Quantitation of prolactin receptor mRNA in the maternal rat brain during pregnancy and lactation

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Abstract

Prolactin receptor (PRL-R) expression in the brain is increased in lactating rats compared with non-pregnant animals. The aim of the present study was to determine the time-course of changes in PRL-R mRNA levels during pregnancy and/or lactation, and to determine relative levels of the two forms (short and/or long form) of receptor mRNA in specific brain regions. Brains were collected from female rats on dioestrus, days 7, 14 or 21 of pregnancy, day 7 of lactation or day 7 post-weaning. Frozen, coronal sections were cut (300 µm) and specific hypothalamic nuclei and the choroid plexus were microdissected using a punch technique. Total RNA was extracted and reverse transcribed, then first strand cDNA was amplified using quantitative real-time PCR. Results showed an up-regulation of long-form PRL-R mRNA in the choroid plexus by day 7 of pregnancy compared with dioestrus, which further increased on days 14 and 21 of pregnancy and day 7 of lactation, and then decreased to dioestrous levels on day 7 post-weaning. Short-form PRL-R mRNA levels increased on day 14 of pregnancy relative to dioestrus, increased further on day 7 of lactation and decreased on day 7 post-weaning. Changes in mRNA were reflected in increased levels of PRL-R immunoreactivity in the choroid plexus during pregnancy and lactation, compared with dioestrus. In the arcuate nucleus, long-form PRL-R mRNA was increased during pregnancy. In contrast to earlier work, no significant changes in short- or long-form PRL-R mRNA expression were detected in several other hypothalamic nuclei, suggesting that changes in hypothalamic mRNA levels may not be as marked as previously thought. The up-regulation of PRL-R mRNA and protein expression in the choroid plexus during pregnancy and lactation suggest a possible mechanism whereby increasing levels of peripheral prolactin during pregnancy may have access to the central nervous system. Together with expression of long-form PRL-R mRNA in specific hypothalamic nuclei, these results support a role for prolactin in regulating neuroendocrine and behavioural adaptations in the maternal brain.

Introduction

The anterior pituitary hormone prolactin has numerous actions in the body, one of the most important being the initiation and maintenance of lactation in the female (Bole-Feyset et al. 1998). In addition to its effects in the mammary gland, prolactin is thought to have important neuroendocrine actions in the brain. Prolactin binds with high affinity to prolactin receptors (PRL-R) (Bole-Feyset et al. 1998) that have been identified on the cell surface of many organs, including the mammary glands, ovaries, testes, liver and kidneys (Posner et al. 1974, Djiane et al. 1977, Kelly et al. 1980, Boutin et al. 1988, Shiroma et al. 1990). Further studies have detected the presence of prolactin-binding sites in the rat brain using in vitro autoradiography (Muccioli et al. 1991, Crumeyrolle-Arias et al. 1993, Mustafa et al. 1994, 1995), and identified PRL-R expression by immunohistochemistry (Roky et al. 1996, Pi & Grattan 1998a, 1999a). Similarly, mRNA for PRL-R has been detected in the brain using both RT-PCR (Chiu et al. 1992, Sugiyama et al. 1994, Pi & Grattan 1998b, 1999a) and in situ hybridization (Chiu & Wise 1994, Bakowska & Morrell 1997, Mann & Bridges 2002). Prolactin has been implicated in the regulation of a variety of brain...
functions, including the suppression of adrenocorticotrophin secretion during the stress response (Torner et al. 2001), an increase in feeding and appetite (Sauve & Woodside 1996, 2000), and the suppression of fertility (Cohen-Becker et al. 1986), as well as the well-characterized short-loop negative feedback regulation of its own secretion by stimulating tuberoinfundibular dopamine (TIDA) neuronal activity (Freeman et al. 2000). In addition, prolactin is known to be involved in further brain functions during lactation, such as the release of oxytocin for the milk ejection reflex (Parker et al. 1991, Ghosh & Sladek 1995) and induction of maternal behaviour (Bridges 1994). The presence of PRL-R in the brain suggests a direct action of prolactin in the central nervous system (CNS) to regulate these functions.

