

Sex differences in prolactin and its receptor expression in pituitary, hypothalamus, and hippocampus of the rat

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ABSTRACT

Prolactin, via its receptor variants, plays an important role in various tissues including brain. Many studies have been performed to define the regulation of prolactin and its receptor in the pituitary gland, both in females and males of different species. However, gender differences at transcriptional level have not been completely established nor has its expression in different brain regions. The aim of this study was to determine expression of prolactin and its receptor in the pituitary of male and female rats during the estrous cycle, and compare it with the expression regulation in selected brain areas such as hypothalamus and hippocampus. Quantitative polymerase chain reaction (PCR) was performed to assess expression of prolactin and its receptor. The results indicate that prolactin and its receptor are more abundant in the pituitary gland of female rats, and it varies during the estrous cycle, presenting its maximal expression on proestrus day. Interestingly, in hypothalamus and hippocampus, prolactin expression was more abundant in male than in female rats and did not vary significantly during the estrous cycle. However, prolactin receptor was consistently more abundant in female tissues

RESUMEN

La prolactina (PRL) a través de las variantes de su receptor (PRL-R) desempeña un papel importante en varios tejidos, incluyendo el cerebro. Se han realizado diversos estudios para definir la regulación de PRL y PRL-R en la glándula hipofisaria, en hembras y machos de diversas especies; sin embargo, las diferencias de género a nivel transcripcional no han sido completamente establecidas, ni tampoco su expresión en diferentes regiones del cerebro. El objetivo de este estudio fue determinar la expresión de PRL y PRL-R en la hipófisis de ratas machos y hembras durante el ciclo estral y compararla con la regulación de la expresión en áreas del cerebro como el hipotálamo y el hipocampo. Se realizó PCR cuantitativo para evaluar la expresión de PRL y PRL-R. Los resultados indicaron que la PRL y el PRL-R son más abundantes en la glándula hipofisaria de ratas hembra, y que su expresión varió durante el ciclo estral, presentando su máxima expresión en el día del proestro. De manera importante, en el hipotálamo y el hipocampo, la expresión de PRL fue más abundante en las ratas macho que en las hembras y no tuvo variaciones significativas durante el ciclo estral. Sin embargo,

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Received for publication: 20-04-2015

Accepted for publication: 06-05-2015

in all studied regions, suggesting that females have more availability of prolactin receptor for physiological functions. The overall results indicated that prolactin and its receptor have a differential expression regulation in male and female rats, suggesting a sexual dimorphic role of prolactin in pituitary, hypothalamus, and hippocampus. (REV MEX ENDOCRINOL METAB NUTR. 2015;2:60-7)

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Key words: Prolactin. Prolactin receptor. Pituitary. Hypothalamus. Hippocampus. Sexual dimorphism.

PRL-R fue consistentemente más abundante en los tejidos de las hembras en todas las regiones estudiadas, sugiriendo que las hembras tienen más disponibilidad de PRL-R para sus funciones. Los resultados globales indican que la PRL y su receptor tienen regulación diferencial en su expresión en ratas machos y hembras, lo cual sugiere que PRL tiene funciones diferentes en machos y hembras en la glándula hipofisaria, así como en el hipotálamo y en el hipocampo.

Palabras clave: Prolactina. Receptor de la prolactina. Dimorfismo sexual. Hipófisis. Hipotálamo. Hipocampo.

INTRODUCTION

Prolactin (PRL) is produced by the anterior pituitary gland and it has been primarily identified as a major stimulating factor for lactogenesis. Apart from its classical functions, this hormone participates in many other aspects of mammalian homeostasis, including osmoregulation, reproduction, sexual behavior^{1,2}, and maternal behavior³. Besides, PRL acts at a hypothalamic level to regulate gonadotropin-releasing hormone secretion, the firing rate of neurons, the metabolism of neurotransmitters and neuropeptides, oxytocin release, enzyme activities, and glial cellular proliferation⁴. Some effects of PRL in the metabolism and regulation of both the immune and central nervous system (CNS) have been described⁴⁻⁷. In addition, PRL-trophic actions on the CNS include mediation in the development and maturation of tuberoinfundibular dopaminergic neurons, regulation of cell proliferation, neurogenesis, and mitogenic actions in glial cells and precursor cells of oligodendrocytes. Furthermore, PRL regulates the generation of new oligodendrocytes in the maternal CNS of rodents, enhances repair of white matter, remyelination, and protects hippocampal neurons in the dentate gyrus of chronically stressed mice^{3,8} and against metabolic stress in cortical neurons^{4,9}.

