Induction of Central Leptin Resistance in Hyperphagic Pseudopregnant Rats by Chronic Prolactin Infusion

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Pregnant rats are hyperphagic, leading to increased fat deposition and a rise in plasma concentrations of the adipose-derived hormone leptin (1). Elevated leptin would normally act to inhibit food intake, but hypothalamic leptin resistance develops around midpregnancy, allowing hyperphagia to be maintained and excess energy to be stored as fat in preparation for future metabolic demands of lactation. To investigate the hormonal mechanisms inducing leptin resistance during pregnancy, the anorectic response to leptin was examined during pseudopregnancy. Pseudopregnant rats have identical hormonal profiles to early pregnancy, but no placenta formation, allowing differentiation of maternal and placental hormone effects on appetite. To investigate the effect of leptin on food intake, d-9 pseudopregnant rats were injected with leptin (4 μg) via an intracerebroventricular (icv) cannula, and then food intake was measured 24 h later. Pseudopregnant rats were hyperphagic but had normal anorectic responses to leptin. We therefore hypothesized that a longer exposure time to high concentrations of progesterone might be required to mimic the leptin resistance that occurs on d 14 of pregnancy. Pseudopregnant rats were given progesterone to prolong pseudopregnancy beyond the time that leptin resistance develops during pregnancy. However, rats remained responsive to icv leptin. To model the placental lactogen secretion that occurs during pregnancy, pseudopregnant rats were given progesterone and chronic icv ovine prolactin infusion. Central icv injection of leptin had no effect on food intake in pseudopregnant rats receiving chronic ovine prolactin. These results suggest that chronically high lactogen levels, secreted by the placenta during the second half of pregnancy, induce central leptin resistance. (Endocrinology 149: 1049–1055, 2008)
these times. Prolactin infusion induces hyperphagia in virgin female rats in a dose-dependent manner (22, 23), and this also occurs in the absence of ovarian hormones (23, 24). This is a central effect, involving direct actions of prolactin in the hypothalamus (25), but a role of prolactin in mediating changes in leptin responsiveness during pregnancy has not been previously studied.

To investigate the hormonal mechanisms that induce leptin resistance during pregnancy, we have used the pseudopregnant rat as a pregnancy-like model in which individual hormones could be manipulated without the risk of terminating the pregnancy. Pseudopregnancy in the rat is characterized by hormone changes that are essentially identical to those observed during early pregnancy (26–30). Like pregnancy, pseudopregnancy is associated with high progesterone, hyperprolactinemia (28), and hyperphagia (14), but there is no metabolic load from fetal development, and hormonal contribution from the placenta is absent. Therefore, pseudopregnancy provides an ideal model to investigate the effect of maternal hormones on appetite regulation, without the confounding influence of pregnancy. The aim of our study, therefore, was to investigate whether maternal hormone secretion is able to induce central leptin resistance as seen in pregnant rats. By introducing pregnancy-like patterns of hormones into pseudopregnant females, the role of individual hormones could be established.

Materials and Methods

Animal preparation

Ten-week-old Sprague Dawley rats weighing between 200 and 300 g were purchased from the Animal Facility, University of Otago, Dunedin. All procedures were approved by the University of Otago Animal Ethics Committee. Rats were given free access to standard rat chow and water in controlled temperature (22 ± 1 C) and lighting conditions (14-h light, 10-h dark cycle). A group of male rats were vasectomized under sterile conditions using halothane anesthesia. The female estrous cycle was monitored by daily vaginal smears, and after two consecutive estrous cycles, rats were used for the various manipulations. To induce pseudopregnancy, proestrous rats were individually placed overnight in a wire-bottom cage with a vasectomized male. The presence of plugs on the cervix was confirmed by daily vaginal smears, and after two consecutive cycles, rats were used for the various manipulations. To induce pseudopregnancy, on the day of pseudopregnancy, and immediately after pseudopregnancy in additional groups of rats, and blood samples were collected for assessment of hormone levels by RIA, as described above.