Before prolactin can exert actions in the CNS, it must gain access into the brain. As a large polypeptide hormone, it would not be expected to pass through the blood–brain barrier. Prolactin appears to be actively transported into the brain, however, by a carrier-mediated transport mechanism in the choroid plexus, located in the cerebral ventricles (Walsh et al. 1987). The choroid plexus contains the highest levels of prolactin-binding sites (Di Carlo et al. 1992, Mustafa et al. 1994), PRL-R immunoreactivity (Roky et al. 1996, Pi & Grattan 1998a) and PRL-R mRNA (Sugiyama et al. 1994) in the brain. It has been suggested that these binding sites might facilitate prolactin entry into the ventricular system and hence the cerebrospinal fluid (CSF), from where it could readily diffuse to various brain regions. Alternatively, or perhaps in addition, there is evidence that prolactin is produced by neurons, and hence may be released within the brain to modulate hypothalamic function (DeVito et al. 1992, Torner & Neumann 2002, Torner et al. 2002).

We have recently reported the marked up-regulation of PRL-R expression in the hypothalamus and choroid plexus during lactation (Pi & Grattan 1999a). PRL-R’s belong to the cytokine family of receptors and exist as two isoforms in the rat, a short and a long form, produced by alternative splicing of a single gene (Boutin et al. 1988). The two forms have identical extracellular portions, but vary in the length of their intracellular domains. Using RT-PCR, an increase in mRNA for both isoforms of the PRL-R has been detected in the choroid plexus and in restricted regions of the hypothalamus in lactating rats compared with non-pregnant rats (Pi & Grattan 1999b,c, Mann & Bridges 2002). The aim of this study was to investigate the time-course for changes in receptor expression during pregnancy and lactation by quantifying the relative levels of mRNA for the two isoforms of the receptor in specific brain regions using real-time PCR (Bustin 2000).

Materials and Methods

Animal preparation

Female, 10-week-old Sprague–Dawley rats weighing between 200 and 250 g were purchased from the Taieri Resource Unit, University of Otago. All procedures were approved by the University of Otago Animal Ethics committee. Rats were given free access to water and food. Controlled temperature (22 ± 1 °C) and lighting conditions (14 h light:10 h darkness cycles, lights on at 0500 h) were maintained throughout the experiment. Oestrous cyclicity was monitored by daily vaginal smears and dioestrous rats were selected after at least two consecutive 4-day oestrous cycles. For timed pregnancies, rats exhibiting pro-oestrous smears were placed in a cage overnight with a fertile male. The presence of sperm in the vaginal smear on the following morning provided confirmation of mating (day 0 of pregnancy). Groups of pregnant rats were killed on days 7, 14 and 21 of pregnancy. Rats give birth in our colony on day 22 of pregnancy (day 0 lactation) and, after birth, litter numbers were normalized to ten pups per mother. A group of lactating rats (with pups present continuously) was killed on day 7 of lactation. A final group of post-weaning rats was prepared by removing pups from lactating dams on day 14 of lactation and then killing the mothers 7 days later. For microdissection, animals were killed between 0800 and 1000 h by decapitation using a guillotine. The brains were rapidly removed and frozen on dry ice (n=6–8 per group). For immunohistochemistry, rats were injected with an overdose of sodium pentobarbital, 17 mg/100 g body weight for late pregnant and lactating rats or 6 mg/100 g body weight for dioestrous and early pregnant rats. Rats were perfused intracardiacally with 50 ml ice-cold heparinized saline, followed by 300 ml 2% paraformaldehyde in 0·1 M phosphate buffer. Brains were removed, post-fixed for 1 h in the same
fixative and further soaked in sucrose solution (30% sucrose in 0·1 M phosphate buffer) until the brain had sunk. Brains were then frozen in isopentane cooled by liquid nitrogen and stored at −80 °C until further processing (n = 4 per group).

Microdissection of specific brain regions

Thick, coronal sections (300 µm) were cut through the brain in a cryostat at −9 °C (see Table 1), thaw-mounted onto glass slides and rapidly refrozen. The frozen sections were placed onto an aluminium block cooled with dry ice. Using a dissecting microscope, five areas were microdissected (see Table 1) using a blunt-ended microdissection needle (Palkovits & Brownstein 1988). Separate, sterile needles were used to punch each area, to eliminate the possibility of contamination by tissue carry-over. At the completion of microdissection, sections were thawed and photographed to allow confirmation of accurate dissection.

