Expression of the long form of the prolactin receptor in magnocellular oxytocin neurons is associated with specific prolactin regulation of oxytocin neurons

I.C. Kokay¹, P.M. Bull², R.L. Davis¹, M. Ludwig², D.R. Grattan¹

¹Centre for Neuroendocrinology and Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand
²Centre for Integrative Physiology, Edinburgh University, Edinburgh, UK

Corresponding Author:

Dr David Grattan
Department of Anatomy and Structural Biology
University of Otago
P.O. Box 913
Dunedin, New Zealand.

phone:+64 3 4797442
fax: +64 3 4797254
email: dave.grattan@anatomy.otago.ac.nz

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ABSTRACT

Magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei show considerable plasticity during pregnancy and lactation. Prolactin receptors (PRL-R) have been identified in both these nuclei. The aim of this study was to investigate the cell type(s) expressing mRNA for the long form of prolactin receptor (PRL-R_L), and to determine whether patterns of expression change during pregnancy and lactation. In addition, we examined effects of prolactin on excitability of oxytocin and vasopressin neurons. Sections from brains of non-pregnant, pregnant and lactating rats were hybridized with an 35S-labeled probe to label PRL-R_L mRNA together with digoxigenin-labeled probes to detect either oxytocin or vasopressin mRNA. In the SON, PRL-R_L mRNA was predominantly co-localised with oxytocin mRNA, with over 80% of oxytocin neurons positive for PRL-R_L mRNA. Very few (<10%) vasopressin neurons expressed PRL-R_L mRNA. In the PVN, PRL-R_L mRNA was also predominantly found in oxytocin neurons, and the proportion of PRL-R_L-positive oxytocin neurons increased significantly during pregnancy and lactation. As in the SON, relatively few vasopressin cells contained PRL-R_L mRNA. For in vivo electrophysiology, non-pregnant rats were anesthetized, then extracellular single neuron activity recorded in identified oxytocin and vasopressin neurons. Following a period of baseline recording, the effect of prolactin (1 µg i.c.v.) on firing rate was examined. Prolactin treatment of non-pregnant rats induced a significant decrease in firing rates of oxytocin neurons. There was no effect of prolactin on the activity of vasopressin neurons. Together, these data provide strong evidence that prolactin directly and specifically regulates activity of oxytocin neurons.

Key words: oxytocin, pregnancy, lactation, magnocellular neurons, prolactin receptor
INTRODUCTION

In addition to its critical actions in mammary gland function during pregnancy and lactation, the anterior pituitary hormone prolactin exerts important actions within the brain. Prolactin is thought to gain access to the brain through a carrier-mediated transport system (74), likely involving prolactin receptors in the choroid plexus (51, 64). Prolactin receptor (PRL-R) mRNA (3, 4, 11, 45) and protein (13, 46) have been identified in many hypothalamic nuclei. Interestingly, PRL-R expression in the choroid plexus is markedly increased during pregnancy and lactation (2), suggesting increased access of prolactin to brain structures during these conditions. Similarly, levels of PRL-R protein in the hypothalamus appear to increase during lactation compared with non-pregnant rats (47-49). These observations suggest that prolactin may be a major regulator of hypothalamic function, particularly during pregnancy and lactation, when prolactin levels are elevated (22, 23).

Within the hypothalamus, the neurons that undergo one of the most dramatic changes during pregnancy and lactation are the magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei (26). PRL-R mRNA has been identified in both of these nuclei (3, 4, 11, 45), while PRL-R protein is observed in the SON in non-pregnant animals (46), and in both the SON and PVN during lactation (47). Intracerebroventricular (i.c.v.) administration of prolactin induces activation of cFos in the SON (8). Prolactin has been shown to specifically increase oxytocin mRNA expression (20, 21, 50) and to stimulate oxytocin secretion from hypothalamic explants.
(43) or into portal blood, in vivo (62). By contrast, hyperprolactinaemia can also inhibit
the stress-induced release of oxytocin (7). While most research suggests that prolactin
influences oxytocin neurons, prolactin and a 16 kDa fragment were recently reported to
increase vasopressin secretion from hypothalamic explants, although it is possible that
this action was not mediated through the PRL-R (39). Interestingly, the magnocellular
nuclei have also been reported to produce prolactin (12, 38, 67), and levels of prolactin
synthesis in the hypothalamus may increase during lactation (69). Hence, oxytocin
and/or vasopressin neurons may be regulated by prolactin released locally or by prolactin
entering the brain from the systemic circulation.

