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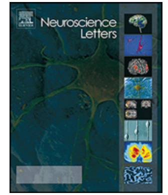

Hypothalamic and pituitary expression of ghrelin receptor message is increased during lactation

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ABSTRACT

In the lactating rat there is a dramatic increase in food intake that peaks at around day 15 postpartum, a time when pups are near weaning age, yet still fully dependant on maternal nourishment. We examined whether the orexigenic hormone ghrelin plays a role in increasing food intake during lactation. To do this, we compared plasma levels ghrelin, as well as brain and pituitary expression of the growth hormone secretagogue receptor (GHS-R 1a) rats in one of three groups: (1) dams whose litters were removed the day after giving birth (non-lactating); (2) dams whose litters were removed on day 13 postpartum (litter removed), and dams allowed keeping their litters (lactating). On day 15 postpartum, all dams were decapitated and trunk blood collected for plasma analysis of active ghrelin levels. Also, brain and pituitaries were collected and snap frozen using liquid nitrogen and stored at −80 °C before mRNA extraction and RT-PCR analysis. Results show no differences in ghrelin concentrations between lactating and non-lactating rats. Hypothalamic and pituitary expression of GHS-R 1a, however, was significantly increased in lactating animals compared to non-lactating animals. Interestingly, litter removed dams had higher levels of plasma ghrelin concentrations than either lactating or non-lactating females. Furthermore, GHS-R mRNA expression in these animals remained elevated in the pituitary but not the hypothalamus. These data suggest that the hypothalamus and pituitary of lactating rats are more sensitive to the effects of ghrelin, and that hypothalamic sensitivity to ghrelin depends on the presence of a suckling litter.
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Ghrelin is a 28 amino acid peptide hormone implicated in the regulation of energy homeostasis [16,17,34]. Ghrelin is produced primarily in the stomach, and in neurons located in hypothalamic regions associated with the regulation of food intake and energy balance [9,21]. Plasma ghrelin and ghrelin mRNA expression in the stomach and hypothalamus increase just prior to the onset of feeding and after fasting [11,15,30,32], and both peripheral and central ghrelin administration increase adiposity and food intake in rodents [31], and effect independent from its ability to secrete growth hormone (GH) [31]. These data suggest that ghrelin, whether secreted from the stomach or within the brain, acts at central sites to regulate energy balance.

Lactation is the most energetically demanding period during the life of a female mammal. During this period, mothers increase their food intake and use their adipose reserves to obtain sufficient nourishment for themselves and their young [12,39]. Lactating rats show a threefold increase in food intake and a dramatic loss of fat pad weight [13,22]. These changes correlate with decreases in leptin levels, and with changes in the expression of hypothalamic peptides regulating food intake and energy balance [3,5,7,19,24,27,37]. Paradoxically, ghrelin levels diminish during the first week of lactation in rats, perhaps as a mechanism that enables the animal to divert energy for milk production [25]. Nevertheless, it remains possible that hypothalamic ghrelin levels are altered in lactation, and or that ghrelin sensitivity increases to produce the changes in food intake and energy expenditure seen during this period. To examine this hypothesis, we compared plasma ghrelin concentrations between lactating rats allowed to nurse their litters with those of lactating rats having their litters removed at different times of lactation. Given that changes in plasma ghrelin concentrations may also affect sensitivity to this hormone, we also evaluated the expression of the message for the active form of the ghrelin receptor, the growth hormone secretagogue receptor 1a (GHS-R 1a), in the brain, pituitary, stomach and mammary glands. Finally, because ghrelin is also synthesized in the hypothalamus [9,15,16], and its expression is modulated by changes in metabolic state [15], we analyzed differences in the expression of the ghrelin message in

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hypothalamic and stomach explants from the same experimental animals.

Timed-pregnant Sprague–Dawley rats purchased from Charles River Farms (Wilmington, MA) were housed individually in plastic cages under controlled conditions (12:12 h light/dark, lights on at 08.00 h; 24–26 °C room temperature; 70–80% humidity). All rats received water and rat chow ad libitum throughout the duration of the study. All procedures were performed under the guidelines prescribed by the Yale Animal Care Committee.