Extraction of total RNA and reverse transcription

Total cellular RNA was extracted from microdissected brain regions using Qiagen’s RNeasy Mini Kit (Valencia, CA, USA), as previously described (Pi & Grattan 1999b,c). Levels of RNA were measured using UV spectrophotometry. As total RNA levels were low (typically one choroid plexus punch contained approximately 30 ng total RNA, one medial preoptic nucleus punch contained 50 ng and one arcuate nucleus punch contained 60 ng), it was impractical to quantify total RNA in each sample and still have sufficient sample volume remaining for the reverse transcription. Instead, equal volumes of total RNA were transcribed into first strand cDNA for each sample, using PE Applied Biosystems GeneAmp Gold RNA PCR reagent kit (PE Biosystems, Foster City, CA, USA), and subsequent assay results analysed relative to a housekeeping gene (β-actin) within the same sample to normalize for possible variations in starting RNA quality and quantity, and RT efficiency. β-Actin levels were analysed independently, and did not vary in any of the experimental groups.

Real-time quantitative PCR

Oligonucleotide primers and probes specific for the two isoforms of the rat PRL-R (accession numbers: M57668 and NM012630) (Boutin et al. 1988, Shirota et al. 1990) were designed for use in TaqMan real-time PCR using Primer Express software (PE Applied Biosystems). A forward (sense) primer directed against the extracellular segment (common to both forms) and two reverse (antisense) primers directed against intracellular segments (unique to each form) provided a distinction between the two isoforms (see Table 2). A fluorogenic TaqMan probe was designed to hybridize with part of the extracellular domain of the PRL-R between the primers, common to both forms of the receptor mRNA. At either end of the probe was attached one of two fluorescent dyes; for the PRL-R primers the quencher dye was TAMRA and the reporter dye was 6FAM. β-Actin probe and primers were designed in the same way, except the probe reporter dye was VIC.

Real-time PCR was completed using the TaqMan system (PE Applied Biosystems 1998). A reaction mix was prepared containing primers and probes at optimized final concentrations of 300 nM and 200 nM respectively, TaqMan Universal PCR
Master Mix (1 ×) and RNase-free water. Template cDNA (2·5 µl) was added, in duplicate, to each well of the MicroAmp optical 96-well reaction plates (PE Biosystems), such that the final reaction volume in each well was 25 µl. No-template controls, consisting of 22·5 µl reaction mix and 2·5 µl water instead of template cDNA, were run on each plate. A stock of cDNA containing relatively high levels of both long- and short-form PRL-R mRNA was created by pooling many punches of choroid plexus, and then twofold dilution series were run on each plate as external standards.

An ABI PRISM 7700 Sequence Detection System (Centre for Gene Research, University of Otago) was used to detect fluorescence during each PCR cycle. The thermal cycling conditions were set at 50 °C for 2 min and 95 °C for 10 min initially, followed by 15 s at 95 °C (melting step) and 1 min at 60 °C (anneal/extend step) for 40 cycles (Harrison et al. 2000). The initial steps were important to activate the AmpErase UNG enzyme, which removes potential contamination from previous TaqMan PCR reactions, and to activate the AmpliTaq Gold DNA polymerase present in the TaqMan Universal PCR Master Mix. The levels of fluorescence in each well were monitored continuously throughout 40 cycles of amplification. Data were analysed using sequence detection systems software (PE Applied Biosystems) and displayed as an amplification plot showing change in fluorescence relative to an internal standard reference (LogRn) versus cycle number. A threshold value was placed in the exponential phase of the amplification plot, where the reagents were not rate limiting and levels of fluorescence were increasing linearly. The cycle number during which fluorescence first exceeded this threshold (C_T) was calculated for each sample. Data were analyzed using the relative quantification technique (PE Applied Biosystems 1998), which provided a means for comparing the amount of mRNA in each sample group with the amount in the same brain region from an arbitrary reference group without needing to determine the precise amount of RNA present in each sample. This involved first correcting each sample for levels of β-actin mRNA in that sample by calculating ΔC_T (C_T for PRL-R mRNA minus C_T for β-actin mRNA in the same sample). The mean level of expression from each time-point was then compared with the reference group (in this case, dioestrus) using the formula:

\[
\text{relative quantification} = 2^{-\Delta C_T} \text{ (the average dioestrus } \Delta C_T \text{ minus the average experimental group } \Delta C_T \text{ values).}
\]

The exponential nature of the amplification means that a ΔC_T of 1 equates to a twofold difference in starting concentration of cDNA. Hence, the above formula converts ΔC_T into a linear fold-difference number. For each brain region, statistical analysis between experimental groups was carried out on the original ΔC_T data using the non-parametric Kruskal–Wallis test. If a significant H-statistic was detected, the Mann–Whitney U test was used to compare each group with dioestrus. The significance level was set at P<0·05. Data are presented as means ± s.e.m. Error bars are uneven, as they represent exponential variation plotted on a linear scale.