Although the majority of studies that have examined functional effects of prolactin
on magnocellular neurons report preferential actions on oxytocin neurons, preliminary
evidence using immunohistochemistry suggests that the PRL-R might be expressed on
both oxytocin and vasopressin neurons (24, 39). The PRL-R gene can be alternatively
transcribed into a long or short isoform, however, and the above immunohistochemistry
studies did not distinguish between them. Both isoforms of the receptor have been
identified in the SON and PVN (3, 4, 45), yet they have different signaling capacities and
this may underlie different functional effects of prolactin on oxytocin and vasopressin
neurons. Only the long form retains full signal transduction capability through the
JAK/STAT pathway (5). Hence, in the present study, we have used dual label in situ
hybridization to identify whether long form PRL-R mRNA is expressed on oxytocin and
vasopressin neurons in the SON and PVN. In addition, we have examined whether there
are changes in the patterns of expression during pregnancy and lactation. Finally, to
determine whether observed patterns of receptor expression correlate with functional activation of the magnocellular neurons by prolactin, we have examined acute electrophysiological responses of oxytocin and vasopressin neurons to prolactin in an in vivo preparation.

**METHODS**

**Animals**

_Neuroanatomical studies._ Adult, female, Sprague-Dawley rats weighing 220 - 300 g were purchased from the Taieri Resource Unit, University of Otago. All animals were housed under a 14:10 (L:D) light cycle with unrestricted food and water. Vaginal smears were taken each morning to assess the reproductive cycle stage and rats exhibiting proestrus were mated with a single male overnight. The presence of sperm in the vaginal smear the following morning indicated a successful mating and was designated as day 0 of pregnancy. Pregnant rats (n = 5) were killed on day 21 of pregnancy (parturition occurs on the morning of day 22 in our colony). For the lactating rats (n = 4), litters were normalized to 10 pups on day 2 of lactation and mothers sacrificed on day 7 of lactation. A non-pregnant control group (n = 5) were rats exhibiting diestrus following at least two normal estrous cycles. Animals were housed in individual cages from approximately day 18 of pregnancy and while lactating. All procedures were approved by the University of Otago animal ethics committee.

_Electrophysiological studies._ Adult, female Sprague-Dawley rats weighing 235 - 413 g were obtained from Bantin and Kingman, UK and group-housed at the University of Edinburgh in controlled temperature conditions under a 12:12 (L:D) light cycle with
continuous access to food and water. All protocols were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines.

**Double-label in situ hybridization**

_Tissue preparation._ Following deep sodium pentobarbitone anesthesia (60 mg/kg for diestrous rats; 120 mg/kg for pregnant and lactating rats), animals were transcardially perfused with 50 ml of ice-cold saline followed by 250 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed, post-fixed for 1 hr then infiltrated with 30% sucrose till the brains sank. Brains were then frozen on powdered dry ice and stored at -80 °C till processed. Sets of coronal sections (18 µm), through the SON and PVN regions of the hypothalamus (bregma - 0.80 - 2.12 mm)(44) were cut in a cryostat at -22 °C and mounted onto RNase-free aminopropyltriethoxy-silane-coated glass microscope slides.

_RNA probe preparation._ Forward and reverse primers were designed from Genbank mRNA sequences specific for the long form of the PRL-R (accession number M57668), from exon 3 of the rat arginine vasopressin-neurophysin precursor gene sequence (accession number NM 016992.1) and from rat oxytocin gene specific sequence (accession number M25649). T7 or SP6 promoter sequences were incorporated on the ends of the primer nucleotide sequences and the PCR products were generated from rat hypothalamic total RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA templates were then transcribed directly to make either sense or antisense RNA probes. A 301-bp RNA probe labeled with 35 S-UTP that targeted the mRNA for the PRL-R long isoform was prepared using a RiboProbe® _in vitro_ transcription kit
(Promega) with T7 RNA polymerase. Sense and antisense digoxigenin-11-UTP-labelled RNA probes directed against vasopressin mRNA (170-bp probes) and oxytocin mRNA (66-bp probes) were transcribed using the appropriate T7 or SP6 polymerases following the protocol outlined in the Roche Applied Science DIG application manual. All probes were purified using Mini Quick spin columns (Roche Applied Science) and run on agarose gels to confirm the integrity and size of the transcripts.