On day 1 postpartum (pp; day 0 pp = day of parturition) rats were assigned to one of three groups. The first group of dams had their litters removed on day 1 pp (NL), the second group had their litters removed on day 13 pp (D13), and the last group was allowed to remain with their litters until all animals were sacrificed (day 15 pp; D15). Animals in the latter two groups had their litters culled on day 1 pp so that they each nursed 12 pups. Animals whose litters were removed on day 1 pp had shown a full estrous cycle by day 15 pp. Only 3 dams from the D13 group showed a vaginal smear showing epithelial cell cornification prior to sacrifice. All day 15 dams remained acyclic. On day 15 pp, dams were deeply anesthetized by isofluorane inhalation, and decapitated after a blood sample was collected from the atrium of the heart. Animals were killed between 10:00 am and noon. The brain, pituitary, and samples of stomach and mammary gland were immediately removed, frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Blood samples were collected in tubes containing 50 μl 1N HCl and addition of 10 μl of phenylmethylsulfonyl fluoride (PMSF) per 1 ml of plasma to decrease sample degradation, and centrifuged. Plasma was collected, and stored at −20 °C until assayed. Plasma ghrelin levels were measured using a commercially available RIA kit (linco) with antibodies specific for the 29-residue octanoylted active form of the ghrelin peptide. All plasma samples were assayed in duplicate yielding a within assay variability of 6%.

Stomach and hypothalamic samples were homogenized for total RNA extraction by the guanidium thiocyanate–phenol–chloroform method using TRIzol reagent (Invitrogen) and 1 μg of total RNA was reverse transcribed using first-strand cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ, USA). The total volume was adjusted to 15 μl with distilled H_2O. The hypothalamic sample included both the arcuate nucleus (ARC) and the subparaventricular zone of the mediobasal hypothalamus, and was dissected so as to encompass about 1 mm of tissue on each side of the third ventricle, and the rostrocaudal extent of the ARC. A 326 bp fragment of ghrelin cDNA was amplified based on reverse transcription polymerase chain reaction (RT-PCR) using specific oligonucleotide primers derived from the coding region of the rat ghrelin sequence (GenBank accession no. ABO29433). These primers had the following sequences: (sense 5′-TTGAGCCCAGAGCACCAGAAA-3′ and antisense 5′-AGTTGCAGAGGAGGCAGAAGCT-3′). The reaction was performed for 1 h at 37 °C. Half of the RT reaction mixture was used directly for the PCR reaction in a total volume of 20 μl, containing, 0.25 U of Taq DNA polymerase, 0.5× PCR buffer, 2.5 mM $MgCl_2$ (Invitrogen), 2.0 mM dNTP (Roche) and 0.3 pmol of the relevant oligonucleotide primers. As an internal control, the same cDNAs were amplified using cyclophilin oligonucleotide primers (sense 5′-AGCACTGGGGAGAAAGGATT-3′ and antisense 5′-CATGCCTTCTTTCACCTTCC-3′, GenBank accession no. M19533), generating a fragment of 252 bp. Parallel amplifications (20, 25, 30, and 35 cycles) of the same cDNA were used to determine the optimum number of cycles. After 35 cycles, a readily detectable signal within the linear range was observed. For the actual analysis, samples were heated for 5 min at 94 °C, and then 35 cycles were performed, each consisting of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. This was followed by a final 10 min extension at 72 °C. PCR reaction products were separated on a 2% agarose gel containing ethidium bromide.

A cDNA transcript containing a 312 bp fragment of cDNA of GHS-R 1a from hypothalamus, stomach pituitary and mammary gland were amplified using RT-PCR using specific oligonucleotide primers derived from the coding region of the rat GHS-R 1a sequence. The primers had the following sequences: (sense 5′-GAGATCGCTCAGATCAGCCAGTAC-3′ and antisense 5′-TAATCCCCAAACTGAGGTTCTGC-3′, GenBank accession no. AB001982) [20]. Primers for cyclophilin were used as controls. Amplification was conducted as described in the ghrelin PCR protocol (see above).

Visualized images of the gels were captured with a digital camera (Kodak), imported into a computer, and analyzed using *Image Tool* software (University of Texas at San Antonio) by comparing the ratio of optical densities of the bands expressing the ghrelin or the GHS-R 1a message over the expression of the control transcript (cyclophilin).

Fig. 1 shows plasma ghrelin concentrations in dams in all experimental groups. A one-way ANOVA showed that lactating rats allowed keeping their litters had plasma ghrelin concentrations that were not different from dams whose litters were removed on the day after parturition ($p > 0.05$). However, plasma ghrelin concentrations were significantly elevated in dams whose litters were removed on day 13 pp and sacrificed on day 15 pp ($p < 0.05$; see Fig. 1).