**Immunohistochemistry**

Frozen coronal sections (35 µm) were cut through each perfused brain in a cryostat at −19 °C.

### Table 2 Oligonucleotides used for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (rat PRL-R mRNA)</th>
<th>Nucleotide number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense (common)</td>
<td>5′-CTG-GGC-AGT-GGC-TTT-GAA-G-3′</td>
<td>940–958</td>
</tr>
<tr>
<td>Antisense (long form)</td>
<td>5′-C-CAG-GCC-ART-CAG-GAG-CTC-T-3′</td>
<td>1070–1051</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5′-AGA-TGA-CCC-AGA-TCA-TGT-TTG-AGA-3′</td>
<td>152–175</td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-ACC-AGA-GGC-ATA-CAG-GGA-CAA-3′</td>
<td>237–217</td>
</tr>
<tr>
<td>TaqMan probe</td>
<td>VIC-TCA-AAC-CCC-CAG-CCA-TCT-ACG-TAG-CC-TAMRA</td>
<td>179–204</td>
</tr>
</tbody>
</table>

[www.endocrinology.org](http://www.endocrinology.org)
Sections were stored at $-20^\circ$C in 24-well cell culture plates containing cryoprotectant until further use. Sections through the lateral ventricle (bregma $-0.1$ mm to bregma $-0.7$ mm) were selected from each experimental group and processed for immunofluorescence in a single run. To remove the cryoprotectant, the sections were washed six times (5 min each time) in phosphate-buffered saline (PBS), pH 7.3. They were then treated twice (5 min each time) with PBS containing 1.5% glycine to remove residual aldehydes from fixation, followed by one wash with PBS containing 0.3% Triton X-100 (PBS-T). Non-specific binding was blocked by incubating sections in PBS containing 10% normal horse serum (NHS) and 1% bovine serum albumin overnight at 4°C. Sections were then incubated with an anti-PRL-R primary antibody (MA1–610; Affinity Bioreagents, Inc., Golden, CO, USA) diluted 1:4000 in PBS-T containing 1% NHS for 48 h at 4°C. This antibody is directed against the extracellular domain of the PRL-R, and hence can detect both isoforms of the receptor. This step was followed by incubation in secondary antibody, fluorescein horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in PBS containing 1% NHS for 3 h at room temperature. Sections were washed twice in PBS-T and once in PBS and mounted onto gelatin-coated slides and allowed to dry in a light-proof box. Once dry, sections were coverslipped using Vectashield mounting medium (Vector Laboratories).

PRL-R immunofluorescence was viewed under a confocal laser scanning microscope (LSM 510; Zeiss, Auckland, NZ). Fluorescein-labelled sections were viewed using pre-configured fluorescein isothiocyanate and rhodamine filters, and all sections were viewed with identical settings (pinhole diaphragm 90 µm, detector gain 902). The laser scanned the frame in one direction and the same scan speed was used to capture all images. Images were collected at 512 × 512 pixels, and then processed with Adobe Photoshop 5.5 using identical settings for each image.

**Results**

Standard dilutions (twofold) of stock cDNA were run on each plate as external standards. Plotting $C_T$ (threshold cycle, where levels of fluorescence cross the threshold line) against the dilution series generates a linear standard curve ($r^2=0.996$), demonstrating the quantitative nature of the assay. Two-fold dilutions were detected one cycle apart as predicted by the exponential nature of PCR amplification and were clearly distinguishable, with tight replicates (coefficient of variation <2%), demonstrating the precision of the method over a wide range of starting cDNA concentrations.

mRNA for both isoforms of the PRL-R were found in the choroid plexus (Fig. 1). There was a marked up-regulation of mRNA for the long-form PRL-R in the choroid plexus (2.2-fold) by day 7 of pregnancy, lactation and post-weaning. Data show fold differences, relative to dioestrus. Long-form PRL-R mRNA expression was significantly higher (2.2-, 2.6- and 3.4-fold) on days (D) 7, 14 and 21 of pregnancy respectively (*$P<0.05$) relative to dioestrus, and 5.1-fold higher than dioestrous levels at day 7 of lactation (*$P<0.05$). On day 7 after weaning there was no significant difference compared with dioestrus. Significant increases in short-form PRL-R mRNA were seen on day 14 of pregnancy (2.5-fold) (*$P<0.05$) and day 7 of lactation (3.8-fold) (*$P<0.05$) relative to dioestrus (*$P<0.05$).

