Cellular Mechanisms of Prolactin Regulation of Oxytocin Neurons in Reproduction

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Abstract

The hormone oxytocin is secreted from nerve terminals of oxytocin magnocellular cells (MNCs) in the posterior pituitary gland and is important in the timing of birth and is essential for milk secretion. Another reproductive hormone, prolactin, is secreted from the anterior pituitary gland and is critical for breast development during pregnancy, as well as for milk synthesis during lactation. Oxytocin MNCs of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus undergo significant plasticity during pregnancy and lactation. Prolactin receptors are expressed by oxytocin neurons in both of these nuclei and prolactin has been shown to inhibit oxytocin MNCs in virgin rats. This project aimed to test two hypotheses. The first hypothesis was that the inhibitory effects of endogenous prolactin on the electrical activity of oxytocin MNCs will be reduced over the course of pregnancy or early lactation. To test this hypothesis, virgin (dioestrous) and lactating (day 6-12 post-partum) female rats were anaesthetised with urethane and extracellular single-unit recordings were made from identified oxytocin (and vasopressin) MNCs. Prolactin (1 µg in 1 µl intracerebroventricular) reduced the firing rate of oxytocin MNCs in virgin, but not lactating, rats. The second hypothesis was that reproduction-induced adaptations in oxytocin MNC responses to prolactin might be mediated by changes in second messenger systems downstream of the prolactin receptor. Double labelled (for oxytocin and phosphorylated signal transducer and activator of transcription 5 (pSTAT5)) immunohistochemistry was used to examine prolactin-induced activation of the Jak/STAT5 pathway in oxytocin MNCs. pSTAT5 expression was significantly increased in oxytocin MNCs of virgin rats treated with prolactin, while both the vehicle and prolactin treated lactating females had high levels of pSTAT5 in their oxytocin MNCs. Together, these data provide evidence that prolactin may directly and specifically regulates activity of oxytocin MNCs. However, the significance of this regulation remains to be elucidated.
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List of Abbreviations

AEC#................................................................. Animal Ethics Committee number
ANOVA.................................................................................................. Analysis of variance
Ca.............................................................................................................. Calcium
CCK............................................................................................................... Cholecystokinin
CIS ........................................................... Cytokine-inducible SH2-containing protein
Cl. .................................................................................................................. Chloride
CNS............................................................................................................ Central nervous system
CO2.. ........................................................................................................... Carbon dioxide
CRH............................................................................................................ Cytokine receptor homology domain
CSF ............................................................................................................... Cerebrospinal fluid
aCSF........................................................................................................... Artificial cerebrospinal fluid
DA .................................................................................................................. Dopamine
DAB.. ............................................................................................................. Diaminobenzidine
E2................................................................................................................................ Estradiol
ERK.. ............................................................................................................ Extracellular signal regulated kinase
GABA........................................................................................................... γ –aminobutyric acid
GH.................................................................................................................. Growth hormone
H..................................................................................................................... Hydrogen
HNS............................................................................................................... Hypothalamo-neurohypophysial system
I.c.v… ............................................................................................................. Intracerebroventricular
Ins. ................................................................................................................ Inositol
I.p................................................................................................................... Intraperitoneal
I.v................................................................................................................... Intravenous
Jak................................................................................................................ Janus kinase
K… ................................................................................................................... Potassium
LH.................................................................................................................. Luteinizing hormone
MAPK . ....................................................................................................... Mitogen-activated protein kinase
Magnocellular neurosecretory cells
mRNA.............................. Messenger ribonucleic acid
Na.............................................. Sodium
NGS........................................... Normal goat serum
P.............................. The proestrus stage of the ovarian cycle
PKC........................................... Protein kinase C
PL........................................ Placental lactogen
PL-I...................................... First isoform PL
PL-II.................................. Second isoform PL
PRFs.................................. Prolactin releasing factors
PRL..................................... Prolactin
PRL-R ..................................... Prolactin receptor
PRL-RL ................................... Long form PRL-R
PRL_Rs .................................. Short form PRL-R
PVN.............................. Paraventricular nucleus
S.c......................................... Subcutaneous
SEM........................................ Standard error of the mean
SOCS................................. Suppressors of cytokine signaling
SON...................................... Supraoptic nucleus
STAT.................................... Signal transducer and activator of transcription
TBS...................................... Tri-buffered Saline
TIDA.............................. Tuberoinfundibular dopaminergic
TRH...................................... Thyrotrophin releasing hormone
Tyr.......................................... Tyrosine
VIP...................................... Vasoactive intestinal peptide
Chapter 1: Introduction

1.1 Pregnancy, Lactation and Motherhood

During reproduction, the physiology of the female body is altered to support the developing fetus and infant, and also to meet the new demands of the carrying mother. Successful pregnancy, lactation and motherhood require a range of physiological adaptations to occur in the mother, including changes involving the maternal brain. Many of these adaptations are linked to changes of a number of hormonal systems that occur during pregnancy and lactation. These adaptations play an important role to facilitate fetal growth, parturition, lactation, maternal behaviour as well as maternal care of offspring. Two hormonal systems have been implicated in mediating a number of these adaptive physiological and behavioural changes; the prolactin and oxytocin hormonal systems, both of which also show adaptive changes during this period. However, the interactions between those two hormonal systems to prepare for birth and motherhood have not been fully elucidated.

1.2 Prolactin

1.2.1 Anterior Pituitary Prolactin

The hormone prolactin is a polypeptide released mainly from the anterior pituitary gland and was the first of the anterior pituitary gland hormones to be identified and purified [1]. The amino acid sequence and tertiary structure of prolactin is closely related to that of growth hormone [2], which is also secreted from the anterior pituitary gland. The hormone was named “pro-lactin” for its actions on mammary gland function and development during pregnancy and lactation. Although most circulating prolactin is synthesised in, and secreted from, specialised cells of the anterior pituitary, the lactotrophs, the prolactin gene is also expressed in some mammals in extrapituitary sites (extrapituitary prolactin), including the mammary gland [3, 4], immune cells [5, 6], adipose tissue [7], decidua [8], prostate [9, 10], and umbilical vein endothelial cells [11]. In addition to its effect on peripheral tissues,
prolactin is thought to gain access to the brain through a carrier mediated transport system [12] probably involving prolactin receptors (PRL-Rs) in the choroid plexus [13, 14] to exert important actions within the brain itself. PRL-R protein [15-17] and PRL-R mRNA [17-20] have been identified in many hypothalamic nuclei.

The lactotrophs, or mammotrophs, form up to 50% of the cellular population of the anterior pituitary gland. The number of anterior pituitary lactotrophs depends on the sex and physiological status of the animal. Using species specific prolactin antibodies, lactotrophs were identified by immunocytochemistry in the anterior pituitary gland of the mouse [21], rat [22], and human [23, 24]. The number of lactotrophs in the anterior pituitary gland was higher in females than in males, and the number increases significantly during pregnancy and lactation, both states characterized by hyperprolactinaemia [25, 26]. Prolactin release is normally under inhibitory control by dopamine (DA) released by tuberoinfundibular DA (TIDA) neurons from the median eminence, and to a lesser extent stimulatory, factors. Thus, to allow a state of hyperprolactinaemia the regulation of prolactin secretion has to be altered during these times of reproduction (see section 1.2.6).

1.2.2 Brain Prolactin

In addition to being transported into the brain, prolactin may also be produced locally within the brain. The first observation of prolactin production in the brain was made when prolactin immunoreactivity in hypothalamic axon terminals was found by Fuxe et al. [27]. Prolactin immunoreactivity was subsequently described in different areas of the brain, including the hypothalamus, the cerebral cortex, hippocampus, amygdala, septum [28], caudate putamen [29, 30], brainstem [28, 30], cerebellum [31], spinal cord [30, 32] and the choroid plexus of the cerebral ventricles [33]. Numerous hypothalamic areas were found to express prolactin immunoreactivity in a variety of mammals [30, 32, 33]. Notably, prolactin immunoreactivity was detectable within the rat supraoptic (SON), and paraventricular (PVN) nuclei of the hypothalamus [34], both nuclei house the cell bodies of oxytocin neurons. Oxytocin neurons secrete the hormone oxytocin, which is involved in regulating many reproductive functions, along with prolactin. Therefore, prolactin produced in the hypothalamus, might play a role in modulating oxytocin cell activity during the various stages of reproduction. On the other hand, pituitary prolactin secreted into the peripheral circulation bypasses the blood-brain
barrier and enters the brain through the choroid plexus of the cerebral ventricles. Therefore, it is difficult to differentiate between the effects of prolactin of pituitary versus hypothalamic origin in the central nervous system (CNS) and thus ascribing a role for prolactin of neural origin can be challenging.