Characterizing food intake and hormonal changes during pseudopregnancy

Female rats were individually housed to allow daily monitoring of food and water intake and body weight throughout two estrous cycles, pseudopregnancy, and the subsequent two estrous cycles. Food and water intake were measured after the subsequent 24 h. To confirm effectiveness of hormone treatments, jugular cannulas were inserted on d 9 of pseudopregnancy in additional groups of rats, and blood samples were collected for assessment of hormone levels by RIA, as described above.

Phasic vs. chronic prolactin secretion. To mimic the chronic pattern of placental lactogen secretion seen during the second half of pregnancy, as opposed to the phasic prolactin secretion of early pregnancy, another group of d-9 pseudopregnant rats had progesterone implants placed sc, together with an Alzet osmotic minipump (model 2001) administering ovine prolactin (oPRL) in aCSF into the lateral ventricle (2.5 μg/ml for 7 d). A 10-cm piece of SILASTIC brand tubing was attached to the flow modulator of the osmotic minipump and connected to a single connector/guide cannula (26 gauge, cut 4 mm below pedestal, side connector 22 gauge, 5 mm long; Plastics One) that had two side-by-side tubes, one for chronic infusion of oPRL from the minipump and a second to allow for an acute icv injection. The tubing and infusion cannulas were filled with oPRL or aCSF and primed along with the minipump, before insertion into the rat. After a 4-d recovery, rats were fasted for 24 h on d 13 of extended pseudopregnancy, and then leptin (4 μg in 2 μl aCSF) or

Feeding response to intracerebroventricular (icv) leptin administration

To measure feeding responses to icv leptin during pseudopregnancy, rats were anesthetized on either d 0 or 1 of pseudopregnancy with an ip injection of ketamine hydrochloride (80 mg/kg) and 2% xylazine hydrochloride (4 mg/kg) and placed into a stereotaxic frame. Cannulas (22 gauge, cut 4 mm below the pedestal; Plastics One Inc., Roanoke, VA) were inserted 1.3 mm lateral to bregma and 3 mm below the level of the skull to sit approximately 1 mm superior to the left lateral ventricle. Dummy cannulas (32 gauge; Plastics One) were inserted into the guide cannulas to prevent blockages. Rats were allowed to fully recover from the surgery for approximately 1 wk before further experimentation. To confirm correct placement of cannulas, all rats were injected icv with 10 ng angiotensin II (Sigma-Aldrich, St. Louis, MO), and water intake measured 30 min later. Rats that drank less than 5 ml water were removed from the study.

On metestrus or d 6 of pseudopregnancy, rats were fasted for 24 h to reduce proestrus leptin concentrations (14), surgically placed in just before lights off. The following evening, 1 h before lights off, artificial CSF (aCSF) or recombinant mouse leptin (4 μg in 2 μl aCSF; National Hormone and Peptide Program, Torrance, CA) was injected via the icv cannulas using a 2-μl Hamilton syringe (Hamilton Co., Reno, NV) attached via SILASTIC brand tubing (Dow Corning Corp., Midland, MI) to a stainless steel injection needle (28 gauge; Plastics One) that extended 2 mm below the base of the guide cannula. Injections were carried out over at least 30 sec and needles held in place to ensure dispersion of the hormone into the ventricle. Dummy cannulas were replaced into the cannulas and rats returned to their wire-bottom cages. At lights off, a preweighed amount of food (rat pellets) was returned to the food carrier, with a tray underneath the wire bottom to capture any spilled food. Twenty-four hours after food was returned, remaining food in the carrier and in the spill tray was weighed and food intake was calculated.
vehicle (aCSF alone) was injected via the icv cannulas (as described above), and food intake was measured after the subsequent 24 h. To confirm effectiveness of hormone treatments, jugular cannulas were inserted on d 9 of pseudopregnancy in additional groups, and blood samples were collected for assessment of hormone levels by RIA, as described above.

**Statistical analysis**

Data were analyzed by one- and two-way ANOVA with Fisher’s and Bonferroni post hoc tests where appropriate. The significance level was set at $P < 0.05$. All data are presented as the mean ± SEM.