Apart from PRL pituitary production, there are other extrapituitary sources of PRL, including the brain, lymphocytes, skin fibroblasts, breast, the decidua, prostate, and adipose tissue cells^{3,4}. In the brain,

mRNA and protein of PRL have been detected in several nuclei of the hypothalamus and extra-hypothalamic regions such as the cerebral cortex^{8,9}; PRL is also released by hypothalamic neurons. Besides local PRL sources, this hormone can enter into the CNS through a receptor-mediated transporter located in the membranes of the choroid plexus³.

The actions of PRL are mainly mediated by the high-affinity PRL receptor (PRL-R) that belongs to class 1 of the cytokine receptor superfamily. There are at least two isoforms of PRL-R that may be the result of alternative splicing of the primary gene. The rat contains short, intermediate, and long forms of the receptor^{4,5}. Consistent with various actions of PRL in the brain, the presence of PRL-R protein has been identified in several brain regions of female rats by autoradiography or immunohistochemistry^{4,5}. The expression of short and long forms of PRL-R mRNA has also been detected in various brain regions of the female rat by real-time polymerase chain reaction (RT-PCR)¹⁰⁻¹² and *in situ* hybridization¹³. The PRL-R and PRL binding sites are widely distributed in the brain and are particularly abundant in the hypothalamus and anterior pituitary gland¹⁴. Part of the mechanism whereby PRL acts in the brain may involve changes in the abundance of PRL-R mRNA.

Observations in the rat suggest that the concentrations of PRL-R mRNA in the brain are differently regulated in distinct brain tissues by PRL and/or ovarian steroids¹⁵. The expression of PRL and its receptors in CNS is upregulated by different physiological events such as lactation, childbirth, stress, and exposure to injury induced by hypoxia or ischemia⁴.

In the male rat, data on expression of PRL-R in different brain regions is very limited. While one study reported relatively high levels of PRL-R immunoreactivity¹⁶, a second report found lower expression of PRL-R protein when compared with ovariectomized plus estrogen-treated rats¹⁷. Besides, one study reported that the long form of PRL-R mRNA in the brain is induced by pup contact¹⁸, while another showed that the long form in the choroid plexus is increased by restraint stress¹⁰. However, none of these studies investigated the expression of PRL-R mRNA in individual brain regions, including the hypothalamus and pituitary, nor the possible gender differences in expression of PRL-R at transcriptional level. Hence, the aim of the present study was to investigate the expression of PRL and PRL-R mRNA in the hypothalamus, hippocampus, and pituitary gland and their differences in male and female rats during the estrous cycle.

MATERIALS AND METHODS

Animals

Adult male and virgin female Wistar rats (250-300 g) were individually housed under controlled temperature and lighting conditions (12:12 hour light: dark cycle, lights on at 06:00 h), with food and water available *ad libitum*. Animals in each experiment were three males and three females for each day of estrous cycle (n = 12) for experiment and the experiments were duplicated (n = 24 total). The Institutional Animal Care and Use Committees of the School of Chemistry at the National Autonomous University of Mexico approved all experimental protocols. Animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Official Mexican Guide of the Ministry of Agriculture (SAGARPA NOM-062-Z00-1999) published in 2001. All efforts were made to minimize the number of animals used and their suffering.

Tissue collection

Tissues were collected from male and female rats during estrous cycle. The estrous cycle was determined assessing vaginal smears according to modification

made recently by Cora, et al.¹⁹. Hypothalamus, hippocampus, and pituitary tissues from male and female rats were obtained according to Paxinos, et al.²⁰.

Extraction of total RNA

Tissues from each brain region were immediately homogenized in a guanidine thiocyanate-containing TRIzol® (Gibco) solution. Total cellular RNA was isolated individually from the homogenized samples according to the instruction provided by Gibco. Briefly, following extraction by adding chloroform, the total cellular RNA was then precipitated with isopropyl alcohol, washed with ethanol, air-dried and re-suspended. The concentration and purity of RNA were estimated by measuring the absorbance at 260 nm (A260) and 280 nm (A280).