**1.2.3 Placental Lactogens**

During pregnancy, the placenta also contributes to total levels of lactogenic hormones in the circulation. The placenta is an organ found inside the uterus during pregnancy that is attached to the embryo via the umbilical cord and is essential for growth and development of the embryo. It has a wide range of endocrine functions in addition to its metabolic transport role between the fetal and maternal circulations. The placental lactogens (PLs) are hormones synthesised in the placenta during pregnancy in the placental trophoblast cells, and named for their lactogenic activity. PLs belong to the lactogenic hormone family along with prolactin, and are found in many animals, including the rat and human [35, 36] [37]. Placental lactogen-I (PL-I) appears immediately after implantation and reaches maximum expression levels at about mid-gestation, followed by a steep decline when replaced by predominant secretion of PL-II. PL-II secretion falls towards the end of pregnancy and declines rapidly following delivery of the placenta (Fig 1.1) [38]. Each of these prolactin like hormones, PL-I and PL-II, can bind to, and activate, the PRL-Rs [39]. Prolactin is low during most of pregnancy, and most of the “prolactin” actions during this time, are actually mediated by PLs acting on PRL-Rs.
Figure 1-1 Lactogenic hormone levels during rat pregnancy

Prolactin (PRL) surges twice daily for the first 10-11 days of pregnancy in rats. The rapidly rising placental lactogen-I (PL-I) results in suppression of pituitary prolactin. On day 12 of pregnancy, PL-I levels drop and PL-II levels increase steadily until birth. On the day before parturition, pregnancy day 21, pituitary prolactin release increases significantly. Reproduced from Ben-Jonathan et al, 2008 [40].
1.2.4 Episodic Prolactin Release Associated with Female Reproduction

Prolactin is released episodically, or in surges, during various reproductive states in the female mammal. These states include menstrual cycles, mating and pregnancy and lactation. However, there is no common stimulator of prolactin release during these different states. A rise in circulating oestrogen stimulates the mid-cycle prolactin surge, while cervical stimulation initiates the prolactin surges of early pregnancy in the rodent and a suckling stimulus induces prolactin release in lactation. Common to all three states, however, is a withdrawal of DA inhibition of lactotrophs, although, the ultimate hypothalamic control leading to this reduction in DA input is not well understood. While the integrated actions of DA and oestrogen appear to be the major regulatory factors of lactotroph functions, the means coordinating this integration are yet to be defined [41].

A) Oestrus Cycle

The secretion of prolactin throughout most of the oestrus cycle in rodents is low and stable. During the afternoon of the pro-oestrus stage of the cycle, however, the secretion of prolactin rises to produce a preovulatory surge that is coincident with the luteinizing hormone surge (Fig. 1-2) [42, 43]. Both DA and oestradiol have been shown to play an important role in regulating this surge of prolactin [44-46].

B) Pregnancy

Cervical stimulation, like mating or artificial stimulation, switches the pattern of prolactin secretion to a pattern of twice-daily surges, which occur in the latter half of the light and dark phases of the light-dark cycle and are called the diurnal and nocturnal surges, respectively [47]. These daily surges occur for nine to ten days after mating and are required for luteal progesterone secretion during this period of the pregnancy of the female rodent. Subsequent to the termination of these daily prolactin surges, PLs, produced by the placenta, support luteal function to the end of pregnancy [48].
Reproductive hormones fluctuate throughout the ovarian cycle. Prolactin (PRL) produces a preovulatory surge on the pro-oestrus stage (P) of the cycle, which is concurrent with the luteinizing hormone surge (LH). Oestadiol (E2), an oestrogenic hormone, produces a surge that precedes, and thought to contribute to, the prolactin surge. Reproduced from Ben-Jonathan et al, 2008 [40].
Some evidence suggests that DA plays a role in regulating both the nocturnal and diurnal surges of prolactin. The involvement of DA is supported by the low DA concentrations in the hypophyseal portal blood during both surges compared to the DA concentration during the inter-surge period [49]. However, the prolactin secretory response to blocking the D2 receptor at similar times of the day is significantly smaller than the surges seen following cervical stimulation [47]. Thus, the role of DA in mediating the nocturnal and diurnal surges is not clear, and other factors could be involved.

C) Lactation

During lactation, the suckling stimulus induces prolactin secretion in an episodic manner, as a classical neuroendocrine reflex. The amount of prolactin released is proportional to the intensity of the suckling stimulus (i.e., number of pups) [50]. It has been shown that the suckling stimulus reduces TIDA neuron activity and transiently decreases the concentration of DA in the portal blood [51], thus, withdrawing the inhibitory tone from lactotrophs (disinhibition). However, the amount of prolactin released in response to suckling lasts much longer than the observed decrease in DA input to the anterior pituitary. In addition, pharmacological or surgical disruption of DA input to the anterior pituitary results in less prolactin secretion compared to that induced by suckling. Some evidence suggests that the release of DA inhibitory input plays an important role in sensitizing the lactotrophs to subsequent stimulation by prolactin releasing factors (PRFs) from the brain [51]. Although DA remains the main known regulator of prolactin secretion, other regulatory inputs, such as oxytocin hormone, seem to play an important role during pregnancy and lactation [41].
1.2.5 Prolactin Receptors and Prolactin Signalling

A) Prolactin Receptor

The PRL-R is a single membrane-bound protein that belongs to the type 1 cytokine receptor family [52], which also includes the receptors for growth hormone (GH), erythropoietin and most of the interleukins [53-55]. The receptor protein contains extracellular (ligand-binding), transmembrane and intracellular domains [56]. The gene encoding the human PRL-R is located on chromosome 5 and contains at least 10 exons [57, 58]. A number of PRL-R isoforms have been described in different tissues. The receptor isoforms vary in length and composition of their cytoplasmic domains, while their extracellular domains are identical within a species [52, 59, 60]. There are three key PRL-R isoforms described in rats; the short (291 amino acids), intermediate (393 amino acids), and long (591 amino acids) forms (Fig. 1-3 and 1-4) [59].

Stimulation of the short form of the PRL-R does not result in activation of the classical pathways associated with prolactin signalling (described below), and the functional significance of the short form of the PRL-R is not well described (Fig. 1-4). It is thought that the short form of the PRL-R may act as a transport molecule because the choroid plexus predominantly expresses the short form of the PRL-R and the levels of prolactin circulating in the blood are positively correlated to the levels of the hormone in cerebrospinal fluid (CSF) [61]. Therefore, it appears likely that the short form of the PRL-R acts as a transporter, at least in the choroid plexus, to transfer prolactin from the circulation into the brain.

The PRL-R is expressed in virtually every organ in the body, in one or a combination of isoforms, and it has been suggested that some tissues may need to be protected from exaggerated prolactin signalling during periods of physiological hyperprolactinemia, such as pregnancy and lactation. The expression of the various isoforms in many tissues changes during these physiological states, specifically the expression of the short form of the PRL-R increases, compared to the long form. This supports the idea that these isoforms play a dynamic role in the regulation of prolactin signalling. As the short form of the PRL-R has limited signalling capacity, it has been suggested that the short form of the PRL-R acts as a ‘decoy’ receptor that protects tissues from exaggerated prolactin signalling during physiological hyperprolactinemic states (See below).
B) Activation of Prolactin Receptor and the Associated Signal Transduction Pathways

I) Extracellular Domain and Ligand Induced Dimerization

The extracellular domain of all PRL-R isoforms, in both rat and human, consists of 210 amino acids [62] and shows sequence homology with other cytokine receptors (cytokine receptor homology domain, CRH) [63]. Activation of the PRL-R involves ligand-induced receptor dimerization [59]. The prolactin molecule contains two binding sites, binding site 1 and 2. First, prolactin’s binding site 1 interacts with the extracellular domain of a PRL-R molecule [64]. The initial formation of this hormone-receptor interaction is required for the interaction of binding site 2 on the same prolactin molecule with a second PRL- R molecule to form a trimeric complex (2 receptors, 1 hormone), which is essential for the activation of the PRL-R [59, 64]. Classical signal transduction requires homodimerization of the long-form PRL-R molecules, while heterodimers of short and long receptors, or homodimers of the short form PRL-R, do not activate normal signalling (Fig. 1-3) [65, 66].
Figure. 1-3 Activation of the prolactin receptor