Measures of the relative transcription of the ghrelin message using RT-PCR showed that the message for the ghrelin gene was slightly elevated in both the hypothalamus and stomach of lactating animals, but these increases were not statistically significant ($p > 0.05$, see Fig. 2).

Analyses of the relative concentrations of GHS-R 1a transcript showed a significant increase in the levels of GHS-R 1a transcript in the hypothalamus of lactating rats allowed to keep their litters throughout the study ($F(2, 14) = 4.23$, $p < 0.05$) compared to those who had their litters removed soon after birth. Animals whose litters were removed two days prior to sacrifice had levels of GHS-R 1a mRNA that were not different from those that had their litters removed soon after birth (see Fig. 3). Stomach levels of GHS-R 1a were not affected by the experimental manipulations ($p > 0.05$; see Fig. 3). The message for GHS-R in the pituitary was significantly elevated in lactating rats ($F(2, 14) = 14.46$, $p < 0.05$)

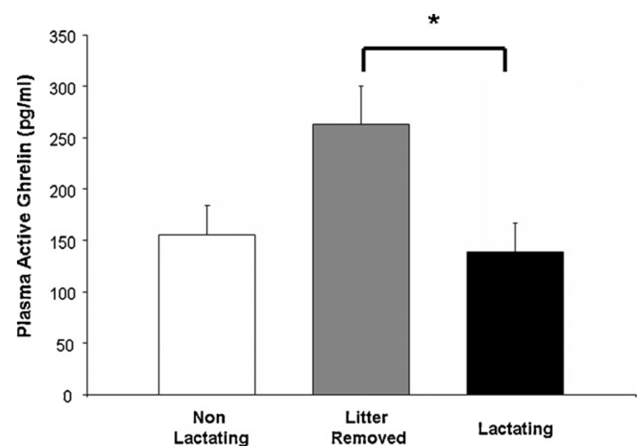

Fig. 1. Plasma acylated ghrelin concentrations in non-lactating rats (NL), litter removed (LR), and dams nursing their young until sacrifice at day 15 pp (L). Ghrelin concentrations in L did not differ from those of NL rats. Removal of the litter, however, resulted in a significant rise in ghrelin concentrations. *: significant from lactating ($p < 0.05$).

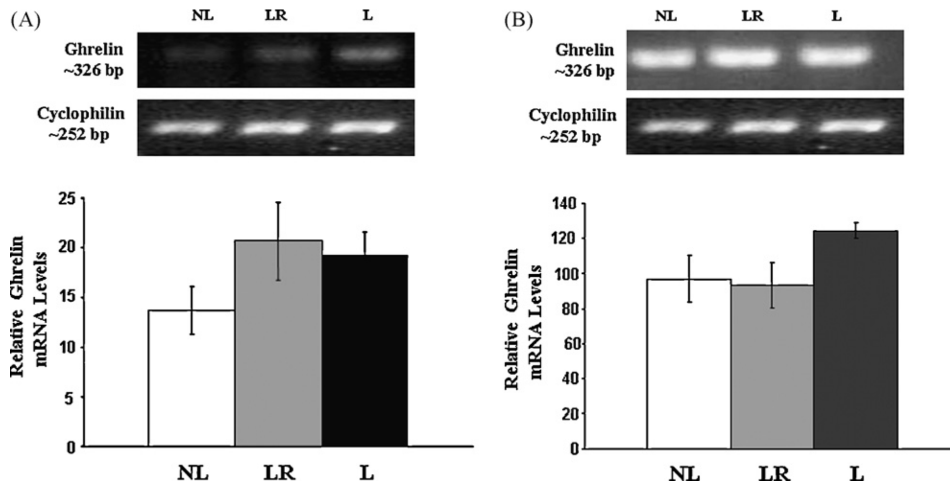

Fig. 2. RT-PCR analysis of ghrelin gene expression in (A) hypothalamus and (B) stomach from NL dams ($n = 6$), LR dams ($n = 6$), and L dams ($n = 6$), all sacrificed on day 15 pp. There were no significant differences in hypothalamic or stomach ghrelin mRNA expression between the groups ($p > 0.05$).