**Results**

**Characterizing food intake and hormonal changes during pseudopregnancy**

As described previously (14, 32), daily food intake measurements over the rat estrous cycle (Fig. 1A) showed a characteristic cyclical pattern with a decrease in food intake at estrus and an increase during metestrus and diestrus. This was reflected in a cyclical pattern in body weight changes (Fig. 1B), with a decrease in body weight at the time of estrus. When the luteal phase of the estrous cycle was extended by pseudopregnancy, food intake increased, resulting in a marked and immediate increase in body weight. Once pseudopregnancy ceased after 11–12 d and normal estrous cycles resumed, food intake returned to pre-pseudopregnancy levels and rate of body weight gain slowed. Body weight remained higher in the pseudopregnant rats after the resumption of cycles compared with continually cycling rats. Plasma prolactin concentrations (Fig. 2A) during the estrous cycle showed the characteristic proestrous surge of prolactin followed by low levels across the rest of the cycle. During pseudopregnancy, there were twice-daily prolactin surges that persisted for 10–11 d, and then the rats started cycling again and exhibited only the proestrus surge of prolactin. Serum progesterone concentrations (Fig. 2B) increased significantly during pseudopregnancy, compared with diestrous animals, due to the enhanced production of progesterone by the corpus luteum. A decline in corpus luteum progesterone secretion occurred after 11–12 d, and the rats started to show normal estrous cycles again.

![Figure 1](https://example.com/image1.png)

**Fig. 1.** A, Daily food intake before, during, and after pseudopregnancy ($n = 9$); D, Diestrus; E, estrus. B, Cumulative body weight gain in non-pseudopregnant ($n = 8$) and pseudopregnant ($n = 9$) rats. Pseudopregnancy lasted for 11–12 d (shaded region). *, Significant difference with respect to food intake in the same group of animals, on the first diestrus before being mated ($P < 0.001$). **, Significant difference with respect to cycling rats at the equivalent time ($P < 0.001$). Food intake was also significantly increased on d 4 of pseudopregnancy ($P < 0.01$) compared with food intake at equivalent time points in rats with continual estrous cycles (data not shown).

![Figure 2](https://example.com/image2.png)

**Fig. 2.** A, Plasma prolactin concentrations (nanograms per milliliter) were measured from serial blood samples taken four times daily from four groups of animals: non-pseudopregnant rats (P, proestrus; E, estrus; M, metestrus; D, diestrus; $n = 5$), pseudopregnant rats (d 1–4, $n = 8$; d 7–9, $n = 10$), and rats that had been pseudopregnant and had resumed normal estrous cycles ($n = 3–8$). B, Serum progesterone concentrations (nanograms per milliliter) were measured from terminal blood samples collected from diestrous rats (D, $n = 6$); d 3 ($n = 6$), 6 ($n = 5$), and 9 ($n = 5$) pseudopregnant rats; and diestrous rats ($n = 5$) that had been pseudopregnant and resumed cycling (D2). *, Significant difference with respect to non-pseudopregnant values ($P < 0.05$).
Effect of leptin on food intake during pseudopregnancy

Pseudopregnant rats were hyperphagic, eating significantly more in 24 h than non-pseudopregnant rats (Fig. 3). In both cycling and d-9 pseudopregnant rats, vehicle-injected rats ate significantly more 24 h after the fast than they did under normal conditions, suggestive of a compensatory post-fasting hyperphagic mechanism. Leptin significantly suppressed food intake in both cycling and pseudopregnant rats when compared with vehicle-injected rats in the same physiological state, by essentially preventing the postfasting hyperphagia.

Hormonal manipulations to mimic pregnancy

Prolonging duration of exposure to progesterone and prolactin. To address the hypothesis that a longer duration of exposure to high levels of progesterone and prolactin may induce leptin resistance, pseudopregnancy was extended beyond the time when leptin resistance normally develops during pregnancy. As described previously (30), progesterone implants extended pseudopregnancy beyond d 11, with rats continuing to exhibit diestrous smears until approximately d 16, whereas blank-implanted rats resumed normal estrous cycles on approximately d 11. Pseudopregnant rats treated with progesterone implants continued to have prolactin surges (Fig. 4A), with the nocturnal surge more predominant than the diurnal surge, until at least d 15 of the extended pseudopregnancy. Pseudopregnant rats containing blank implants showed a cessation of prolactin surges by d 11, as seen previously. Progesterone concentrations in extended pseudopregnant rats were identical to those in pregnant rats (Fig. 4B), and both groups had significantly higher progesterone than pseudopregnant and diestrous rats. Leptin significantly suppressed food intake in rats receiving blank implants, which had resumed cycling, and also in the pseudopregnant rats receiving progesterone implants (Fig. 5).