Hybridization protocol. To simultaneously detect PRL-R_L mRNA and oxytocin or vasopressin mRNA in the same tissue sections, double-label in situ hybridizations were performed using a previously described protocol (28). Brain sections were fixed for 5 min in 2 % paraformaldehyde at 4 °C, washed in 0.5 X SSC (75 mM NaCl, 7.5 mM sodium citrate) subjected to proteinase K (2 µg/ml) digestion and acetylated with 0.25 % acetic anhydride. Each section was covered with 100 µl of hybridization buffer (100 mM DDT, 0.3 M NaCl, 20 mM Tris pH 8, 5 mM EDTA, 1 X Denhardt’s solution, 10 % dextran sulphate, 50 % formamide) and pre-hybridized for 3 hrs at 42 °C. Probes were denatured at 95 °C for 3 min, then a mixture of isotopic and non-isotopic probes (1.2 x 10^6 cpm/section of ^35^S-labelled PRL-R probe and approximately 5 ng/section of oxytocin or alternately 25 ng/section of vasopressin digoxigenin-labeled probe) in 20 µl of hybridization buffer was pipetted onto the sections. Hybridizations were carried out overnight at 55 °C then slides were washed (most stringent wash, 0.1 X SSC at 55 °C for 2 hrs) and treated with Ribonuclease A (20 µg/L) for 30 min at room temperature. Following a 48 hr incubation, with anti-digoxigenin antibody conjugated to alkaline phosphatase (diluted 1:2000), sections were washed 3 times, then subjected to a levamisole (1 mg/ml) blocking step. The digoxigenin-labeled probes were detected by
incubation with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4 chloro-3-indolyl-phosphate) substrate. Color development of the visible product was periodically monitored under the microscope and the reaction stopped after 2 - 2.5 hr by 4 consecutive 30 min washes in buffer (150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA) to eliminate residual NBT and BCIP. Sections were then dipped briefly in distilled water followed by 70 % ethanol and dried. A selection of slides were exposed to scientific imaging film (Kodak BioMax MR) for 3 -4 days to generate autoradiograms. All slides were then coated with LM-1 Hypercoat emulsion (Amersham Biosciences), placed in light-proof slide boxes containing desiccant and stored at 4 ºC for 5 weeks. Slides were developed in Kodak D19, fixed with Ilford Hypan, then dehydrated through graded ethanols and dried at 42 ºC for 1 hr. Finally, sections were cleared in xylene and coverslipped with VectaMount™ mounting medium.

To confirm the specificity of the oxytocin and vasopressin probes, a selection of tissue sections were hybridized with sense RNA probes whose transcripts were identical to specific oxytocin or vasopressin cellular mRNA sequences. The specificity of the ³⁵S-labelled PRL-R₇ probe has been confirmed previously (28). In addition, well characterized expression of PRL-R₇ mRNA in the epithelial cells of the choroid plexus (2), was used as a positive control.

In situ hybridization data analysis. Sections were photographed and analyzed under brightfield illumination using an Olympus BX51 microscope equipped with a Spot RT digital camera (Diagnostic Instruments). Digoxigenin-labeled oxytocin and vasopressin
cells were identified by the presence of a blue/violet precipitate within the cytoplasm of the cell. Clusters of silver grains in the emulsion layer over neuronal cell bodies in the PVN and SON identified PRL-R mRNA. For both nuclei (PVN and SON), the total number of DIG-labeled oxytocin and vasopressin mRNA-positive cells present on one side of the brain were counted in 3-4 sections from each rat under 40 X objective using the unprocessed images. Only those cells in which a nucleus was visible in the plane of focus were counted. Captured images were then converted in Adobe Photoshop® and a threshold set such that exposed silver grains were detected whereas digoxigenin labeling did not contribute to the detected signal. NIH image was then used for analysis of silver grain distribution. A template was manually drawn around each individual cell and positively-labeled PRL-R mRNA was defined as signal greater than 3 X background values. The total number of cells expressing PRL-R mRNA were identified and the percentage co-localization of PRL-R mRNA on oxytocin and vasopressin mRNA-expressing cells was calculated using the original unprocessed image as a reference.