Quantification of nucleic acids

Quantification was performed using a NanoDrop 1000 Spectrophotometer V3.7 from Thermo SCIENTIFIC. The RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. Total RNA was re-suspended in either RNase-free water for reverse transcription or in hybridization buffer for RNase protection assay.

Genomic sequences and oligonucleotides

Rattus norvegicus sequence (*Rattus norvegicus* Rnor_5.0.73) was obtained from the ENSEMBL database PRL gene (ENSRNOG00000017374) and PRL-R gene (ENSRNOG00000017727).

The oligonucleotide were synthesized at Integrated DNA Technologies with TaqMan® probes; PRL gene oligos are located in exons 3-4 (oligo1 CCT CCA CCA GTT ATT AGT AGT TGA, oligo 2 CTA TAG CCC ACA CTC CTG AAG and probe / 56-FAM / ACA AGC CCA / ZEN / GAA AGT CCC TCC G / 3IABkFQ /), for PRL-R gene oligonucleotides are located in exons 3-4 (oligo1 GGT GCA CTT GAA TTC GTC TGT, oligo2 CTT GCT CTA CTT GTC TTC GTT C and probe / 56-FAM / TGG TGG TGA / ZEN / CTG TCC CTT CAG G / 3IABkFQ /), for the oligonucleotide were synthesized at Applief

Biosystems with TaqMan® probes; HPRT gene oligonucleotides are located in exons 8-9 (oligo1 GAC CGG TTC TGT CAT GTC G oligo2 ACC TGG TTC ATC ATC ACT AAT CAC and probe / R95-FAM-MGB / NM_012583).

Synthesis of complementary DNA

Complementary DNA (cDNA) synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific (K1622) as per protocol. Two micrograms of total RNA was gently mixed with 100 ng of oligo (dT)₁₈, briefly centrifuged and incubated at 65 °C for five minutes. The reaction mix was placed on ice and 5X reaction buffer, RNase inhibitor Ribo-Lock (20 U/l), 10 mM dNTP and M-MuLV RT RevertAid (200 U/l) was added. The reaction was gently mixed and briefly centrifuged. The reaction was incubated for 60 minutes at 42 °C and thereafter terminated by heating at 70 °C for five minutes. The samples were stored at -20 °C until further use.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using Applied Biosystems thermocycler StepOnePlus™ 96-well plates. The reaction mixtures were prepared using the GoTaq® 2-Step RT-qPCR System from Promega in a final volume of 10 µl. Oligonucleotides were used at 500 nM final concentration. Probes were used at 250 nM final concentrations and 1.5 µl of the cDNA reaction mixture was added in each PCR. Thermocycling conditions were: one cycle at 95 °C for 20 seconds and 40 cycles of 95 °C for one second, 62 °C for 20 seconds.

Prolactin and prolactin receptor mRNA content

The optical density values for genes of PRL and PRL-R mRNA and hypoxanthine phosphoribosyltransferase (HPRT) mRNA from each brain region of different animal models were compared to the respective standard curve, and mRNA PRL and PRL-R genes were then normalized with HPRT mRNA.

DATA ANALYSES

Results are expressed as mean, statistical analysis of data was performed using analysis of variance followed by a t-test. These statistical tests were conducted by using the GraphPad Prism Software 6.0. Differences are regarded to be significant if $p \leq 0.05$.

RESULTS

Differential prolactin mRNA expression between male and female rats

The relative amount of PRL and PRL-R mRNA in the pituitary, hypothalamus, and hippocampus presented a sexual dimorphism. The mRNA of PRL gene in female pituitary was 6.34, 27.24, and 53.82 times higher in females during estrus, metestrus-diestrus, and proestrus, respectively, than in males ($p < 0.001$) (Fig. 1 A). The pituitary of the estrus rat expressed the lowest level of PRL mRNA, whereas the proestrus rat expressed the highest level. The PRL-R mRNA expression in male pituitary was 0.44 times higher than female on estrus ($p < 0.05$). On the other hand, the expression of PRL-R mRNA in females on metestrus-diestrus and proestrus was 3.77 and 5.56 times, respectively, higher than in males ($p < 0.001$) (Fig. 1 B).