Prolactin molecule, containing two binding sites, induces sequential receptor dimerization to activate the prolactin receptor (PRL-R). First, prolactin’s binding site 1 interacts with a PRL-R molecule (step 1). The formation of the initial hormone receptor complex leads to the interaction of binding site 2 on the same prolactin molecule with a second PRL-R (step 2). Successful dimerization of two PRL-R molecules, of the same isoform, induces activation of a tyrosine kinase termed Janus kinase2 (Jak2), which is associated with the intracellular domain of the PRL-R. Jak2 kinases transphosphorylate each other (step 2) and phosphorylate (P) the Tyr residues (Y) of the PRL-R itself (step 3). However, Tyr phosphorylation of the receptor itself does not occur upon activation of the short form of the PRL-R. Reproduced from Freeman (2000) [41].
II) Intracellular Domain and Activation of Jak2 and Receptor Phosphorylation

a) Transmembrane and intracellular domains

The role of the transmembrane domain in the activation of PRL-R is still unknown. The intracellular domain, however, is a major player in the initiation of the signal transduction mechanisms downstream of the PRL-R. In contrast to the extracellular domain, the intracellular domain varies in length and composition between the various isoforms of the PRL-R, and also shows little sequence similarities with other receptors of the cytokine family [59]. There are, however, two relatively conserved regions of the intracellular domain of the cytokine receptor family, termed box 1 and box 2 [67]. Box 1 (Fig. 1.3) is an 8-amino acid proline-rich motif, which interacts directly with the tyrosine kinases that are activated upon ligand binding, and is also necessary for the correct folding of the receptor molecule [59]. Box 2 is missing in the short form of the PRL-R and less conserved compared to Box 1 [52].

b) Activation of Jak2

The intracellular domain of the PRL-R lacks any intrinsic enzymatic activity, like all cytokine receptors, and transduces its signal through numerous kinases that in turn activate other downstream pathways. The ligand-induced activation of PRL-R results in tyrosine phosphorylation of several cellular proteins [68], including the receptor itself [52], to trigger a cascade of intracellular events. The Box 1 region of the intracellular domain is associated with a tyrosine kinase, independent of ligand binding, termed Janus kinase 2 (Jak2) [69-71]. Two major prerequisites for Jak2 activation have been described; the presence of the proline-rich motif (Box 1) in the intracellular domain of the PRL-R [72] and the ligand mediated, homodimerization of the long form PRL-R [66, 73-75]. Activation of Jak2 occurs by transphosphorylation upon receptor dimerization, which brings two Jak2 molecules close to each other. Homology of the intracellular domain between the two receptor dimers is required for activation of the Jak2 kinases, suggesting possible role of the COOH-terminal [74].
c) Phosphorylation of the PRL-R

Following receptor activation, Jak2 kinases transphosphorylate each other and are also involved in the phosphorylation of tyrosine (Tyr) residues of the PRL-R itself [76], mainly the long form of the PRL-R [77] (Fig. 1.3). Phosphotyrosines of the PRL-R are of interest because they are potential binding sites for second messenger system transducer molecules. Phosphorylation of Jak2 enzymes occurs in all activated PRL-R isoforms, while Tyr phosphorylation of the receptor itself does not occur following activation of the short form of the PRL-R [78].

C) Signal Transduction Pathways Associated with the PRL-R

I) STAT Proteins

The signal transducer and activator of transcription (STAT) protein family has been shown to be a major signal transduction mechanism downstream of cytokine receptors [70]. The recognition for a role of these factors in prolactin signalling came from the study of PRL-induced genes [79, 80]. Eight members of the STAT family have been described, four of them, STAT1, STAT3, and especially STAT5a and STAT5b, have been implicated as signal transducer molecules downstream of the PRL-R [81, 82]. A phosphorylated Tyr residue of the activated PRL-R acts as a docking site to interact with a STAT molecule (Fig. 1.4) [59, 83]. While docked at the receptor, the STAT protein is phosphorylated by the receptor-associated Jak kinase enzyme. Then, the phosphorylated STAT detaches from the receptor and forms a hetero- or homodimer with another phosphorylated STAT molecule through its phosphotyrosine residues (Fig. 1.4). The STAT dimer then translocates to the nucleus and activates the transcription of target genes [59, 84]. The exact mechanism by which STAT dimers are transported into the nucleus and how they interact with the transcription machinery remain to be elucidated.

Of the STAT proteins, STAT5 (earlier known as mammary gland factor, MGF) is recognized as the major signal transducer of the long and intermediate isoforms of the PRL-R [84]. Two isoforms of the STAT5 protein have been described, STAT5a and STAT5b, which are encoded by two closely related genes. Targeted disruption of these genes in mice results in phenotypes remarkably similar to those of mice in which either prolactin or the PRL-R have been knocked out [85-88]. Hence, the STAT5 proteins were established as primary mediators of the physiological actions of
prolactin. STAT5a plays a predominant role in mediating prolactin effects in the mammary gland [86], while STAT5b plays a more important role in mediating prolactin effects in the ovaries [88, 89].

The two STAT5 protein isoforms show 95% sequence homology and differ only in the COOH-terminal domain. Both isoforms possess a Tyr-694 residue, which is phosphorylated by Jak2 [90]. Activation of STAT involves serine/threonine phosphorylation, in addition to Tyr phosphorylation. The major difference between the STAT5a and -b isoforms lies in their serine/threonine phosphorylation sites. Protein kinase C (PKC), and casein kinase II, have been proposed to be serine/threonine kinases that activate STAT5 [91].

II) Other Signalling Pathways

The physiological relevance of the STAT5-independent signalling pathways is not yet clear, although they have been suggested as mediators for the mitogenic actions of prolactin [92]. The existence of subtle differences between the prolactin or PRL-R knockout mice and STAT5 knockout mice, supports the notion that non-STAT5 pathways play some role in prolactin signalling in mammalian cells. Another signalling pathway for prolactin recently proposed involves the direct transport of the prolactin–PRL-R complex to the nucleus, which remains controversial. One group reported anti-prolactin immunoreactivity in the nuclei of target cells [93], while others failed to detect either the ligand or the receptor within the nucleus [94].

a) Ras/Raf/MAP kinase pathway.

Although the Jak/STAT pathway is the classical signalling pathway initiated by activation of the PRL-R, other pathways have also been implicated, such as the mitogen-activated protein kinase (ERK1/2-MAPK) cascade [95-102]. As explained earlier, phosphotyrosine residues of the PRL-R can serve as docking sites for intracellular proteins. To activate the MAPK pathway, the phosphotyrosine residues of the PRL-R act as binding sites for adapter proteins (Shc/Grb2/SOS) connecting the receptor to the Ras/Raf/MAPK cascade [84] (Fig. 1.4). It has been suggested that the Jak/STAT and the MAPK pathways might be interconnected, rather than independent, parallel pathways [103].
Figure 1-4 Signal transduction pathways downstream of the prolactin receptors

The cellular pathways through which the long and short forms of the prolactin receptor (PRL-R) are thought to activate cells. The classical pathway initiated following activation of the long form PRL-R is the Jak/STAT pathway. Jak is a janus kinase enzyme associated with the receptor, and STAT is a protein family called the signal transducer and activator of transcription (STAT) protein family, which includes STAT1, STAT3, STAT5a, and STAT5b. A STAT protein binds to a phosphorylated Tyr residue (Y) of the activated long PRL-R and is phosphorylated by the receptor-associated Jak kinase. Then, phosphorylated STAT forms a dimer with another phosphorylated STAT molecule, which then translocates to the nucleus and activates target genes. Activation of the PRL-R also activates the mitogen-activated protein kinase (MAPK) cascade, which is involved in the activation of a wide range of transcription factors. The intracellular domain of PRL-R is also involved, through Jak2, in the activation of a tyrosine kinase-dependent, calcium-sensitive potassium channels (K'). The COOH terminal of PRL-R's intracellular domain is involved in the regulation of an intracellular pathway that opens voltage-independent Ca\(^{2+}\) channels, using intracellular enzymes, like Fyn and PI3K, and also signalling messengers, such as IP4 and IP6. The Jak/STAT pathways can be inhibited by SOCS (suppressors of cytokine signaling) which inhibit Jak kinases or CIS (cytokine-inducible SH2-containing protein), which compete with STAT for docking sites on PRL-R. The tyrosine residues of the short form of PRL-R are not phosphorylated by Jak2, but the phosphotyrosine of Jak2 can serve as docking site for Stat1. Though, the downstream pathways of the short form are not well understood. Reproduced from Freeman (2000) [41].
b) Intracellular ion concentration

Two regions of the PRL-R involved in prolactin-induced intracellular ionic changes have been described. Box 1 of the intracellular domain of the PRL-R has been implicated in the activation of tyrosine kinase-dependent K\(^+\) channels by Jak2 [104], whereas the COOH terminal of the intracellular domain has been shown to be involved in the production of the intracellular messengers, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P4] and inositol hexakisphosphate (InsP6) that open voltage-independent Ca\(^{2+}\) channels [105-107] (Fig. 1.4).