Statistical analysis. Results were analyzed using analysis of variance followed by post hoc analysis using Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant at p < 0.05. Data are presented as means ± SE.

Electrophysiology

Surgical procedures and cannula placement. Rats were anesthetized with urethane (ethyl carbamate; 1.25g/kg, i.p.) and the right femoral vein and trachea cannulated. For i.c.v. hormone administration, a 22 gauge stainless steel guide cannula (Bilaney Consultants Ltd, Kent, UK) was stereotaxically implanted into the right lateral cerebral
ventricle (0.6 mm posterior to bregma, 1.6 mm lateral, 4 mm below the skull surface) and secured with two small screws fixed in the skull and dental cement. The pituitary stalk and right SON was then exposed transpharyngeally. A glass micropipette filled with saline (20-40 MΩ) was introduced into the SON to record extracellular single neuron activity via a CED 1401 interface attached to a PC running Spike 2 software (Cambridge Electronic Design, Cambridge, UK). A bipolar stimulating electrode (Snex-200X, Harvard Apparatus, UK) was placed on the pituitary stalk and set to deliver single matched biphasic pulses (1 msec, < 1 mA peak to peak) for antidromic identification of SON neurons. Collision of antidromic action potentials triggered by spontaneous orthodromic action potentials was subsequently used to confirm identification. Oxytocin neurons were distinguished from vasopressin neurons by their continuous firing pattern and by a transient excitation in response to cholecystokinin (20 µg/kg, i.v.) (57).

Following a period of stable basal recording, prolactin (L6520, Sigma, UK) dissolved in artificial cerebrospinal fluid (pH 7.2, 138 mM NaCl, 3.36 mM KCl, 9.52 mM NaHCO₃, 0.49 mM Na₂HPO₄, 1.26 mM CaCl₂, 1.18 mM MgCl₂ and 2.16 mM urea) was administered via the i.c.v. cannula (1-10 µg in a volume of 2.5 µl). For quantitative analysis, all comparisons were made at the lowest doses of prolactin used (1 µg per injection).

Statistical analysis. The effect of seventeen 1 µg prolactin injections were analysed in eleven rats. The mean firing rate of identified oxytocin and vasopressin cells was calculated for 5 min periods immediately before, 0-5, 5-10 and 10-15 minutes after
each prolactin injection. Results were analyzed using a one-way repeated measures analysis of variance to isolate differences within groups. Where the F ratio was significant this was followed by post hoc analysis with Dunnett's Method using SigmaStat® software (SPSS Science, Chicago, USA) and differences were considered statistically significant at $p \leq 0.05$. Data are presented as means ± SE.

RESULTS

Analysis of autoradiograms showed that the most intense labeling of PRL-R$_L$ mRNA in rat brain sections was present over the cells of the choroid plexus. In addition, in all experimental groups, there was moderate expression of PRL-R$_L$ mRNA within the hypothalamus, with discrete localizations over the PVN and the SON (Fig. 1A). At this level of the brain, light PRL-R$_L$ mRNA expression also was evident in additional hypothalamic nuclei including the periventricular nucleus and the anterior hypothalamus.

