dams compared with those in the other lactation groups, there is little support for the notion that low leptin concentrations play a major role in the reduced sensitivity to exogenous kisspeptin at this time. The results of other studies examining the effect of food restriction or fasting, and the associated decrease in circulating leptin concentrations, have provided no evidence of a reduction in the ability of kisspeptin to induce LH secretion. For example, in prepubertal fasted rats (Castellano *et al.* 2005) and food-restricted male rats (Castellano *et al.* 2010), i.c.v. kisspeptin administration has been shown to result in an increased release of LH compared with that in the AL-fed controls. Furthermore, food restriction in nonlactating female rats has been shown to result in a prolonged LH response to chronic kisspeptin compared with a similar treatment in AL-fed rats that become desensitized to chronic kisspeptin after ~24 h (Roa *et al.* 2008). It should be noted, however, that in these studies food availability was restricted for a shorter period of time than in the food-restricted lactating model and hence the duration of reduced leptin concentrations was also shorter.

Consistent with previous reports, the results of the present study indicate that progesterone concentrations are significantly elevated in the food restriction model (Walker *et al.* 1995). The full effect of these high progesterone concentrations is yet to be definitively established. Although high progesterone concentrations do not appear to play a defining role in the suppression of basal LH secretion in food-restricted dams (Walker *et al.* 1995), previous work has implicated high concentrations of progesterone to be contributing to the lack of sensitivity to the positive feedback effect of estrogen that accompanies the prolonged period of lactational diestrus observed in the food restriction model (Abizaid *et al.* 2003). It seems unlikely, however, that high progesterone concentrations play a major role in the decreased LH sensitivity to exogenous kisspeptin on day 15 pp in food-restricted dams. First, on day 20 pp, kisspeptin-induced LH release was similar in the food-restricted dams and AL-fed dams, while progesterone concentrations remained significantly higher in the previously food-restricted group. Furthermore, it has been shown that the LH response to kisspeptin is robust in OVX rats treated with progesterone (Roa *et al.* 2006).

The mechanism underlying the lactation-induced suppression of fertility is not well established.

The present study demonstrates that *Kiss1* mRNA expression in the arcuate nucleus is suppressed during late lactation, even when LH concentrations begin to increase significantly, indicating a dissociation between LH concentrations and arcuate nucleus *Kiss1* mRNA during late lactation. It is possible that this suppression of arcuate nucleus kisspeptin expression contributes to the reduction in pulsatile LH release that is still present in these late stages of lactation (Walker *et al.* 1995). Food restriction during lactation produces a useful model in which the length of lactational diestrus can reliably be extended. The results of the present study indicate that this prolongation of lactational diestrus is associated with reduced *Kiss1* mRNA expression in the AVPV, similar to that observed at an earlier time period *postpartum* in AL-fed dams (True *et al.* 2011) and an attenuated LH response to exogenous kisspeptin on day 15 of lactation. The reduced AVPV *Kiss1* mRNA expression observed in the day 15 food-restricted dams may represent a key factor involved in the delayed restoration of HPG axis function during the *postpartum* period resulting from the food restriction manipulation.

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.

Funding

This study was supported by the Canadian Institutes of Health Research and Fonds de la recherche en santé Québec.

Acknowledgements

The authors thank Tammie Quinn and Lin Dufresne for their assistance in animal care and procedures and Dr Alain Caraty for gifting the kisspeptin antibody.

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Received 5 September 2013

First decision 27 September 2013

Revised manuscript received 15 January 2014

Accepted 3 February 2014