Phasic vs. chronic prolactin secretion. Figure 6 shows plasma rat prolactin concentrations in extended pseudopregnant rats receiving either vehicle or oPRL infusions. As seen previously, vehicle-infused rats continued to exhibit the twice-daily surges of prolactin, whereas endogenous prolactin secretion was inhibited by oPRL infusion. These results confirm that oPRL was functionally effective in the brain, inhibiting prolactin secretion via negative feedback. In extended pseudopregnant rats, prolactin treatment significantly reduced the response to leptin compared with control rats (Fig. 7). These animals displayed total leptin insensitivity, similar to that previously seen in pregnant animals, characterized by an absence of postfasting hyperphagia and an inability to suppress food intake in response to leptin.
Pregnancy and lactation are both states of physiological hyperphagia, an adaptive response that supports the growing conceptus and provides adequate energy for lactation. In rats, food intake increases by up to 50% during pregnancy, decreases the day before parturition, and then increases by up to 300% during lactation compared with nonpregnant rats (33, 34). Increased food intake during pregnancy precedes the metabolic demand, resulting in an increase in maternal body fat (34), which is later depleted due to suckling-induced mobilization of fat stores (35). Plasma leptin concentrations increase as pregnancy advances, reaching peak levels on d 19 followed by a rapid decline before parturition (1, 7, 8). Despite elevated plasma leptin concentrations during pregnancy, increased food consumption is maintained due to the development of leptin resistance in the hypothalamus, with a region-specific loss in leptin receptors and leptin-induced phosphorylation of signal transducer and activator of transcription 3 proteins (1, 4, 36). Like pregnant rats, pseudopregnant rats were hyperphagic, confirming this as a good model for the investigation of hormonal actions on appetite and body weight. Thus, the aim of this study was to use the pseudopregnant model to evaluate the role of pregnancy hormones in modulating the hypothalamic response to leptin. Using a series of hormone manipulations, we have established that a pregnancy-like state of hypothalamic leptin resistance could be induced in the absence of pregnancy, by prolonged elevations in progesterone and prolactin.

The hormonal changes characterized in the present study are consistent with an extensive body of previous work (26–28, 37). Food intake and body weight measurements in nonpregnant animals showed characteristic cyclical changes, predominantly due to the anorectic effect of estradiol (10, 12, 32). This has also been described previously (32, 38, 39) and is particularly prominent in Long Evans rats (10). Although differences on estrus were not always statistically significant in the Sprague Dawley rats used in the present study, the general trend was apparent. Pseudopregnant rats were hyperphagic and lost the cyclical pattern of weight change. To investigate whether this hyperphagia was due to a loss in...
responsiveness to leptin, as occurs during midpregnancy, the anorectic response to exogenous leptin (icv) was measured. Leptin significantly suppressed food intake in both non-pseudopregnant and pseudopregnant rats compared with the control rats in the same physiological state, suggesting that pseudopregnant rats do not become leptin resistant.