Oxytocin and vasopressin-secreting neurons were identified by visualization of anti-digoxigenin antibody conjugates. The specificities of the hybridized oxytocin and vasopressin probes were confirmed by the excellent match of the digoxigenin-labeled oxytocin and vasopressin mRNA neuronal distributions (Fig. 1B) with the known anatomical localization of the corresponding proteins that has been previously determined by immunohistochemical studies. There appeared to be negligible cross-hybridization between the two digoxigenin-labeled probes. In addition, sections used in control experiments hybridized with sense RNA probes to sequences unique to oxytocin or vasopressin resulted in the complete absence of hybridization signal.
Figure 2 illustrates results following double-label in situ hybridization using an antisense $^{35}$S-labelled PRL-RL probe together with RNA probes specific for either oxytocin mRNA (left panels) or vasopressin mRNA (right panels), in the PVN from sections at approximately the same anatomical level. Double-labeled cells co-expressing PRL-RL mRNA and oxytocin or vasopressin mRNA are revealed as neuronal cell bodies containing a blue/purple precipitate within the cytoplasm of the cell, with clusters of reduced silver grains in the layer of autoradiographic emulsion overlying the tissue. In all three groups, PRL-RL mRNA in the PVN was predominantly colocalized with oxytocin mRNA-containing cells (Fig. 2). In contrast, it was relatively uncommon for vasopressin-positive cells to be double-labeled in any group (Fig. 2), and in these sections hybridized with probes recognizing vasopressin mRNA, abundant expression of PRL-RL mRNA was seen in non-digoxigenin-labeled cells. Presumably, the majority of these non-digoxigenin-labeled cells are oxytocinergic.

Combined data for the PVN are shown in figure 3. The proportion of neurons that expressed both oxytocin mRNA and PRL-RL mRNA increased significantly during pregnancy and lactation compared with non-pregnant controls. In the SON, PRL-RL mRNA was also predominately co-localized with oxytocin mRNA with little evidence of co-expression of vasopressin and PRL-RL mRNA (Fig. 4). In contrast to the PVN, no significant changes were observed between the different groups. Most oxytocin-labeled neurons (> 84 %) were double-labeled for PRL-RL mRNA in all three conditions.
Within many PVN sections that were hybridized with a mixture of oxytocin and PRL-RL probes, there were a number of cells that were found to express PRL-RL mRNA but were oxytocin-negative. Figure 5 illustrates examples of these cells which were present in most sections, typically in the parvocellular region of the PVN near the ventricle. As very few vasopressin neurons were double-labeled in either nucleus (see figs. 3 & 4), these PRL-RL-mRNA-expressing cells are unlikely to be vasopressin-secreting cells. Further studies are necessary to identify the neurochemical phenotype of this population of PVN cells.

Figure 6 shows representative examples from two oxytocin neurons (A, B) and one vasopressin neuron (C) in which in vivo electrophysiological recordings were made. The transient excitatory response to CCK (Fig. 6A, B) was indicative that the recordings were being made from an oxytocin neuron. Combined data from all animals treated with the 1 µg dose of prolactin are presented in Figure 6D. Administration of prolactin induced a rapid suppression in firing rates of oxytocin neurons in the SON in vivo. The basal firing rate (averaged in 5 min bins) of oxytocin neurons (n = 9) decreased from an initial rate of 2.76 ± 0.93 Hz to 1.96 ± 0.94 Hz 0 - 5 min after prolactin injection, remained at 1.98 ± 0.95 Hz at 5 - 10 min, then increased to 2.25 ± 1.01 Hz by 10 -15 min. Prolactin treatment of identified vasopressin cells, however, did not induce any change in firing rate. The mean basal firing rates of vasopressin neurons (n = 8) was 7.05 ± 1.13 Hz. Following prolactin injection, mean firing rates did not alter significantly (7.25 ± 1.23 Hz at 0 -5 min, 7.40 ± 1.20 Hz at 5 - 10 min and 7.0 ± 1.40 Hz).
DISCUSSION