D) Downregulation of PRL-R Signal by Tyrosine Phosphatases and Inhibitor Proteins

In addition to dephosphorylation by the numerous intracellular tyrosine (Tyr) phosphatases, STAT activation by prolactin is regulated by an intracellular negative feedback mechanism. Receptor associated Tyr phosphatases play less of a role in the down regulation of prolactin signalling than in the closely related GH or cytokines [108-113]. Src kinase homology domain 2 (SH2) contains protein families that have been linked to cytokine receptor signalling and implicated in inhibiting the Jak/STAT pathways. CIS (cytokine-inducible SH2 protein), and SOCS (suppressor of cytokine signalling) are members of this class of proteins that are transcriptionally regulated by activated STAT proteins. CIS and SOCS feedback on the PRL-R complex to inhibit the coupling of Jak to either the receptor or to the STAT proteins [114-122]. In addition, SOCS-2 seems to restore the cells’ sensitivity to PRL-R stimulation, probably by suppressing the inhibitory effect of other SOCS proteins, such as SOCS-1 and SOCS-3 [111] (Fig. 1.4).

1.2.6 Regulation of Prolactin Secretion

Prolactin secretion is regulated in a complex manner by a variety of hormones and neurotransmitters [41, 123] and is also influenced by the light-dark cycle, sleep patterns and stress, among other factors. In mammals, lactotrophs spontaneously secrete prolactin, requiring no acute stimulatory input to maintain secretion. Indeed synthesis and release of large amounts of prolactin occurs from anterior pituitary tissue that has been removed from hypothalamic control, such as removal to cell
culture or in transplants to ectopic sites [42]. The major regulatory input to anterior pituitary lactotrophs is inhibitory. This inhibitory input is mediated almost entirely by DA secreted from the hypothalamus.

Superimposed onto the dopaminergic system is the influence of other factors, such as oxytocin and oestrogen, which can stimulate prolactin release [41], but none of these releasing factors has been shown to be essential for normal regulation of prolactin secretion. Rather, a reduction in the dopaminergic inhibitory control is associated with the periodic physiological surges of prolactin in females [41, 124, 125].

A) Dopamine

As introduced above (Section 1.2.1), DA is produced by the TIDA neurons of the arcuate nucleus of the hypothalamus, and released from nerve terminals in the median eminence where it is delivered to the anterior pituitary gland via the hypophysial portal vessels [42]. Prolactin, in turn, feeds back on the TIDA neurons to regulate the release of DA and, ultimately, its own release [43, 126] (Fig. 1.5). DA acts on the lactotroph at various levels of cell function. In addition to inhibiting acute release of prolactin, DA also inhibits transcription of the hormone gene [127]. DA is also a potent antimitotic factor in pituitary lactotrophs [128, 129]. Although there is broad diversity of potential prolactin regulators, DA has impressively diverse actions as a modulator of lactotroph function, acting on multiple levels of prolactin and lactotroph regulation. Therefore, DA is considered to be the prime regulator of prolactin secretion.

I) DA Receptors

Dopaminergic actions on the lactotroph are mediated via D2-like DA receptors [130, 131]. Five structurally distinct DA receptors have been identified, D1 to D5 [132]. These receptors are grouped into two families (D1-like and D2-like) based on molecular, pharmacological and functional characteristic features. D1-like receptors (D1 and D5) are linked to the stimulation of adenylate cyclase, whereas D2-like receptors (D2, D3, and D4) either inhibit or have no effect on this enzyme. Only D2-like DA receptors are present in the anterior pituitary gland [133-135], and these receptors are coupled to a G-protein, which plays a necessary role in the inhibitory actions of DA on the lactotroph mainly by regulating adenylate cyclase and potassium channels [136-138].
B) Other Inhibitors of Prolactin

In addition to DA, a number of factors have also been shown to inhibit prolactin secretion from the anterior pituitary. Somatostatin, secreted from the hypothalamus, and calcitonin, which might be of hypothalamic origin, have been shown to inhibit prolactin secretion [139-141]. Interestingly, endothelin-1 produced by lactotrophs themselves also inhibits prolactin secretion through an autocrine mechanism [142]. However, hypothalamic DA is still required for the normal control of anterior pituitary prolactin secretion, whereas the physiological role of these other factors is yet to be established.

C) Prolactin Releasing Factors

Oestrogen is another major regulator of lactotroph activity in mammals and stimulates anterior pituitary prolactin secretion. Oestrogen is the reason for the greater serum levels, and pituitary content, of prolactin in females compared to males [143]. Although it has not been proven that oestrogen directly stimulates the release of prolactin, oestrogen dramatically increases prolactin gene expression, which can lead to both a higher rate of spontaneous prolactin release and a larger reservoir of prolactin that can be released upon removal of DA inhibition or via stimulation by releasing factors.

A wide variety of other prolactin releasing factors (PRFs) has been described in mammals [41]. A number of hypothalamic peptides have been associated with acute prolactin release, including, but not limited to, oxytocin, thyrotrophin releasing hormone (TRH) and vasoactive intestinal peptide (VIP) [144, 145]. Also some local pituitary factors, such as epidermal growth factor and fibroblast growth factor-2, angiotensin II and pituitary adenylate cyclase activating peptide stimulate the acute release of prolactin from the anterior pituitary gland [64, 146-148]. Despite all the efforts described in the literature, a clear physiological role of those PRFs is yet to be elucidated.
Figure 1-5 Short-loop negative feedback of prolactin on TIDA neurons

Dopamine (DA) released from TIDA neurons at the median eminence is delivered to the anterior pituitary gland via the hypophyseal portal vessels (red), to inhibit prolactin secretion. Prolactin secreted from lactotrophs of the anterior pituitary gland enters the brain via the cerebral ventricles and have a positive effect on DA secretion, and therefore inhibiting its own secretion. This interplay between the two hormones forms the negative feedback loop of prolactin secretion. Reproduced from Grattan (2002) [149].
D) Oxytocin

Of the possible prolactin-releasing factors discussed above, for the present thesis it is important to consider the putative role of oxytocin further. Oxytocin has been shown to stimulate prolactin release in vitro and exhibits a similar secretion pattern to prolactin during lactation, possibly because both hormones are secreted in response to the suckling stimulus. Oxytocin, released from the posterior pituitary gland, can reach the anterior pituitary through both the short portal vessels and the general circulation. Studies using oxytocin antagonists show partial suppression of prolactin secretion, so oxytocin appears to contribute to the physiological regulation of prolactin release [150-152]. This might be important during lactation, where oxytocin and prolactin regulate milk ejection and synthesis, respectively, both functions are essential for the survival of the young.

1.2.7 Prolactin Functions in the Hypothalamus during Pregnancy and Lactation

During pregnancy and lactation there are increased levels of circulating lactogenic hormones, mainly prolactin secreted from the anterior pituitary gland and placental lactogens secreted from the placenta (Fig. 1.1). Additionally, the expression of the PRL-R in the brain increases during pregnancy and lactation [153]. Both suggest that prolactin signalling in the brain is likely to be increased during these period of reproduction. This increased exposure to prolactin has been linked to a number of behavioural and physiological changes that occur during pregnancy and lactation [154]. These changes include induction and maintenance of maternal behaviour [155-158], increased food intake [159-161] and altered response to stress [158, 162]. These reproductive functions are also modulated by the hormone oxytocin, secreted from the posterior pituitary (see section 1.3).
1.3 Oxytocin

At the start of the 20th century, extracts from the posterior pituitary gland were shown to have powerful stimulatory actions on the contractility of the uterus. Due to this stimulatory effect on the uterus, and therefore the birth process, it was called “oxytocin”, the Greek term for “rapid birth”[163, 164]. Shortly afterwards, oxytocin was shown to have stimulatory effects on mammary glands milk secretion[165]. Oxytocin is a peptide hormone produced by oxytocin neurons in the hypothalamus and released from nerve terminals at the posterior lobe of the pituitary gland. The primary structure of oxytocin is closely related to another posterior pituitary hormone, vasopressin. Vasopressin, or anti-diuretic hormone (ADH), is mainly concerned with the body water balance. Both oxytocin and vasopressin are generally referred to as the neurohypophysial (posterior pituitary) peptides, based on their storage location in the body.

1.3.1 Oxytocin Systems

A) Endocrine Oxytocin System

Oxytocin, secreted from nerve terminals of oxytocin cells in the posterior pituitary gland, is important in the timing of birth and is essential for milk secretion. Oxytocin release into the blood is governed by oxytocin cell bodies, found in the hypothalamus, which integrate information from multiple sources to regulate hormone secretion. For this reason, the electrical activity of oxytocin cells determines the secretory profile of this hormone. At birth, and during lactation, oxytocin cells fire in pulsatile and coordinated bursts that generate oxytocin secretion to promote rhythmic contraction of the uterus at birth and induce milk let-down during suckling, each of which is essential for offspring survival [166].