The PRL mRNA expression in the female hypothalamus was approximately 10 times lower throughout the estrous cycle than in males ($p < 0.001$) (Fig. 2 A). In contrast, PRL-R mRNA in female hypothalamus was 7.7, 23.4, and 55.3 times higher in females on estrus, metestrus-diestrus, and proestrus, respectively, than in males ($p < 0.001$) (Fig. 2 B).

As in the case of hypothalamus, in hippocampus, PRL mRNA expression in females was 9.4, 9.7, and 9.7 times lower during estrus, metestrus-diestrus, and proestrus, respectively, than in male rats ($p < 0.001$) (Fig. 3 A). Also, as in hypothalamus, PRL-R mRNA in hippocampus was 1.42 times higher in female on estrus than in males ($p < 0.05$), and 2.87 and 3.9 times higher in metestrus-diestrus and proestrus than in males (Fig. 3). In the pituitary, hypothalamus, and hippocampus

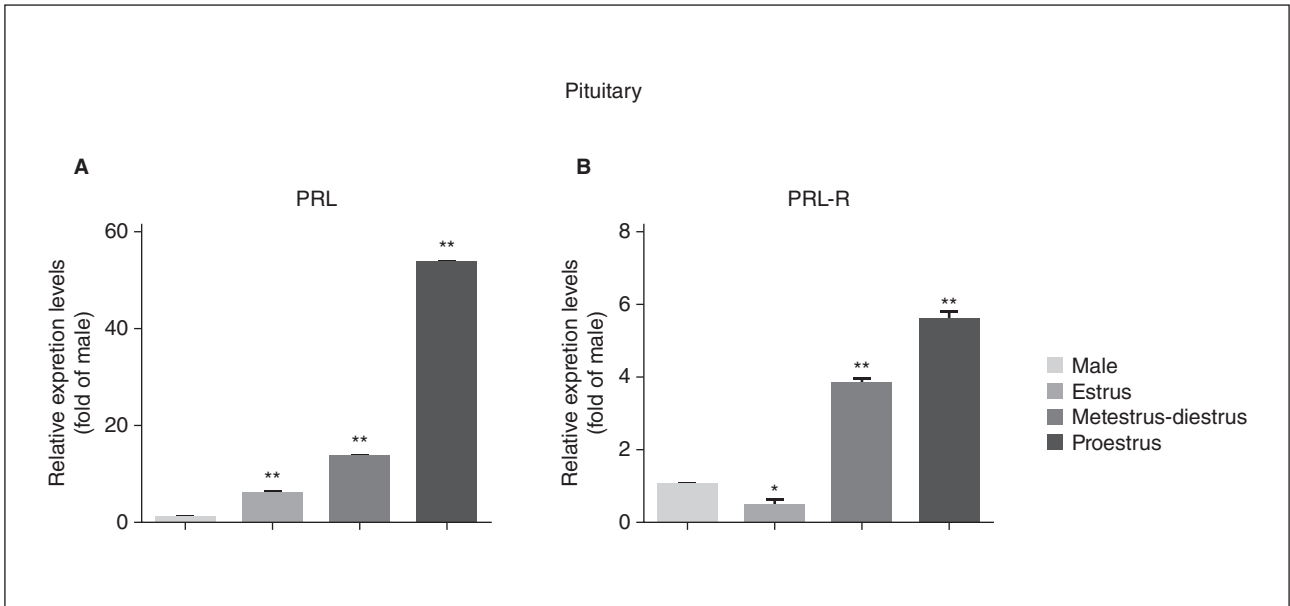


Figure 1. Sex differences and changes during the estrous cycle in the expression of prolactin and prolactin-receptor mRNA in the rat pituitary rat. Animals in each experiment were three males and three female for each day of estrous cycle, n = 12 for experiment and the experiments were performed by duplicate (n = 24). Data are presented as mean and standard deviation of two isolate experiments. The variance analysis between male vs. estrus, metestrus-diestrus and proestrus was followed by a t-test analysis. Statistical significances are represented as p-value * < 0.05 and ** < 0.001.

PRL: prolactin; PRL-R: prolactin-receptor.