This study has confirmed previous work documenting expression of prolactin receptor mRNA in the magnocellular hypothalamic nuclei (3, 4, 11), and has extended these observations by demonstrating a preferential expression of the long form of the PRL-R mRNA in oxytocin neurons compared with vasopressin neurons. Furthermore, we have observed that expression of PRL-R\textsubscript{L} mRNA is associated with specific prolactin-induced changes in the firing rate of oxytocin neurons in non-pregnant rats \textit{in vivo}. Vasopressin neurons, which express little or no PRL-R\textsubscript{L} mRNA, do not show changes in firing rate in response to prolactin. Finally, we have shown that levels of expression of PRL-R\textsubscript{L} mRNA in oxytocin neurons in the PVN are significantly increased during pregnancy and lactation. Hypothalamic oxytocin neurons undergo dramatic morphological and functional adaptation during late pregnancy and lactation, resulting in changes in electrical activity, and patterns of oxytocin secretion (32). The neurochemical systems mediating these changes in function have not been fully elucidated (32). The present data suggest that prolactin may be an important regulatory factor influencing the function of hypothalamic oxytocin neurons. Given the changes associated with prolactin secretion during pregnancy and lactation (22), prolactin could be considered as a prime candidate to mediate some of the effects of pregnancy on oxytocin neuronal function. In addition, placental lactogen is secreted at high levels during the second half of pregnancy (22) in rats and is known to cross the blood-brain barrier and activate PRL-Rs in the brain (6, 15). Hence, placental lactogen is also likely to influence oxytocin neurons during pregnancy.
The PRL-R protein (46, 55) and $^{125}$I-labelled prolactin binding sites (13) have previously been identified in both SON and PVN, and consistent with the present data, levels of PRL-R in the PVN appear to be significantly increased during lactation (47). Only one previous study has specifically identified the neuronal cell types expressing the PRL-R in these nuclei, and interestingly, PRL-R protein was found on both oxytocin and vasopressin neurons (39). In preliminary studies, we also have observed similar patterns of staining with the same antibody (24). The presence of PRL-R immunoreactivity on vasopressin neurons (39) clearly contrasts with the observation in the present study that most vasopressin neurons do not contain detectable levels of PRL-RL mRNA. The antibody used in these previous studies, however, is directed against the extracellular domain of the PRL-R which is common between the short and long forms of the receptor. Hence, a reasonable interpretation of the data is that vasopressin neurons may predominantly express the short form of the receptor. The functional consequences of this pattern of expression are not clear. As outlined in the introduction, most previous data suggests that prolactin exerts specific actions on oxytocin neurons, supported by our observation of specific actions of prolactin on firing rate of oxytocin and not vasopressin neurons. The long form of the receptor is required for full activation of signaling pathways downstream of the PRL-R (31, 73), in particular the activation of the JAK/STAT signal transduction cascade (5, 19). Recently, prolactin-induced activation of STAT5 in the hypothalamic magnocellular nuclei has been demonstrated (71), supporting the presence of a functional PRL-R$_L$ in these neurons. Hence, it is possible that the PRL-R$_L$ mediates reported stimulatory actions of prolactin on oxytocin mRNA (20) and oxytocin release (43, 62), through transcriptional activation of the JAK/STAT
pathway. In contrast, the short form of the receptor, which may be present on both oxytocin and vasopressin neurons, is reported to retain some signaling capacity, predominately mediated through the MAP kinase pathway. Perhaps this receptor mediates the reported actions of prolactin (and 16 kDa prolactin) on vasopressin secretion (39).

Our data show a rapid action of prolactin in suppressing the firing rate of oxytocin neurons \textit{in vivo}, with no effect on vasopressin neuronal firing rates. These data are broadly consistent with the \textit{in vitro} brain slice studies of Townsend et al., (71), who reported both stimulatory and inhibitory actions of prolactin on oxytocin cells, with no effect on identified vasopressin neurons. The physiological consequences of prolactin-induced suppression of the endogenous firing rate of oxytocin neurons (present study), coupled with stimulation of oxytocin synthesis (20, 21) and secretion (43) are not clear at present. A similar dissociation between stimulation of oxytocin release and suppression of firing has been reported for \(\alpha\)-melanocyte-stimulating hormone (58), postulated to result in differential regulation of dendritic versus nerve terminal release of oxytocin. Hence, it is possible that prolactin might be involved in patterning of oxytocin neuronal firing patterns or regulating dendritic release of oxytocin, particularly during periods of high prolactin secretion such as occur during lactation.

In several recent studies, however, we have observed different effects of peptides on the electrical activity of magnocellular neurons that are dependent on the route of administration and the physiological conditions of the animals. For example, retrodialysis
of apelin onto SON vasopressin neurons of virgin female rats increases the firing rate of vasopressin cells (34), but, when given into the cerebral ventricle apelin has either no effect in virgin female rats (34) or decreases vasopressin cell activity in pregnant rats (14). Similarly, i.c.v. administration of somatostatin increases SON oxytocin neuron firing rate, but when retrodialysed directly onto the SON somatostatin decreases oxytocin cell activity (37). Large molecules such as prolactin can not be given by microdialysis administration directly onto SON neurons and thus in the current study prolactin was injected into the lateral ventricle. The i.c.v. injection may mimic the effects of prolactin entering the brain affecting prolactin-sensitive inhibitory afferents to the SON oxytocin neurons. The actions of prolactin released locally within the SON may be different. Furthermore, we have only examined non-pregnant animals, and it is possible that responses to prolactin may change during pregnancy and/or lactation.