The major endocrine oxytocin system is classically called the magnocellular hypothalamo-neurohypophysial system (HNS). The magnocellular neurons, 20 to 40 µm cell body diameter, are located mainly in the SON and PVN nuclei of the hypothalamus and have axonal fibre tracts that project to the posterior lobe of the pituitary gland and terminate on capillaries, where oxytocin is released into the peripheral circulation [167].
The SON exclusively contains magnocellular neurons that project to the posterior pituitary gland, whereas the PVN is divided into a lateral magnocellular subdivision and a more medial parvocellular subdivision that contains smaller neurons (10–15 µm cell body diameter) that project to other areas of the CNS, including the median eminence of the hypothalamus. Although there is some topographical segregation, both oxytocin and vasopressin magnocellular neurons are found intermingled in the SON and PVN [168, 169]. Oxytocin magnocellular neurons are mainly found rostral and dorsal in the SON and predominantly rostral in the PVN.

B) Central Oxytocin Systems and Release

Parvocellular hypothalamic oxytocin neurons give rise to numerous central projections, including projections to the hypothalamus, brainstem and spinal cord [170]. It has been suggested that those projections are mainly involved in modulating behaviours, especially maternal, reproductive and social behaviours. Oxytocin release within the CNS has been extensively studied in the sheep in relation to the regulation of maternal and sexual behaviour [171-173]. In vivo microdialysis studies have demonstrated an increase in extracellular oxytocin in various areas of the brain as a result of a number of stimuli, including parturition and suckling [174, 175].

I) Somatodendritic Oxytocin Release

The release of oxytocin from the dendrites and cell bodies of oxytocin neurons (somatodendritic release) was first suggested from the finding that burst firing of oxytocin neurons during suckling in lactation depends on a central stimulatory action of oxytocin [176]. The most important source of this oxytocin was deduced to be the magnocellular nuclei, from measurements of release of oxytocin in vivo and in vitro [177]. The dendrites, and the cell bodies, are now considered as an important source of oxytocin release that have autocrine and paracrine actions in various brain regions [177-180]. This oxytocin is produced and packaged in the cell body and then the oxytocin containing secretory granules are transported into the dendrites where the hormone can be stored or released, into the extracellular space, by exocytosis [180].
The concentration of oxytocin in the PVN and SON, measured by microdialysis and radioimmunoassay, can be 100 to 1,000 fold greater than circulating oxytocin concentrations [181]. During parturition, the extracellular concentration of oxytocin in the SON and PVN increases 2.5-fold [182]. In addition, oxytocin in the SON increases by about 75% in pregnancy [183]. Microdialysis application of an oxytocin antagonist into the hypothalamus disrupts the progress of parturition, implying an important role of somatodendritically released oxytocin in regulating parturition [65]. Furthermore, intracerebroventricular injection of oxytocin can facilitate burst firing of oxytocin neurons during suckling [184]. Therefore, somatodendritic oxytocin release seems to have many reproduction-related modulatory actions in the brain.

### 1.3.2 Regulation of Oxytocin Secretion

#### A) Action Potential Generation and Regulation.

As for any neuron, oxytocin neurons discharge action potentials (spikes) from their cell bodies when they are depolarized above threshold. The depolarization of the cell body is primarily the result of summation of excitatory and inhibitory postsynaptic potentials that follow the release of excitatory or inhibitory neurotransmitter from synaptic terminals on the dendrites and soma. Whenever the sum of ongoing excitatory and inhibitory postsynaptic potentials exceeds threshold, an action potential is triggered, ultimately leading to oxytocin release from nerve terminals [185, 186].

#### B) Pulsatile Oxytocin Secretion

During continuous suckling in lactating rats, a pulse of oxytocin is secreted every few minutes to cause ejection of milk. A brief high frequency spike discharge of the cell bodies of oxytocin neurons, synchronized among the magnocellular nuclei, precedes the release of an oxytocin hormone pulse [187]. It has also been shown that a similar high frequency bursting discharge of oxytocin neurons occurs during parturition, shortly before the birth of each pup, where each burst leads to the secretion of a pulse of oxytocin hormone into the circulation [188].
Each pulse of oxytocin then stimulates uterine contractions to expel the next pup. The contracting uterus and the birth canal, as the fetus passes through it, send neural signals to the hypothalamus to stimulate oxytocin release. Since oxytocin stimulates further uterine contractions, which in turn, generates additional neural signals, this process involves positive feedback regulation of oxytocin secretion that ends with delivery of the newborn. A similar secretion pattern of oxytocin has been observed in the pig [189], and in women [190].

In humans and rats, this pulsatile pattern of oxytocin secretion has been shown, to be more efficient for the birth process in that less oxytocin is required to achieve delivery, compared to continuous oxytocin secretion [191, 192]. The intermittent stimulation of the uterus, by pulsatile oxytocin secretion, prevents oxytocin receptor desensitization in the myometrium (uterine muscle) that occurs in response to continuous exposure to oxytocin. It has also been suggested that the pulsatile secretion of oxytocin conserves the stores of oxytocin in the posterior pituitary gland, of which about one third is used during a normal parturition, for possible further secretion in the event of dystocia, i.e. abnormal or difficult childbirth or labour [193].

In the last few days of pregnancy, oxytocin neurons are already capable of displaying burst firing in response to suckling and vagino-cervical stimulation [194, 195], so it seems that either of these two types of afferent input are capable of inducing burst firing. No other known natural stimuli have been shown to induce burst firing of oxytocin neurons [196, 197].

It has been suggested that there may be a hierarchy of mechanisms that ensure coordinated bursting of oxytocin neurons during parturition and lactation. Among those mechanisms, there is a crucial autocrine and paracrine role of somatodendritically released oxytocin (See section 1.3.1 B I), which has presynaptic as well as postsynaptic actions [198, 199]. Also, at the end of pregnancy, the glial processes retract from between oxytocin neuron cell bodies and dendrites, which facilitate interactions among the transmitters and modulators, produced presynaptically or by the oxytocin neurons themselves, and possibly decrease the active clearance of neurotransmitters by glial cells [200, 201]. Therefore, signals from other brain areas, as well as from oxytocin neurons themselves, can be more efficiently conveyed. This might be important to facilitate afferent inputs during parturition and lactation, when oxytocin is required for birth and milk ejection, respectively.
1.3.3 Physiological Functions of Oxytocin in Reproduction

A) Role for Oxytocin in Parturition

A large body of evidence in the literature supports the importance of oxytocin in driving the birth process. Oxytocin infusion was shown to initiate labour and an oxytocin antagonist was shown to be effective in delaying the start of parturition or impeding its progress in several species [202, 203], and also delaying threatened preterm labour in women [204]. Using radioimmunoassay techniques, it was shown that oxytocin concentration increases in the circulation during parturition in several species [189, 205-208]. Nevertheless, genetically modified mice to inactivate the oxytocin hormone, or oxytocin receptor, gene still undergo parturition and give birth. Therefore, there seems to be a redundancy in the mechanisms that bring about parturition, and while oxytocin is important for parturition, it is not essential [209, 210].

B) Lactation

Oxytocin, along with prolactin, is essential for milk delivery to the young during lactation. Suckling stimulates the nipple to send a neural signal through an ascending neuronal pathway from the nipple to the hypothalamus to stimulate oxytocin release from the posterior lobe of the pituitary gland, which in turn acts on the mammary glands to cause milk ejection, this is called the milk ejection reflex. Powerful evidence for an essential role for oxytocin in the milk ejection reflex comes from oxytocin knockout mice that fail to secrete milk to the suckling young, even though the mammary glands are able to produce milk in these animals [211, 212]. Prolactin is the other main hormone regulating lactation, which acts mainly on mammary glands to stimulate milk synthesis (Fig. 1.6). The interplay between these two hormones is believed to be important in regulating lactation, and possibly other reproductive functions.
Suckling of the young during lactation send signals to the hypothalamus to regulate the secretion of both prolactin and oxytocin hormones. The hormone prolactin, secreted from the anterior pituitary gland, is vital for milk synthesis during lactation. Suckling sends neural signal to the hypothalamus to decrease inhibitory input to prolactin secretion, DA secretion (DA), leading to increased plasma prolactin and ultimately increased milk synthesis from the mammary glands. The same neural signal stimulates oxytocin cells to fire in coordinated bursts that generate pulsatile oxytocin secretion to promote rhythmic contraction of the myoepithelial cells of the breast to induce milk let-down.

**Figure 1-6 Hormonal regulation of lactation**

Suckling of the young during lactation send signals to the hypothalamus to regulate the secretion of both prolactin and oxytocin hormones. The hormone prolactin, secreted from the anterior pituitary gland, is vital for milk synthesis during lactation. Suckling sends neural signal to the hypothalamus to decrease inhibitory input to prolactin secretion, DA secretion (DA), leading to increased plasma prolactin and ultimately increased milk synthesis from the mammary glands. The same neural signal stimulates oxytocin cells to fire in coordinated bursts that generate pulsatile oxytocin secretion to promote rhythmic contraction of the myoepithelial cells of the breast to induce milk let-down.
1.4 Interactions between Prolactin and Oxytocin

Oxytocin has been observed to increase prolactin secretion in vitro from anterior pituitary explants and also in vivo [213], but not in the presence of DA, the main prolactin inhibitory hormone [214]. The oxytocin receptor is expressed in lactotrophs of the anterior pituitary gland, and its expression is greatly increased at the end of pregnancy and is stimulated by oestrogen treatment [150-152]. The concentration of oxytocin in hypothalamo-hypophysial portal blood was shown to be correlated with that of prolactin in peripheral blood, suggesting possible interaction between the two hormones [215].