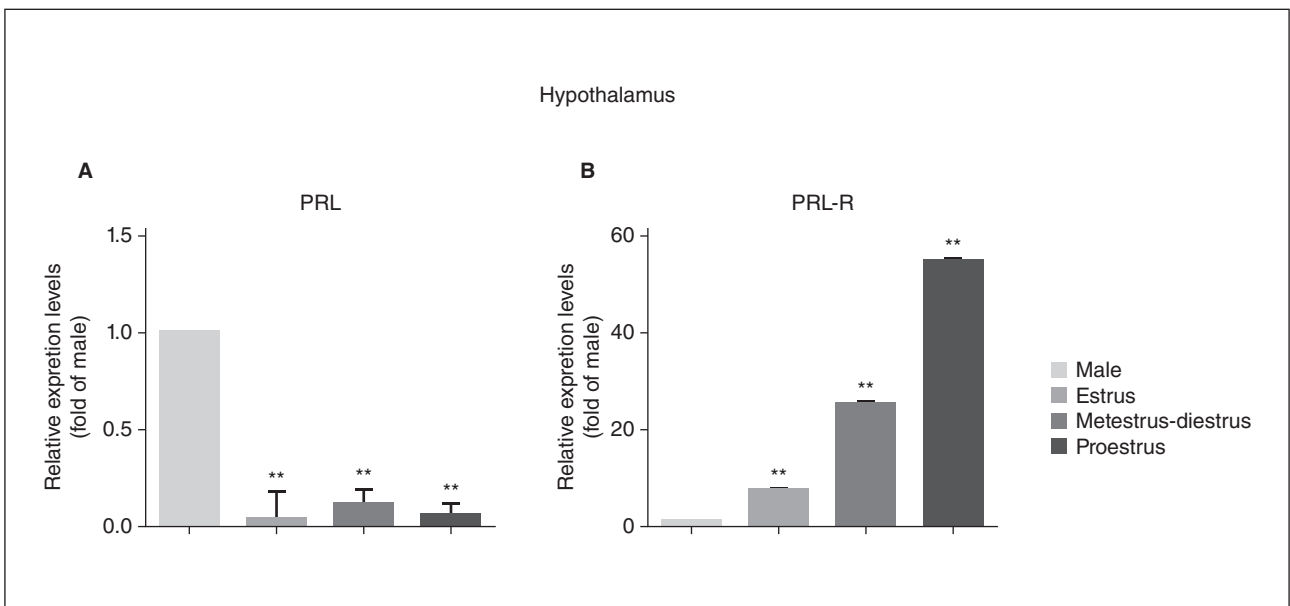


Figure 2. Sex differences and changes during estrous cycle in the expression of prolactin and prolactin-receptor mRNA in the hypothalamus of the rat. Animals in each experiment were three males and three females for each day of estrous cycle, n = 12 for experiment and the experiments were performed by duplicate (n = 24). Data are presented as mean and standard deviation of two isolate experiments. The variance analysis between male vs. estrus, metestrus-diestrus and proestrus was followed by a t-test analysis. Statistical significances are represented as p-value ** < 0.001.

PRL: prolactin; PRL-R: prolactin-receptor.