Alternatively, it is also possible that rapid actions of prolactin on membrane potential are induced by alternative pathways to those involved in regulating gene transcription. There have been a number of previous studies documenting rapid actions of prolactin on excitable cells. Prolactin induces rapid changes in excitability of arcuate neurons (42) and ventromedial hypothalamic neurons (10, 25, 41) in vitro. The mechanism of prolactin action to induce these rapid effects is not fully understood, but it is not likely to involve activation of the JAK/STAT pathway downstream of the PRL-RL, as this signal transduction results in changes in gene transcription over a longer time course. Prolactin-induced JAK2 phosphorylation, however, can also lead to phosphorylation of a range of ion channels (63) and intracellular protein kinases (36)
including ERK1 and 2, and protein kinase C. In CHO cells transfected with PRL-R_L, prolactin activates rapid increase in intracellular Ca\textsuperscript{2+} (72) through a voltage-insensitive influx of extracellular Ca\textsuperscript{2+} as well as a mobilization of intracellular Ca\textsuperscript{2+} stores (52, 66). In addition, prolactin induces a membrane hyperpolarisation, through direct activation of Ca\textsuperscript{2+}-dependent potassium channels (52). This latter effect appears to be dependent on JAK2-mediated tyrosine phosphorylation of intracellular proteins (53), requiring at least two residues on the cytoplasmic domain of the long form of the prolactin receptor (65). It is possible that this response also involves prolactin-induced activation of phosphoinositol-3 kinase and production of a range of inositol phosphate molecules (54). Similar prolactin-induced increases in intracellular Ca\textsuperscript{2+} are also seen in glial cell lines (16, 17) and neurons (29) that endogenously express the PRL-R. Hence, there is precedence for both rapid stimulatory and inhibitory actions of prolactin, and it is conceivable that either response could be induced, depending on the physiological state of the cell.

In addition to possible prolactin actions on oxytocin neurons, there is extensive evidence for a role of oxytocin in the regulation of prolactin secretion. Oxytocin stimulates prolactin secretion both \textit{in vitro} (9, 27, 33, 35) and \textit{in vivo} (60), whereas oxytocin antagonists can block prolactin secretion under certain physiological situations (27, 40, 59). It has been suggested that oxytocin may play a major role in stimulating the proestrous prolactin surge (27), and the prolactin surges of early pregnancy in the rat (1, 18). Hence, it is possible that PRL-Rs on oxytocin neurons might be involved in feedback responses to regulate oxytocin secretion. Both stimulatory (62) and inhibitory
effects of prolactin on oxytocin release have been reported, and it is possible that the specific response to prolactin may be different depending on the physiological state of the animal, as discussed above. In our non-pregnant females, the predominant effect would appear to be inhibitory, which could be interpreted as negative feedback suppression of a potent prolactin releasing factor.

In addition to PRL-R_L mRNA on oxytocin neurons, we also observed PRL-R_L expression in non-oxytocinergic cells in the parvocellular paraventricular nucleus. Based on our data, these are unlikely to be vasopressin-containing neurons, and hence the neurochemical phenotype is unknown at this stage. There is good evidence of prolactin regulation of central stress responses (68, 70), and thus it would be of interest to determine whether corticotrophin-releasing hormone-containing neurons in this region contain PRL-R mRNA. Similarly, with the long postulated, but controversial, role for thyrotrophin-releasing hormone (TRH) in the control of prolactin secretion (61), it would also be of interest to determine whether TRH neurons express PRL-R.

Prolactin and oxytocin are important reproductive hormones. In addition to their critical and specific roles in milk synthesis and milk ejection, respectively, both hormones have been implicated in a range of common adaptive functions during pregnancy and lactation (22, 56), including the onset and maintenance of maternal behavior, regulation of food intake and suppression of the stress response. Taking the present data into consideration, it seems likely that a number of common functions of
prolactin and oxytocin might be underpinned by prolactin interaction with oxytocin neurons.