Moreover, MNCs of the SON are also activated following intracerebroventricular (i.c.v.) administration of prolactin. Prolactin has also been shown to specifically enhance oxytocin mRNA expression in the hypothalamus, as well as stimulate in vivo oxytocin secretion into the portal blood, and in vitro from hypothalamic explants. As mentioned earlier, prolactin can enter the brain via the choroid plexus of cerebral ventricles, and hypothalamic nuclei have also been shown to produce prolactin. Therefore, prolactin acting in the brain could be produced locally, or from the anterior pituitary gland. MNCs of the hypothalamus, mainly SON and PVN, undergo some changes and adaptations during pregnancy and lactation, which include changes involving PRL-R expression. Therefore, the interaction between prolactin and oxytocin is likely to be altered during this time of reproduction, and this project will focus on the altered prolactin signalling in oxytocin MNCs in the SON during lactation.
1.5 Aim

The key objectives of this study are: 1: to determine prolactin effects on oxytocin cells and the cellular mechanisms that underpin these effects; and 2: To determine whether these effects are modified in different reproductive states.

In order to understand the relationship between the hormones prolactin and oxytocin and the adaptations that occur in this relationship during reproduction, I will investigate the effects of the hormone prolactin on the firing rate of oxytocin neurons as well as the activation of the classical second messenger system (Jak/STAT pathway) downstream of the PRL-R. To achieve this, the following experiments will be performed:

**In vivo** electrophysiological recordings will be made from oxytocin neurons of the SON during i.c.v. administration of prolactin. This experiment will be done in both virgin and lactating female rats.

Immunohistochemistry will be carried out on brain sections collected from both virgin and lactating female rats treated i.c.v. with either vehicle or prolactin, to neurochemically identify possible changes in the activation of the classical intracellular messenger system downstream of the PRL-R (Jak/STAT pathway) to aid in determining the cellular mechanisms that mediate prolactin action on oxytocin neurons.

**Hypotheses:**

1/ The inhibitory effects of endogenous prolactin on the electrical activity of oxytocin MNCs will be reduced over the course of pregnancy or early lactation.

2/ The reproduction-induced adaptations in oxytocin MNC responses to prolactin might be mediated by changes in second messenger systems downstream of the prolactin receptor.
Chapter 2: Methodology

All procedures were approved by the University of Otago Animal Ethics Committee (AEC#: 05/07)

2.1 Animals and Preparatory Procedures

Adult female Sprague-Dawley rats (8-10 weeks; 200-275 g) were purchased from the Hercus Taieri Resource Unit, University of Otago. All animals were housed under a 12:12 hour light-dark cycle (lights on: 06:00 am) with unrestricted access to food and water. All rats were individually labelled by a serial number on the base of the tail, using a black marker pen.

A vaginal smear was taken each morning to determine the reproductive stage of each female rat used. The reproductive cycle, or the oestrous cycle, of female rats is composed of four different stages; pro-oestrus, oestrus, dioestrus 1 and dioestrus 2. Ovulation occurs overnight between pro-oestrus and oestrus. Hence, the normal cycle length in the female rat is 4 days [216, 217].

The characterization of each stage of the oestrous cycle is based on the proportion of three types of cells observed in the vaginal smear: round and nucleated cells (epithelial cells); irregular cells without a nucleus (cornified cells); and small round multinucleated cells (leukocytes). The appearance of these cells typically correlates with the status of the vaginal mucosa, uterus, and ovaries and is linked to identifiable alterations in plasma concentrations of the ovarian, as well as anterior pituitary, hormones.

In a standard 4-day cycle, pro-oestrus is identified by the presence of clusters of epithelial cells. Pro-oestrus lasts for 1 day and is followed by vaginal oestrus. Oestrus is consistently identifiable by the presence of large numbers of cornified, or keratinized, cells. The predominance of cornified cells will last one day in a 4-day cycle. Following leukocytic infiltration during the early part of the first day of dioestrus (dioestrus 1), the smear is characterized by a combination of leukocytes and cornified and rounded epithelial cells. These round epithelial cells commonly persist
during days 1 and 2 of dioestrus, when they co-occur with leukocytes in the smear. The concentration of leukocytes can vary, and the smear can often be almost exclusively leukocytic. The second day of dioestrus (dioestrus 2) may also show a few small clumps of nucleated epithelial cells, which is followed by pro-oestrus a day later to complete the cycle [217, 218].

If samples are taken early in the day, one will often see a smear that will reflect a transition between the current day and previous stage. The same is true for a sample taken late in the day, when cells characteristic of the next day are beginning to appear. For this reason, vaginal smears were taken at approximately the same time each day, between 9.00 am and 11.00 am.

All virgin female rats used in these experiments were in the dioestrous stage of their cycle, as determined by vaginal cytology. Rats were observed to undergo at least one normal 4-day cycle before they were used for an experiment.

To prepare lactating rats, the cycle of a group of female rats was established, using vaginal cytology, as detailed above. Animals in the pro-oestrus stage of their cycle were marked as candidates for breeding. For in vivo electrophysiology experiments, only one female rat was mated per day, as only one animal can be used for electrophysiological recording at any one time. For immunohistochemistry, all rats exhibiting pro-oestrus were selected as candidates for mating, since a large number of animals can be processed on any one day. In these experiments, up to 12 female rats were selected each day. A total of 20 female rats were mated, and 18 of those were successful in reaching the targeted stage of lactation, lactation day 7.

Selected female rats were placed with a breeder (stud) male rat in a separate cage overnight. The following day, female rats had a vaginal smear taken and were returned to the female group cage after the serial number on the base of the tail was noted for identification. The presence of sperm in the smear confirmed successful mating, and the day following mating was denoted day zero of pregnancy. At day 16 of pregnancy, 5 days before parturition, pregnant female rats were separated into individual cages for the reminder of the pregnancy to term and during lactation.
Since the number of pups varies between litters, on the day following parturition (day 1 lactating), mothers with more than eight pups had the extra pups removed and euthanized, so that the litter size was normalised for all females.

For *in vivo* electrophysiological recordings, on the day of the experiment, the DA receptor agonist, bromocriptine, was administered following the rat losing consciousness after anaesthesia (see section 2.2.1). Recordings were made at least 4 hours after bromocriptine administration. For lactating rats, pups were removed at the same time as bromocriptine was administered.

For immunohistochemistry experiments, the pups were removed and bromocriptine was administered at least 4 hours before the rats were anaesthetised and perfused. The pups were euthanised by decapitation if less than 5 days old, or by CO₂ gas if older than 5 days of age.

### 2.2 In Vivo Electrophysiology

#### 2.2.1 Anaesthesia and Cannulations

Rats were anesthetized with an intraperitoneal (i.p.) injection of urethane (ethyl carbamate; 1.25 g/kg). Maintenance anaesthesia was not required over the four hours of experimentation as the initial dose of the non-recovery anaesthetic, urethane, was sufficient as indicated by no evidence of a hindlimb withdrawal reflex during this period. Once the animal reached a surgical level of anaesthesia (determined by a complete cessation of the flexor withdrawal reflex), a 22-guage stainless steel guide cannula, for i.c.v. prolactin administration, was stereotaxically implanted into the right lateral cerebral ventricle (0.8 mm anterior to bregma, 1.3 mm lateral to bregma, 4 mm below the surface of the skull) and secured with two small screws fixed into the skull and acrylic dental cement. Next, the animal was removed from the stereotaxic frame and placed supine on a firm polystyrene block for femoral vein and trachea cannulation. The right femoral vein was cannulated using silicon tubing, which was used for intravenous (i.v.) cholecystokinin (CCK) administration, as well as for withdrawal of blood samples (data not analysed). The trachea was cannulated with an L-shaped polyethylene tube to maintain breathing throughout the experiment. Finally, bromocriptine (3 mg/kg) was injected subcutaneously (s.c.)
to inhibit endogenous prolactin secretion. At the end of the experiment, animals were killed by an anaesthetic overdose.