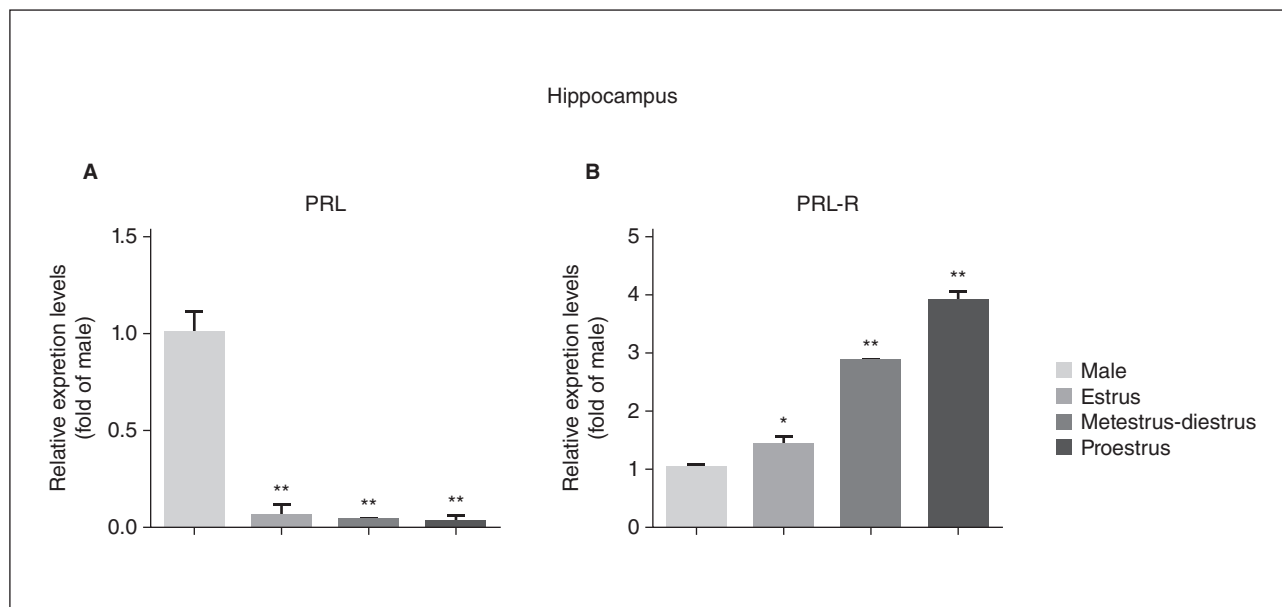


Figure 3. Sex differences and changes during estrous cycle in the expression of prolactin and prolactin-receptor mRNA in the hippocampus of the rat. Animals in each experiment were three males and three female for each day of estrous cycle, $n = 12$ for experiment and the experiments were performed by duplicate ($n = 24$). Data are presented as mean and standard deviation of two isolate experiments. The variance analysis between male vs estrus, metestrus-diestrus and proestrus was followed by a t-test analysis. Statistic significances are represented as p-value * < 0.05 and ** < 0.001 .

PRL: prolactin; PRL-R: prolactin-receptor.

the highest expression of PRL-R mRNA was observed in proestrus rats (Fig. 1-3).

DISCUSSION

The results of this study indicate that the mRNA expression of PRL and PRL-R mRNA in the pituitary, hypothalamus, and hippocampus of male and cycling female rats are differentially expressed. The PRL mRNA was lower in the pituitary gland of males, but higher in hypothalamus and hippocampus. In the cycling female rat, there was a different expression pattern of PRL-R between studied tissues and during the estrous cycle, but PRL changes in females only occurred in the pituitary gland.

The observation that prolactin was significantly lower in male than in female rats is in agreement with previous observations about total PRL content of the protein in the pituitary gland; however, the finding

that PRL mRNA was more abundant in brain tissues such as in male hypothalamus and hippocampus is interesting and has not been previously reported by quantitative PCR.

In the anterior pituitary gland of the female rat there is a constant remodeling during the estrous cycle. Furthermore, during different specific physiological conditions, such as pregnancy and lactation, there are also changes in the structure and functionality of the pituitary gland^{21,22}. A surge of PRL in the pituitary gland has been related with an increase in lactotrope cells apoptosis, which occurs during proestrus²³. This observation is in agreement with the increase in both PRL and PRL-R observed during proestrus day in the present study. Even more, in some fish models, local interactions of PRL via autocrine/paracrine mechanisms could modify PRL production in pituitary cells through differential regulation of PRL mRNA stability and gene transcription^{24,25}. Whether this mechanism could occur in rat pituitary deserves to be established.

Various studies reveal that individual lactotropes present differences in PRL release in time and magnitude, which is gender-specific²⁶. Besides, in the pituitary gland around the time of puberty, PRL mRNA and pituitary transcription factor levels become sexual dimorphic, in which males exhibit lower levels of PRL mRNA than female rats²⁷. Furthermore, the gender- and state-specific differences are involved in Ca²⁺ dynamics and induction of PRL gene expression in the rat pituitary²⁸. It has been demonstrated that a sexually dimorphic PRL expression pattern occurs in the yellow perch; in this species, the male brain tissue shows low expression levels of PRL, while female brain tissue does not show any expression²⁴.