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FIGURE LEGENDS

Figure 1: The upper panel shows a representative autoradiogram illustrating $^{35}$S labeled-PRL-R_L mRNA in the PVN and SON of the rat hypothalamus. Strong PRL-R_L expression is also present in the choroid plexus. The lower panels show low power micrographs of coronal brain sections at the level of the PVN and the SON depicting the distribution of digoxigenin-labeled oxytocin (left hand side) and digoxigenin-labeled vasopressin mRNA (right hand side). Scale bars = 1 mm (upper panel) and 100 µm (lower panel).

Figure 2: Representative images of double label in situ hybridization in the PVN in each of the experimental groups viewed under brightfield microscopy. The panels on the left show PRL-R_L mRNA (black grains overlying cells) plus oxytocin mRNA (blue/purple digoxigenin immunoreactivity). The panels on the right show PRL-R_L mRNA and vasopressin mRNA. Black arrows show examples of double-labeled cells, while red arrows show examples of oxytocin or vasopressin cells that do not contain significant levels of PRL-R_L mRNA. Green arrows show examples of apparent PRL-R_L mRNA positive cells that have not been labeled by the vasopressin probe. Note that while most oxytocin neurons contain PRL-R_L mRNA, it is relatively uncommon to find a vasopressin cell that contains PRL-R_L mRNA in any of the experimental groups. Scale bar = 20 µm.
Figure 3: Quantitative analysis of PRL-R_L mRNA expression in oxytocin (A) and vasopressin (B) neurons in the PVN in diestrus (n=5), pregnant (n=5) and lactating (n=4) rats. PRL-R_L mRNA was predominantly found in oxytocin neurons, and there was a significant increase in expression during pregnancy and lactation (*, P < 0.05). Data show means ± S.E.M.

Figure 4: Quantitative analysis of PRL-R_L mRNA expression in oxytocin (A) and vasopressin (B) neurons in the SON in diestrus (n=5), pregnant (n=5) and lactating (n=4) rats. PRL-R_L mRNA was predominantly found in oxytocin neurons, and expression did not increase significantly during pregnancy and lactation. Data show means ± S.E.M.

Figure 5: High power brightfield image of a PVN section hybridized with an $^5$S-labelled probe to label PRL-R_L mRNA together with a digoxigenin-labeled probe for oxytocin mRNA. Double arrowheads indicate examples of PRL-R_L mRNA expression in non-oxytocinergic cells (discrete clusters of black grains overlying cells that do not contain oxytocin mRNA). The single black arrow shows an example of a cell double-labeled for oxytocin (revealed as dark staining) and PRL-R_L mRNA. Scale bar = 20 µm.
Figure 6: Intracerebroventricular (i.c.v.) injection of prolactin significantly decreased firing rates of oxytocin, but not vasopressin neurons in the SON *in vivo*. Oxytocin neurons were distinguished from vasopressin neurons by their firing rate and by their response to intravenous cholecystokinin (CCK), i.e. transient excitation of oxytocin neurons (A,B) and no effect or short-term inhibition of vasopressin neurons (C). Note the rapid, dose-dependent suppression of oxytocin neuron firing rate in response to i.c.v. prolactin. (A,B). In contrast, i.c.v. administration of prolactin does not affect vasopressin neurons (C). (D) Quantitative data from 17 prolactin injections of the lowest dose of prolactin used (1 µg/injection) in 11 rats. *P<0.05, Repeated measures ANOVA. Data represent means ± S.E.M. of recordings from 9 oxytocin neurons and 8 vasopressin neurons.
References


Figure 2

Oxytocin

Vasopressin

Diestrus

Late pregnancy

Lactation
Figure 4

Supraoptic Nucleus

(A). Co-localisation of oxytocin and PRL-R<sub>L</sub> mRNA

(B). Co-localisation of vasopressin and PRL-R<sub>L</sub> mRNA
Figure 5
Figure 6

A

CCK 10μg Prolactin.

B

CCK 1 μg Prolactin

C

CCK 1 μg Prolactin

D

Oxytocin Cells  Vasopressin cells

% of basal activity

0 40 80 120

Basal 5 10 15

Time (min, 5 min bins)

1 μg Prolactin

*