![Figure 1](https://www.endocrinology.org/)

**Figure 1** Mean ($\pm$S.E.M.) levels of long- and short-form PRL-R mRNA expression (normalized to $\beta$-actin) levels in the choroid plexus during pregnancy, lactation and post-weaning. Data show fold differences, relative to dioestrus. Long-form PRL-R mRNA expression was significantly higher (2.2-, 2.6- and 3.4-fold) on days (D) 7, 14 and 21 of pregnancy respectively (*$P<0.05$) relative to dioestrus, and 5.1-fold higher than dioestrous levels at day 7 of lactation (*$P<0.05$). On day 7 after weaning there was no significant difference compared with dioestrus. Significant increases in short-form PRL-R mRNA were seen on day 14 of pregnancy (2.5-fold) (*$P<0.05$) and day 7 of lactation (3.8-fold) relative to dioestrus (*$P<0.05$).
pregnancy relative to dioestrus \((P < 0.05)\). Levels of long-form PRL-R mRNA increased further on days 14 and 21 of gestation and into lactation such that by day 7 of lactation mRNA expression was 5.1-fold higher than observed on dioestrus \((P < 0.05)\). By day 7 post-weaning, levels of mRNA for the long form were not significantly different from dioestrus. Levels of short-form PRL-R mRNA were significantly higher (2.5-fold) on day 14 of gestation compared with dioestrus \((P < 0.05)\). On day 7 of lactation, levels of short-form PRL-R mRNA were 3.8-fold higher than that observed on dioestrus \((P < 0.05)\). On day 7 post-weaning, short-form PRL-R mRNA levels were not significantly different from dioestrus.

PRL-R immunofluorescence was detected in the choroid plexus in all experimental groups, and the relative intensity of staining observed suggested that PRL-R protein expression changes in a similar manner to that observed for mRNA. Figure 2 shows representative images of PRL-R immunofluorescence in the choroid plexus. PRL-R immunostaining was clearly detected in the dioestrous rat (Fig. 2A). Figure 2B shows the same dioestrous section as Fig. 2A, observed at a lower intensity level when specific immunofluorescence appears only slightly above background. Figure 2C–F shows immunofluorescence at different times through pregnancy and lactation, all collected at the same intensity setting as Fig. 2B. By day 7 of pregnancy (Fig. 2C) there was a notable increase in PRL-R immunofluorescence in the choroid plexus compared with dioestrus (Fig. 2B) and this marked increase in PRL-R immunofluorescence was maintained throughout pregnancy reaching maximal staining at day 21 of pregnancy (Fig. 2E). On day 7 of lactation (Fig. 2F) the immunostaining was similar to that seen on day 21 of pregnancy.

In the arcuate nucleus, there was an up-regulation of mRNA expression for the long-form
PRL-R during pregnancy (Fig. 3), reaching significance (twofold) on day 21 of pregnancy compared with dioestrus ($P < 0.05$). By day 7 of lactation, PRL-R mRNA levels had decreased and were not significantly different from dioestrus. However, on day 7 post-weaning mRNA levels had increased to be 2.4-fold higher than dioestrous levels ($P < 0.05$). The short-form PRL-R mRNA was readily detectable in the arcuate nucleus, but levels did not change significantly during pregnancy or lactation.

Only the long-form PRL-R mRNA was consistently detected in the other hypothalamic nuclei examined: the medial preoptic, paraventricular and ventromedial hypothalamic nuclei (Fig. 4). The paraventricular and ventromedial hypothalamic nuclei showed a similar pattern of mRNA expression. Compared with dioestrus, levels of PRL-R mRNA appeared to be elevated on days 7 and 14 of pregnancy, and during lactation. Due to

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**Figure 3** Data represent mean (±S.E.M.) levels of long- and short-form PRL-R mRNA expression normalized to β-actin levels in the arcuate nucleus during pregnancy, lactation and post-weaning. Data show fold differences, relative to dioestrus. Long-form PRL-R mRNA expression increased in the arcuate nucleus during pregnancy reaching a significantly higher expression (twofold) on day (D) 21 of pregnancy and 2.4-fold higher expression on day 7 post-weaning relative to dioestrus ($^*P < 0.05$). Short-form PRL-R mRNA expression was not significantly different at any time-point relative to dioestrus (Kruskal–Wallis test).