In pituitary lactotropes of rats, the cell density and surface density decreased with age in both males and females. However, cell density as well as surface density appeared to be increased in females when compared to males, but the cell volume was only increased in old female rats as compared to male rats; thus the authors conclude that there exists a clear sexual dimorphism in the age-related changes of pituitary PRL cells²⁹. Our results are in line with this observation; we found lower mRNA expression of PRL in males than in females in pituitary gland.

Expression of PRL-R mRNA was higher in the pituitary gland, hypothalamus, and hippocampus of females than in male rats. The presence of PRL-R mRNA in the hypothalamus at higher concentrations than in pituitary and hippocampus supports previous observations in different animal species including mammals^{16,30}. This interesting finding of PRL-R expression in the hypothalamus supports the idea that PRL exerts important regulatory effects in this brain area. It has been demonstrated that in turkeys, PRL may up-regulate PRL-R in both the anterior pituitary and hypothalamus¹⁴.

Gender-related differences in the abundance of PRL-R mRNA in the rat have been reported in peripheral tissues such as the liver (> female than male), and in kidney and adrenal gland (> male than female), assessed by *in situ* hybridization; nevertheless, the actions of PRL have not been identified in all tissues expressing PRL-R transcripts^{31,32}. Furthermore, demonstration of sex differences in expression of PRL-R gene in the brain has not been conclusive until now.

However, in the present study, a high concentration of PRL-R mRNA was observed in hypothalamus > pituitary > hippocampus, supporting and extending the idea of PRL actions in these tissues.

In birds (bantams), a sex difference has been observed in anterior pituitary and basal hypothalamic PRL-R mRNA, with lower values in both tissues in females than in males¹⁵. In another study using binding assay, the authors observed a trend in sex differences of specific PRL-R in the preoptic area of the hypothalamus but not in any other brain region³³. Furthermore, also using binding assays, another study showed higher PRL binding sites in the hypothalamus of female than male rats, while no significant sex differences in PRL binding sites to other brain regions were observed³⁴. A striking gender difference in expression of both forms of PRL-R mRNA in the mediobasal hypothalamus has been observed, with females presenting four times greater levels than males, and 2.3-fold higher expression of PRL-R mRNA in the choroid plexus and preoptic area in female compared to male rats⁵. Finally, in human subjects, higher PRL-R in the hypothalamus of female than in male subjects was reported³⁵. These results are consistent with the higher levels of PRL-R mRNA levels observed in all studied tissues of females in the present study.

Also, in the present study we observed that expression of PRL-R mRNA reached the highest levels on the day of proestrus and dropped to the lowest levels on the day of estrous, in line with previous reports of Pi, et al.⁵. The factors responsible for upregulation of PRL-R mRNA in the female rat brain during proestrus could be multiple; either sexual steroid hormones or PRL levels have been involved. The highest level of pituitary PRL mRNA is also found at 14:00 h on proestrus³⁶. Considering that the levels of serum PRL, pituitary PRL mRNA, and PRL-R mRNA in the brain coincidentally reach a peak in the afternoon of proestrus, it is possible that this peak of PRL-R mRNA in the brain during proestrus may be induced by PRL.

Increased expression of PRL-R gene observed on proestrus day suggest an increase in PRL function. The PRL surge during the estrous cycle is thought to have several functions such as the enhancement of female sexual behavior⁵. In fact, inhibition of the

PRL surge with dopamine agonist attenuates the lordosis quotient. It has been demonstrated that PRL exerts different functions in brain tissues such as neuroprotection in the hippocampus^{3,37}, regulation of PRL-R expression in hypothalamus¹², maternal behavior⁴, and lactotrope replacement in pituitary gland²³, among many others.

The overall results indicate that there is a gender differential content of both PRL and PRL-R mRNA levels in the pituitary and cerebral tissues, suggesting a differential role of PRL between the sexes. Besides, the differential regulation of PRL and its receptor in pituitary gland and brain areas should be involved in different functions such as the replacement of lactotrope populations in the pituitary gland, neuroprotection and maternal behavior.

ACKNOWLEDGEMENTS

This work was supported by PAPIIT, UNAM grant (IN 210412 and IN220315). CREA, RSNA and VCE are recipient of a CONACYT Ph.D. scholarship grant.

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