Responses to leptin (above) were evaluated on d 9 of pseudopregnancy, whereas leptin resistance in pregnancy develops between d 7 and 14. To investigate whether the development of leptin resistance was simply due to the duration of hormone exposure, pseudopregnancy was extended using progesterone implants. These animals continued to show the early pregnancy-like pattern of prolactin secretion. They remained hyperphagic but did not become leptin resistant. The timing of the onset of leptin resistance in pregnancy, between d 7 and 14 (1), was coincident with the onset of placental lactogen secretion in the rat (40). Thus, we hypothesized that the pattern of exposure to lactogenic hormones may be a critical factor influencing appetite regulatory centers in the brain. Early pregnant, pseudopregnant, and extended pseudopregnant rats were characterized by pulsatile prolactin secretion and hyperphagia but continue to have a relatively normal response to leptin. In contrast, during the second half of pregnancy, when prolactin is chronically elevated, animals show a loss of response to leptin. Hence, to investigate whether the pattern of prolactin secretion causes changes in feeding and hypothalamic responses to leptin, extended pseudopregnant rats were given continuous exposure to high concentrations of prolactin to mimic the pattern of prolactin during the second half of pregnancy. Under these conditions, a total loss in leptin action occurred. As seen during pregnancy, the loss in leptin sensitivity was characterized by an absence of postfasting hyperphagia and an inability to suppress food intake in response to leptin. This suggests that postfasting hyperphagia is mediated by the fasting-induced decrease in leptin, and pregnant and prolactin-treated animals are not sensitive to this decrease.

Central leptin resistance does not develop until midpregnancy in the rat, whereas hyperphagia develops almost immediately. Thus, pregnancy-induced hyperphagia is not primarily caused by leptin resistance. The orexigenic drive is likely to be caused by progesterone, although prolactin surges may also contribute. Systemic prolactin increases body weight and food intake in virgin female rats in a dose-dependent manner (24), which is not dependent on ovarian hormones (41). The mechanism by which lactogenic hormones induce hyperphagia are not clear, but prolactin receptors are expressed in regions of the hypothalamus containing orexigenic neurons (42, 43). Neuropeptide Y (NPY) and agouti-related peptide (AgRP) mRNA levels in the arcuate nucleus (Arc) are elevated during pregnancy (32, 44, 45) and increase further in lactation (44, 46). Prolactin and placental lactogen stimulate an increase in NPY mRNA in a prolactin-responsive cell line (47). During lactation, however, suppression of prolactin levels by bromocriptine treatment did not reduce Arc NPY activity but did reduce NPY mRNA expression in the dorsomedial hypothalamic nucleus (DMH) (46). The DMH has been implicated in the control of food intake and energy balance (48), and the DMH NPY neurons (but not the Arc NPY neurons) were shown to express prolactin receptors (49). Although not required for the initial hyperphagia of pregnancy, the subsequent placental lactogen-induced leptin resistance would facilitate hyperphagia by allowing it to be maintained in the face of high leptin levels. This probably plays a role in the substantially higher food intake observed later in pregnancy (45, 50).

In conclusion, lactogenic hormones may play a key role in metabolic adaptations of pregnancy. We have shown that by mimicking pregnancy-like hormonal changes in the pseudopregnant rat, central leptin resistance can be induced. The exact neuroendocrine changes that are occurring in response to chronic lactogenic infusion are currently unknown but may involve a loss in functional leptin receptors in key hypothalamic nuclei or a loss in leptin signaling via intracellular signaling cascades. Interestingly, we have shown that pregnant rats are also resistant to a central injection of α-MSH (unpublished observation), a potent anorexigenic peptide that is produced by proopiomelanocortin neurons. Therefore, in pregnant and pseudopregnant rats, leptin resistance may be due to a prolactin-induced loss in signaling in second-order leptin-responsive neurons involved in melanocortin signaling. This warrants further investigation.

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References

2. Woodside B 2007 Prolactin and the hyperphagia of lactation. Physiol Behav 91:375–382
32. Eckel LA 2004 Estradiol: a rhythmic, inhibitory, indirect control of meal size. Physiol Behav 82:35–41
37. Smith MS, Neill JD 1976 Termination at midpregnancy of the two daily surges of plasma prolactin initiated by mating in the rat. Endocrinology 98:696–701
43. Kokay IC, Grattan DR 2005 Expression of mRNA for prolactin receptor (long form) in dopamine and pro-opiomelanocortin neurons in the arcuate nucleus of non-pregnant and lactating rats. J Neuroendocrinol 17:827–835
46. Li C, Chen P, Smith MS 1999 Neuropeptide Y and tuberoinfundibular dopaminergic activities are altered during lactation: role of prolactin. Endocrinology 140:118–123

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