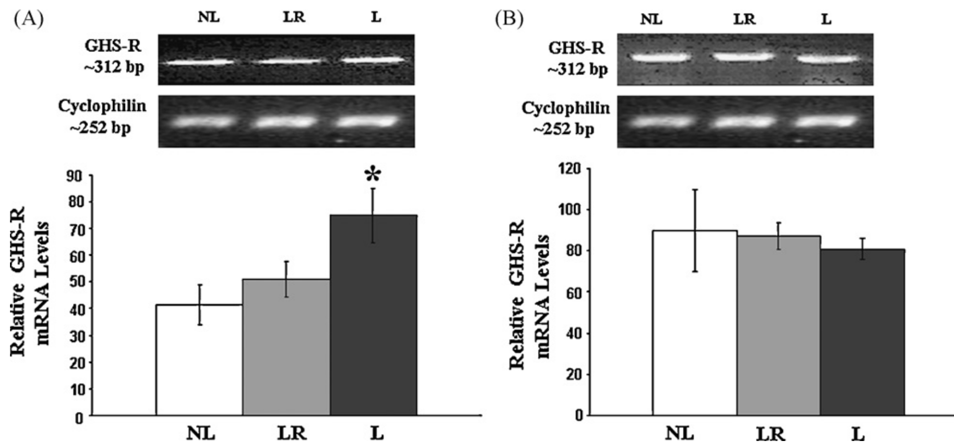

Fig. 3. RT-PCR analysis of GHS-R 1a gene expression in (A) hypothalamus and (B) stomach from NL, LR and L dams. Lactation resulted in increased hypothalamic expression of GHS-R 1a, and the removal of the litters resulted in GHS-R 1a expression that was similar to that of non-lactating animals. Results are expressed as the mean ± S.E.M. *: significant from NL and D13 ($p < 0.05$).

compared to rats whose litter were removed soon after birth and regardless of whether they were allowed to nurse their litters throughout the experiment or if the litters were removed 2 days prior to sacrifice (see Fig. 4). In contrast, relative GHS-R 1a mRNA levels in the mammary glands were reduced in lactating rats allowed to keep their litters for 13 or 15 pp compared to that of rats whose litters were removed after birth ($F(2, 14) = 6.69$, $p < 0.05$).

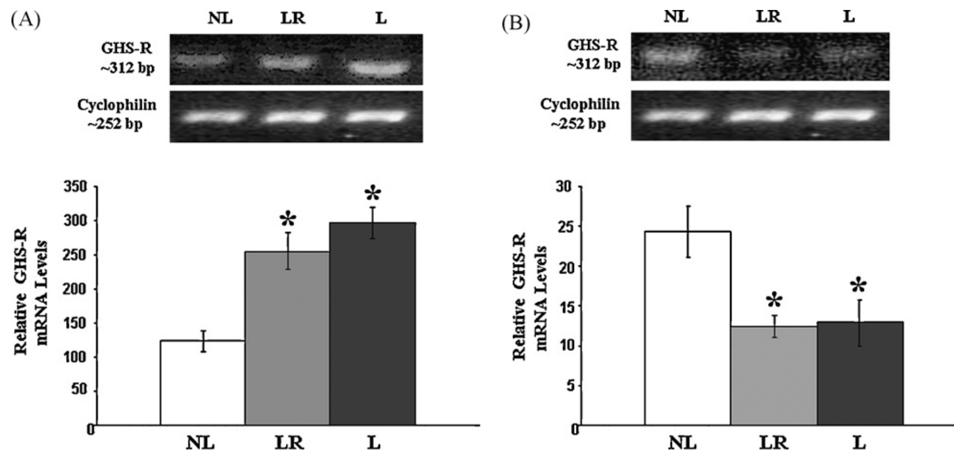

Fig. 4. RT-PCR analysis of GHS-R 1a gene expression in (A) pituitary and (B) mammary gland from NL, LR, and L dams. The expression of the GHS-R 1a transcript was higher in L animals and remained elevated for at least 48 h after the removal of the pups as seen in LR animals. Mammary gland expression of GHS-R 1a mRNA was significantly reduced during lactation and did not change in response to removal of the litter. Results are expressed as the mean ± S.E.M. *: significant from NL ($p < 0.05$).

The present data shows that, while lactating rats have no significant alterations in ghrelin synthesis and secretion at peak lactation (day 15 pp), they do show changes in GHS-R 1a expression in both brain and pituitary. Moreover, our data suggest that both circulating levels of active ghrelin, and the message for the receptor in various tissues vary in response to the presence of a litter.