**Figure 4** Data represent mean (±S.E.M.) long-form PRL-R mRNA expression in the medial preoptic (MPO), paraventricular (Pa) and ventromedial hypothalamic (VHM) nuclei during pregnancy, lactation and post-weaning. Data show fold differences, relative to dioestrus. No significant differences were seen in these areas at the time-points examined (Kruskal–Wallis test).
relatively high variation between samples, however, these differences were not statistically significant. Levels of PRL-R mRNA in the medial preoptic nuclei did not change markedly at any of the time-points examined.

Short-form PRL-R mRNA expression was also investigated in the medial preoptic, paraventricular and ventromedial hypothalamic nuclei (results not shown). Expression was low and often significant fluorescence did not appear until later than 35 cycles of PCR, at which stage it is often possible to see non-specific amplification. Hence, we have regarded the short form as largely undetectable in these regions, using the present techniques, suggesting that only very low levels of this isoform of the PRL-R exist in the hypothalamus, outside of the arcuate nucleus.

Discussion

Real-time PCR is a sensitive and highly reproducible method that allowed us to use low amounts of starting copy DNA and amplify it to detectable levels in a quantitative manner (Bustin 2000). Using this method, it has been possible to investigate the time-course of changes in expression of both the short and long forms of PRL-R mRNA in the choroid plexus and discrete hypothalamic nuclei of the female rat during pregnancy and lactation.

The results demonstrated a marked up-regulation of both the short- and long-form PRL-R in the choroid plexus during pregnancy compared with dioestrus. PRL-R mRNA levels were highest on day 7 of lactation but, following removal of the pups, levels of PRL-R mRNA were similar to those seen in the non-pregnant state. These data extend our previous observations of increased PRL-R mRNA in the choroid plexus during lactation (Pi & Grattan 1999b,c). One previous study that has examined an extensive time-course of PRL-R mRNA expression during pregnancy found large changes in the long form, but not the short form, in the whole brain (Sugiyama et al. 1994). These authors later attributed these changes largely to the choroid plexus (Sugiyama et al. 1996). Our data, however, clearly suggest that both forms are changing in parallel in the choroid plexus. The relative functions of the two forms of the receptor in the choroid plexus are not known. Prolactin binding to the long-form PRL-R activates a number of intracellular signal transduction pathways, most notably affecting gene transcription via the JAK/STAT pathway (Bole-Feyso et al. 1998). The role of the short-form PRL-R, however, has remained somewhat elusive. It cannot activate the JAK/STAT pathway, but can exert mitogenic actions via the mitogen-activated protein kinase pathway (Bole-Feyso et al. 1998). It has been suggested that, in the choroid plexus, the short form of the receptor may have a role in the transport of prolactin into the brain (Kelly et al. 1991). Prolactin of peripheral origin has been detected in the CSF of rats, with levels changing in accordance with changes in the peripheral circulation (Login & MacLeod 1977). Entry for prolactin into the CSF is a saturable carrier-mediated process, thought to involve prolactin-binding sites in the choroid plexus (Walsh et al. 1987). The PRL-R may act as an uptake mechanism that binds prolactin and transports it across the epithelial cells of the choroid plexus and into the CSF where it can diffuse to various areas in the brain (Nilsson et al. 1992). It is not clear which isoform is required for transport into the CSF, but it seems possible that the short-form PRL-R may be involved. In an analogous system, there is evidence that the short form of the leptin receptor may function as a transporter to move leptin into the CSF (Peiser et al. 2000). The long form of the PRL-R may have some other role in the choroid plexus, perhaps regulating gene expression via the activation of the JAK/STAT pathway. Whatever the specific functions of the PRL-R in the choroid plexus, our evidence suggests a marked up-regulation in this function during pregnancy and lactation, and this is likely to result in increased access of prolactin to the brain.

Interestingly, the timing of changes in PRL-R mRNA levels coincides with known changes in prolactin levels in the blood. In early pregnancy, twice daily prolactin surges are present resulting from the mating stimulus (Erskine 1995). By day 14 of pregnancy, pituitary prolactin is low, but elevated placental lactogen production by the placenta provides a more continuous presence of hormones capable of activating the PRL-R (Lee & Voogt 1999). During lactation, high prolactin levels are sustained predominantly due to the suckling stimulus (Freeman et al. 2000). Based on these observations, it seems possible that the pattern of constantly high prolactin stimulates expression of...
its own receptor, at least in the choroid plexus. This hypothesis is supported by a number of studies that show that exogenously administered prolactin can upregulate PRL-R in the brain (Mangurian et al. 1992, Muccioli & Di Carlo 1994, Sugiyama et al. 1994). Importantly, PRL-R mRNA levels on day 7 post-weaning were not significantly different from those seen on dioestrus. This may be due to a removal of the stimulatory effects of hyperprolactinaemia, or due to loss of the suckling stimulus, which also appears to regulate PRL-R expression (Pi & Voogt 2001).