2.2.2 Transpharyngeal Surgery for Supraoptic Nucleus (SON) Exposure

The rat was placed supine in a modified stereotaxic frame that holds the rat’s head stationary, using ear, incisor and nose bars, to allow precision during surgery and recording. The mandible (lower jaw) was split between the incisors using stout scissors, and then both halves, as well as the tongue, were individually retracted to expose the oral cavity. The soft palate and the soft tissue overlying the hard palate were removed by cautery, and then the hard palate was removed using a high speed drill to expose the nasal cavity. Thereafter, the soft tissue around the upper jaw and the base of the right teeth was removed by cautery to expose the zygomatic bone, which approximately overlies the SON. The nasal membrane overlying the sphenoid bone and the medial aspect of the zygomatic bone was removed. A branch of the trigeminal nerve runs below the zygomatic bone, with an underlying vein that runs medially to the nerve. To expose the SON, the right zygomatic bone overlying the SON was drilled away, revealing the branch of the trigeminal nerve. This nerve was then removed using fine forceps (Dumont #3) to expose the meninges over the surface of the right SON. The vein that runs with the trigeminal nerve was occluded caudal and rostral to the underlying SON using small pieces of paper tissue and then the portion of vein between the two pieces of paper tissue was removed using fine forceps. Lastly, a small nick was made in the meninges overlying the SON to allow the insertion of a recording electrode.

Axons of the magnocellular neurons of the SON project to the posterior pituitary gland via the pituitary stalk. Electrical stimulation of the pituitary stalk sends an antidromic action potential that can be detected in the cell bodies of MNCs of the SON. To expose the pituitary stalk, a small hole was drilled at the caudal extent of the venous sinus in the sphenoid bone (visible as a dark portion of bone on the midline caudal to the level of the SON) which was packed with bone wax to occlude the sinus. Then the hole was extended rostrally through the sinus’s visible borders to allow enough room for the stimulating electrode to be positioned. Finally, the remaining bone overlying the surface of the brain was removed to expose the pituitary stalk.
2.2.3 In vivo Electrophysiological Recording

Extracellular single-unit activity of SON neurons was recorded using a glass micropipette, filled with physiological (0.9%) saline, via an Ag/AgCl electrode wire. SON MNCs, namely oxytocin and vasopressin neurons, have their cell bodies located in the SON nucleus and send axons to the pituitary gland via the pituitary stalk. Hence, a bipolar side-by-side stimulating electrode was placed on the pituitary stalk and set to deliver matched biphasic pulses (1 ms. 1-10 mA peak to peak) for antidromic identification of SON neurons. An antidromic spike of constant latency from the stimulus identified MNCs as projecting to the posterior pituitary gland. The neurons were characterized as either oxytocin or vasopressin cells by their activity pattern and, where necessary, by their response to intravenous CCK (20 µg/kg in 0.9% saline). Oxytocin neurons transiently increase their firing rate following a systemic CCK injection, while vasopressin neurons are inhibited, or show no response to CCK [219-223].

2.2.4 Firing Rate Analysis

Neuronal activity was recorded onto a computer and analysed off-line using Spike2 software version 5.13 (Cambridge Electronic Design, UK). The mean firing rate of each neuron was calculated over the five minute period immediately before and after prolactin administration and the mean was obtained by dividing the total number of spikes over the five minute period over 300 seconds. Statistical analysis was performed as detailed in section 2.4 below.
2.3 Double Label Immunohistochemistry for pSTAT5 and Oxytocin after Prolactin or Vehicle Treatment in Virgin and Lactating Female Rats

Female Spargue Dawley rats (270-360 g; n = 7 for vehicle treated virgin group; n = 9 for prolactin treated virgin group, vehicle treated lactating group and prolactin treated lactating group) were implanted with lateral ventricle cannulae (see section 2.3.1) and allowed to recover for 5 days before the animals were injected i.c.v. with 1 µl prolactin (1 µg/µl) or vehicle (aCSF; pH = 7.2, composition in mM: NaCl 138, KCl 3.36, NaHCO₃ 9.52, Na₂HPO₄ 0.49, urea 2.16, CaCl₂ 1.26 and MgCl₂ 1.18). Thirty minutes after injection, animals underwent transcardial perfusion (see section 2.3.2). Fixed brains were sectioned and then an immunohistochemical protocol for free floating brain sections was performed. Immunohistochemistry is used for the localization of antigens or proteins in tissue sections by the use of labelled antibodies through antigen-antibody interactions that can be visualized by a marker such as an enzyme or a fluorescent dye. This project used double-label enzyme based immunohistochemical techniques for nuclear pSTAT5 and cytoplasmic oxytocin.

2.3.1 Implantation of Guide Cannula – Lateral Ventricle Injection

Animals were anaesthetised with 2-3% halothane in 2 l/min oxygen, and anaesthesia was confirmed by a complete cessation of the flexor withdrawal reflex. Rats were positioned in a stereotaxic frame and held in the frame using ear bars positioned on the external auditory meatus and an incisor (nose) bar. This setting holds the head stationary for precise positioning of the guide cannula. Before starting the surgery, rats were administered an anti-inflammatory drug, Carprofen (5mg/kg; s.c.).

The fur from the dorsal portion of the head was shaved and a 2-3 cm incision was made along the midline of the skull exposing the periosteum of the cranium. The periosteum was displaced laterally to reveal the cranial surface and identify landmarks. The guide cannula was positioned 1.3 mm lateral and 0.8 mm posterior to bregma (co-ordinates determined from the Rat Brain Atlas [224]). A guide cannula was inserted to a depth of 3 mm through a hole drilled in the cranium. The cannula was fixed in position using acrylic dental cement adhered to two small screws placed anterior and posterior to the guide cannula. Once the dental cement was dry, animals
were removed from the frame and allowed to recover from anaesthesia in their respective cages, placed on a heating pad, until consciousness was fully regained.

2.3.2 Perfusion Method (Transcardial Perfusion)

Rats were first anaesthetised by intraperitoneal (i.p.) administration of sodium pentabarbitone (6 mg/100 g body weight) containing sodium heparin (1000 IU). Upon reaching surgical anaesthesia, confirmed by loss of flexor withdraw reflex, the thoracic cavity was exposed and transcardial perfusion was carried out via the left cardiac ventricle with 200 ml of heparinised (3000 IU/l) isotonic saline (0.9% NaCl). Next, rats were perfused with 300 ml of the fixative solution containing 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.2 ± 0.1) solution. Brains were then extracted from the skull and post-fixed in 4% paraformaldehyde in 0.1 M PB overnight before cryoprotection in 30% sucrose solution in 0.1 M PB for at least 24 hours at 4°C, to prevent the formation of ice crystals during freezing. Brains were soaked in the 30% sucrose solution until the solution had penetrated the brain and caused it to sink to the bottom of the container. Brains were then frozen on dry ice and stored at -80°C until processed for immunohistochemistry.

2.3.3 Double Label Immunohistochemistry for pSTAT5 and Oxytocin

Perfused brains were sectioned (30 µm) using a freezing-sledge microtome. Sections were collected into cryoprotectant solution in 12-well Petri dishes. Brain sections were collected in three sets, in which each set contained every third section (i.e. 90 µm apart), then sections were stored at -20°C until used. One set was used for the experiment, while the other two sets were kept as backup if the experiment failed. Before performing the pSTAT5 and oxytocin immunohistochemistry protocol for free floating brain sections, the cryoprotectant solutions the sections were bathed in was rinsed in 0.05 M Tris Buffered Saline (TBS; 6.06 % Tris-HCl, 1.39 % Tris Base and 8.7 % NaCl; pH = 7.6 ± 0.1) 6 times. All rinses and incubations were of 10 min duration, using 12-well Petri plates, and occurred on an orbital shaker, set to rotate at 50 rpm, unless otherwise stated.
To start with, tissue underwent an antigen-retrieval procedure, required for pSTAT5 staining, to improve antigenic site availability for antibody binding. This was achieved by placing brain slices in plates containing boiling 0.01 M Tris solution (pH = 10) placed on a heat block (at 110°C) for five min, followed by 10 min cooling time. The sections were then incubated in a blocking serum (0.25 % Bovine Serum Albumin in 0.05 M TBS plus 0.3% Triton X-100) for 10 min to reduce non-specific binding of the antibody. Endogenous peroxidases were deactivated by incubation for 10 min in 0.05 M TBS containing 3% hydrogen peroxide and 40% methanol, followed by three washes in 0.05 M TBS to terminate the reaction. Sections were incubated for 24 h at 4°C in a rabbit anti-pSTAT5 antibody (1:1000, tyr 694, Cell Signalling) diluted in the blocking serum containing 2% normal goat serum (NGS).

Excess antibody was removed by washing the sections three times in 0.05 M TBS, and sections were then incubated for 1½ h at room temperature in a biotinylated secondary goat anti-rabbit antibody (1:300; Vector, BA-1000) diluted in the blocking serum. Sections were washed three times in 0.05 M TBS, followed by a further incubation in avidin-biotin peroxidase complex (1:100 in the blocking serum; Vectastain ) for 1 h at room temperature. The bound antibody-peroxidase complex was visualised using a nickel-enhanced diaminobenzidine (DAB) reaction. The reaction was followed using a microscope until a strong black/purple precipitate was evident in cell nuclei, and halted by a short rinse in 0.05 M TBS followed by three washes in 0.05 M TBS.