Lactation is an energetically expensive phase in female mammals because mothers must generate enough energy to maintain their own energy homeostasis as well as that of their young [13,22,39]. To do so, lactating females increase their food intake, reduce energy expenditure [23], and channel energy stored in fat towards milk synthesis [13]. This is reflected in the drop of circulating levels of plasma leptin and insulin, both indexes of adiposity levels that are seen during lactation [5,6,37]. It is, therefore, counterintuitive that ghrelin levels fail to increase as seen in this study and others [10], and even decrease as previously reported [25]. Nevertheless, it should be noted that ghrelin is an adipogenic hormone [31], and one that is anabolic in nature [34]. It is therefore possible that ghrelin levels are maintained at a basal level in order for the dams to continue the breakdown of fat that is required to maintain milk secretion and energy balance. In support of this contention is the fact that circulating levels of ghrelin increase following the removal of the litters, and are almost twice as high in concentration at the time of sacrifice than those of dams who are allowed to continue to nurse their litters. Presumably, the increase in circulating ghrelin concentrations seen in dams separated from their young could play a role in the rapid decrease in the lipolysis that is reported to occur following the removal of litters in lactating rats, and restore anabolic activity in adipose tissue [13].

Despite unaltered plasma ghrelin concentrations, the hypothalamus and the pituitary showed increased expression for the GHS-R 1a message, suggesting that these structures are more sensitive to ghrelin stimulation during lactation. Previous reports suggest that this is the case. For example, peripheral ghrelin injections significantly increase food intake and body weight in lactating rats [20]. This is notable considering that lactating rats already eat three times more than non-lactating rats, and have a body weight that is about 30% higher than that of control non-lactating rats. In addition, ghrelin receptor antagonists seem reduce hypothalamic neuropeptide Y (NPY) and agouti related peptide (AgRP) mRNA expression in lactating rats, but not in virgin females [10]. Similarly, lactating cows produce a greater increase in plasma glucose, cortisol, and glucagon in response to intravenous ghrelin infusions than non-lactating cows [14].

Dams whose pups were removed at birth or 2 days before sacrifice had lower levels of hypothalamic GHS-R 1a expression than lactating rats. Similarly, pituitary GHS-R 1a was also increased in lactating animals, but in contrast to hypothalamic GHS-R 1a expression, pituitary GHS-R 1a message remained elevated in dams whose litter was removed on D13 pp. Increases in GHS-R 1a expression may result from sensory feedback generated by the suckling stimulus, which is responsible for the release of lactogenic hormones like prolactin, GH, and leptin [4,5,18,28]. Changes in GHS-R 1a expression in lactating rats may also be related to the large energetic drain that comes with nursing a litter [1,37,38]. Both of these factors may be independent and the relative contribution of each on modulating circulating ghrelin and GHS-R 1a mRNA remain to be determined. Regardless of the mechanism, it is likely that the increase in hypothalamic expression of GHS-R 1a is related to enhanced sensitivity to the orexigenic effects of ghrelin [31,33], whereas the increase in GHS-R 1a expression seen in the pituitary generates increased responsiveness of this gland to the stimulatory effects of ghrelin upon the somatotropic axis in favor of milk synthesis [4,20,35].

Recent evidence shows that ovariectomy leads to higher feeding responses after ghrelin in female mice, an effect that is reduced after estrogen replacement treatment [8]. Considering these and other data showing that lactating rats have low circulating estrogen concentrations [29] and diminished sensitivity to exogenous estrogen [2,26], it is possible that the expression of GHS-R 1a is mediated by estrogen, and that the lack of estrogen increases GHS-R 1a expression in the hypothalamus and pituitary of lactating rats.

Finally, levels of GHS-R 1a transcript in the mammary gland were diminished in rats that were allowed to nurse their litters for at least 13 days postpartum and removal of the litters was not a significant factor in restoring GHS-R 1a mRNA levels to those of non-lactating rats. While ghrelin has been previously found to stimulate milk secretion in rats [20], it may do so by increasing the release of GH and insulin growth factor (IgF) which then act upon mammary gland tissues to increase milk synthesis [16,36].

A note of caution to these results is that RT-PCR is only a semi-quantitative technique to evaluate differences in gene expression, and more accurate quantitative PCR (qPCR) is necessary to generate more accuracy. Nevertheless, our data suggest that while ghrelin levels do not change as a result of lactation, relative hypothalamic and pituitary sensitivity to ghrelin is enhanced in correlation with metabolic changes that are necessary for a successful lactation. Enhanced ghrelin sensitivity in the hypothalamus could increase food intake, decrease energy expenditure, and modulate glucose levels. Similar changes in the pituitary may serve to increase levels of lactogenic hormones like prolactin and GH. Finally, the presence of the pups and the metabolic changes associated with milk production during lactation contribute to changes in ghrelin content and sensitivity.

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