In the arcuate nucleus, an up-regulation of the long isoform of PRL-R mRNA relative to dioestrous levels was observed that was initiated during pregnancy but not maintained into lactation. The predominant neuronal type expressing PRL-R in the arcuate nucleus in non-pregnant animals are TIDA neurones (Lerant & Freeman 1998, Grattan 2001, Grattan et al. 2001). Previous data using immunohistochemistry showed a significant two- to threefold increase in number of neurones expressing PRL-R immunoreactivity in the arcuate nucleus in lactating rats compared with dioestrous rats (Pi & Grattan 1999a). Since numbers of TIDA neurones do not increase during lactation (Wang et al. 1993), this suggests that a new population(s) of arcuate neurones are recruited to express the PRL-R at this time. Such a change, however, was not reflected in overall changes in long-form PRL-R mRNA expression in the arcuate nucleus during lactation, raising the possibility that protein and mRNA are differentially regulated. It is also possible that PRL-R mRNA levels are increased in some neurones, but decreased in others. For example, prolactin receptor expression on TIDA neurones may be suppressed during late pregnancy and lactation. The observed increase in the long-form PRL-R mRNA in the arcuate nucleus during mid- to late pregnancy occurs at a time when elevated placental lactogen is maintaining maximal activation of the TIDA neurones (Lee & Voogt 1999). This feedback system becomes insensitive to prolactin and placental lactogen during late pregnancy, however, allowing a large prolactin surge to occur during the night preceding parturition (Grattan & Averill 1995, Fliestra & Voogt 1997, Andrews et al. 2001). The suppression of negative feedback continues into lactation (Demarest et al. 1983, Arbogast & Voogt 1996), an important adaptation allowing a prolonged state of hyperprolactinaemia. We have shown that prolactin stimulation of TIDA neurones involves the transcription factor STAT5b (Grattan et al. 2001, Lerant et al. 2001) and it is known that activation of gene transcription requires the long-form PRL-R (Lesueur et al. 1991). Hence, it is tempting to speculate that the reduction in mRNA for the long-form PRL-R within the arcuate nucleus during lactation might contribute to the loss of responsiveness of TIDA neurones to prolactin. Alternatively, since the PRL-R antibodies used in previous research (Pi & Grattan 1999a, Pi & Voogt 2000) do not distinguish between the two isoforms, it is possible that the observed increase in PRL-R immunoreactivity represents expression of the short form of the receptor. Levels of the short-form PRL-R mRNA remain relatively high in the arcuate nucleus during late pregnancy and lactation. It is possible that an increasing ratio of short- to long-form PRL-R may contribute to the impaired prolactin signalling in these neurones at this time. The short form of the receptor may act as a competitive inhibitor by forming inactive heterodimer receptor complexes with the long form (Perrot-Applanat et al. 1997). Such an effect might contribute to the loss of sensitivity of TIDA neurones to prolactin during late pregnancy and lactation.

On day 7 after weaning, levels of long-form PRL-R mRNA were significantly higher than dioestrous levels. The function of this change remains to be determined, but it may reflect the reactivation of negative feedback regulation of TIDA neurones by prolactin during a physiological time when high prolactin levels are no longer required.

Results from all other hypothalamic regions investigated showed no significant changes in mRNA expression for either the short- or long-form PRL-R at any of the time-points examined. Given our previous data showing changes in PRL-R mRNA expression in specific parts of the hypothalamus (Pi & Grattan 1999b,c), this was particularly surprising. Hence, we have independently repeated the major comparisons using samples from separate groups of rats, and obtained similar results (data not shown). Previously, in the paraventricular and ventromedial hypothalamic nuclei, two- to threefold increases in long-form PRL-R mRNA were observed during lactation (Pi & Grattan 1999b,c). In the present
study, approximately twofold increases in long-form mRNA were observed, but this change was not statistically significant due to inter-sample variation. Consistent with the present data, Bakowska & Morrell (1997) found no changes in expression of long-form PRL-R mRNA during pregnancy in the ventromedial hypothalamic nuclei. Hence, it seems that changes in mRNA expression are not as pronounced as suggested by our earlier data. Protein expression of the PRL-R is increased during lactation in the paraventricular and ventromedial hypothalamic nuclei (Pi & Grattan 1999a), and there is evidence that the changes in receptor expression are biologically significant. The ventromedial hypothalamus has been implicated in increased feeding intake in hyperprolactinaemic and lactating rats (Gerardo-Gettens et al. 1989), suggesting that prolactin may have a role in feeding behaviour during lactation. In the paraventricular and supraoptic nuclei, prolactin may be involved in inducing oxytocin gene expression (Ghosh & Sladek 1995) and oxytocin secretion (Sarkar 1989) during lactation.

The absence of any change in expression in the medial preoptic nuclei is consistent with our previous observations using the micropunch technique (Pi & Grattan 1999c), although changes in anatomically close regions were previously reported (Pi & Grattan 1999c). Other studies, using in situ hybridization, have reported subtle changes in levels of long-form PRL-R mRNA (Bakowska & Morrell 1997, Mann & Bridges 2002), but these may well be specific to certain subregions of the nucleus or fluctuate over a relatively short time-course. For example, Mann & Bridges (2002) found the numbers of cells expressing PRL-R mRNA in the medial preoptic nuclei were significantly increased 2 h postpartum, but then declined back to pre-pregnancy levels throughout the remainder of lactation. The medial preoptic nucleus has been well characterized as a site of prolactin action to induce maternal behaviour in rats (Bridges 1994, Bridges et al. 1997). During pregnancy, the lactogenic hormones are thought to prime the brain for maternal behaviour, so that at parturition there is an immediate onset of maternal responsiveness (Bridges et al. 1996). Hence, while PRL-R mRNA levels in the medial preoptic nuclei are not changing dramatically, receptor expression in this region has important functional significance.

In addition to changes in the long form of the PRL-R, in our previous studies we reported significant increases in short-form PRL-R mRNA expression in the paraventricular and ventromedial hypothalamic nuclei of lactating rats compared with dioestrous rats (Pi & Grattan 1999b). This was based on finding detectable levels of mRNA in lactating rats compared with almost undetectable levels during dioestrous. In the present study, mRNA for the short form of the PRL-R was detected in these brain regions, but at very low levels, and expression levels did not alter significantly during pregnancy and lactation. The present results are consistent with earlier findings from Sugiyama et al. (1994) in which short-form PRL-R mRNA was evident in the brain of pregnant and lactating rats but did not change from basal levels. The discrepancy between our previous studies and the present results may be explained by the different experimental procedures employed. The previous studies used conventional RT-PCR, which measured PCR product at the end of a set number of cycles of amplification, with high numbers of cycles required to produce a detectable signal (Pi & Grattan 1998b, 1999b). End-point measurements can vary in the amount of cDNA detected between duplicates of the same sample. The present real-time method monitored PCR product after each cycle of amplification, relative to an internal standard, providing a clearer and more repeatable measure of the relative amounts of cDNA. Hence, we consider this to be an improved methodology, and the present results do not support the earlier reports suggesting increased short form expression in a number of hypothalamic nuclei during lactation.

In summary, the results from the present study showed marked up-regulation of both long and short forms of PRL-R mRNA in the choroid plexus in pregnant and lactating rats, compared with dioestrous. Protein expression in the choroid plexus, using an antibody that detects both forms of the PRL-R, follows the same pattern of expression as mRNA. It seems likely that the high levels of expression of PRL-R in this region will play a role in transporting prolactin from the peripheral circulation into the CNS. A significant up-regulation of mRNA for both forms of the receptor was also evident in the arcuate nucleus during pregnancy relative to dioestrous levels. Expression of both forms of the PRL-R was observed in other
hypothalamic nuclei but, in contrast to earlier work using conventional RT-PCR, no significant changes were seen across pregnancy and lactation. PRL-Rs are expressed on neurones in numerous hypothalamic nuclei that have been implicated in behavioural and neuroendocrine adaptations seen during pregnancy and lactation (Grattan 2001). This relatively widespread expression of PRL-Rs, coupled with elevated levels of prolactin in the blood and the potential for markedly increased transport into the brain during pregnancy and lactation, are consistent with the hypothesis that prolactin has a role in influencing neuronal function in the maternal brain.

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