Endogenous peroxidases were re-quenched by incubation for 10 min in 0.05 M TBS containing 3% hydrogen peroxide and 40% methanol, followed by three washes in 0.05 M TBS to terminate the reaction. Sections were then incubated for 24 h at 4°C in a monoclonal mouse anti-oxytocin antibody (1:20000) diluted in the blocking serum containing 2% normal goat serum (NGS). Sections were then washed three times in 0.05 M TBS to remove excess antibody, before sections were incubated for 4 h at room temperature in a secondary goat anti-mouse horse radish peroxidase antibody complex (1:300) diluted in the blocking serum. Following three washes in 0.05 M TBS, the antibody-peroxidase complex was visualised using a standard DAB reaction. The reaction was followed under the microscope and terminated, by rinsing the sections three times in 0.05 M TBS, following the formation of a brown cytoplasmic precipitate.
Sections were mounted from 0.05 M TBS onto glass microscope slides coated with gelatine. Slides were allowed to dry overnight before dehydration through a graduated series of alcohol followed by two rinses in xylene. Slides were then allowed to dry overnight, before being cover-slipped using a xylene based mountant.

Brain sections were then viewed under a light microscope (Olympus BX51) for cell counting. All cells labelled for oxytocin (brown staining) in the SON region were counted from three sections for each brain, and considered to be single labelled cells. Next, SON oxytocin cells that were also labelled for pSTAT5 (black staining in the nucleus) were counted in the same brain sections, and considered to be double-labelled cells. The number of all double-labelled cells divided by all oxytocin cells (both single and double labelled), from all three sections, was considered to be the average percentage activation of oxytocin neurons for each brain. The percentage activation was used for subsequent analysis. Images of brain sections were taken with a camera (RTKE, model no. 7.4 Slider, Diagnostic instruments, Inc.) attached to the light microscope (Olympus BX51), using Spot programme (Spot Software, version 4.6, Diagnostic Instruments, Inc.).

2.4 Statistical Analysis

Data are expressed as mean ± standard error of the mean SEM. Pre- and post-prolactin results were compared between virgin and lactating animals by two-way repeated measure ANOVA (analysis of variance) followed by Bonferroni’s post-hoc test where the variance ratio (F ratio) was significant. Statistical significance was taken as P < 0.05. The F ratio is stated when significance was found. All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software Inc, USA)
Chapter 3: Results

3.1 In Vivo Electrophysiology

Recordings were made from 35 female Sprague-Dawley rats (260–400 g), 18 lactating (day 6-12 post-partum) rats and 17 virgin rats in the dioestrous stage of their cycle. The spontaneous activity of 78 cells was recorded. In virgin animals, a total of 16 SON cells were recorded, 10 of which were positively identified as oxytocin cells and of these, six cells were recorded during i.c.v. injection of prolactin. In addition, six vasopressin cells were positively identified and five of these were recorded during prolactin administration. In lactating rats, six out of seven oxytocin cells were recorded during prolactin administration, whereas none of the three identified vasopressin cells were recorded during prolactin injection. The main reason for not recording cells during prolactin injection is early loss of signal. All animals used were treated with the dopamine agonist bromocriptine at least four hours before recording to inhibit endogenous prolactin secretion and eliminate the potential confounding effects of endogenous prolactin.

3.1.1 Dose-Dependent Inhibition of Oxytocin Neuron Firing Rate by Prolactin in Virgin Female Rats

To determine the appropriate dose of prolactin to be used in comparing virgin and lactating rats, the effect of two different doses (0.5 µg and 1.0 µg) of prolactin on the firing rate of oxytocin cells was investigated in virgin rats. Figure 3.1 shows a ratemeter recording of the firing rate of an oxytocin cell over 90 min. The firing rate of oxytocin cells was consistently reduced for several min following the injection of either dose of prolactin, but there was a more profound inhibition by the 1.0 µg dose of prolactin. Figure 3.2 shows the mean firing rate of oxytocin cells five min pre- and five min post-administration of prolactin. This “dose-ranging” experiment was preliminary, and although the number of cells recorded (“n” number) is too low to demonstrate statistical differences, the larger dose was selected for subsequent experiments as it generated the most consistent response.
Figure 3-1 Representative ratemeter recording of oxytocin cell activity in a virgin rat

Ratemeter recording (in 10 s bins) of the firing rate of an identified oxytocin neuron (identified by a transient excitatory response to i.v. CCK) in a urethane-anaesthetized virgin rat. Note the reduction in the firing rate of the oxytocin neuron after an intracerebroventricular (i.c.v.) injection of prolactin (PRL; 1.0 µg and 0.5 µg, respectively; n = 3 per group) that was typical of all cells tested.
Figure 3-2 The effect of different doses of i.c.v. prolactin on oxytocin cell activity in virgin rats

Mean firing rate of SON oxytocin neurons from virgin rats before and after i.c.v. treatment with 0.5 μg or 1.0 μg of prolactin (PRL; n = 3 per group). Each bar represents the mean (± SEM) of the five-min period immediately before and after prolactin administration. F-ratio = 3.3, P-value = 0.14, *, two-way repeated measure ANOVA, * P < 0.05.
3.1.2 Prolactin Inhibits the Firing Rate of Oxytocin Neurons in Virgin, but not Lactating, Female Rats

Based on the data above, 1.0 µg of prolactin was used in subsequent experiments to investigate the effect of prolactin on the firing rate of oxytocin neurons of the SON in virgin and lactating female rats. Fig. 3-3 is a representative ratemeter recording of the firing rate of an oxytocin neuron in virgin female rats and shows a profound depression of the firing rate of the oxytocin neuron in response to 1.0 µg of prolactin injected into the lateral ventricle of a virgin female rat, which was consistent across multiple recordings. On the other hand, Fig 3-4 is a representative ratemeter recording of the firing rate of an oxytocin neuron in a lactating female rat, showing a lack of a significant change of the firing rate of the oxytocin in response to i.c.v. administration of prolactin.

In virgin female rats, the mean firing rate of oxytocin cell of the five min period immediately before prolactin administration was 4.3 ± 0.7 spikes/s, while the mean firing rate of the five min period immediately after prolactin administration was 2.2 ± 0.4 spikes/s. In lactating female rats, oxytocin cell mean firing rate went from 4.3 ± 1.0 spikes/s to 4.5 ± 0.9 spikes/s in response to i.c.v. prolactin (Fig 3-5). Two-way repeated measures ANOVA (analysis of variance) followed by Bonferroni’s post-hoc test demonstrated that the 1.0 µg dose of prolactin produced a significant reduction in the firing rate of oxytocin cells in virgin, but not lactating, female rats. Also, the change in the firing rate of oxytocin neurons in response to prolactin was significantly different between virgin and lactating rats, F-ratio = 5.8, P-value = 0.037 (Fig. 3-5).
Figure 3-3 The effect of 1.0 µg of prolactin on oxytocin cell activity from a virgin rat

Representative ratemeter recording (in 10 s bins) of the firing rate of an identified oxytocin neuron in a urethane-anaesthetized virgin rat. Note the reduction in the firing rate of the oxytocin neuron after an intracerebroventricular (i.c.v.) injection of 1.0 µg prolactin (PRL).
Figure 3-4 The effect of 1.0 µg of prolactin on oxytocin cell activity from a lactating rat

Representative ratemeter recording (in 10 s bins) of the firing rate of an identified oxytocin neuron in a urethane-anaesthetized lactating rat. Note the lack of significant change in the firing rate of the oxytocin neuron after an intracerebroventricular (i.c.v.) injection of 1.0 µg prolactin (PRL).
Figure 3-5 The effect of 1.0 μg of i.c.v. prolactin on oxytocin cell firing rate in virgin and lactating rats

Mean firing rate of SON oxytocin neurons from virgin and lactating (day 6-12 post-partum) rats before and after i.c.v. treatment with 1.0 μg of prolactin (PRL; n = 6 per group). Each bar represents the mean firing rate (± SEM) of the 5-min period immediately before and after prolactin administration. F-ratio = 5.8, P-value = 0.04 two way repeated measures ANOVA, followed by Bonferroni’s post-hoc test; * P < 0.05.
3.1.3 Prolactin Does not Alter the Firing Rate of Vasopressin Cells in Virgin Female Rats

To determine whether the observed effects of prolactin were specific to oxytocin neurons, vasopressin cell activity was recorded from five virgin female rats. Fig 3-5 is a representative ratemeter recording of the firing rate of a vasopressin neuron in a virgin female rat, showing a lack of a significant change of the firing rate of the vasopressin cell in response to i.c.v. administration of 1.0 µg prolactin. The mean firing rate of the five min period immediately before prolactin administration was 8.8 ± 2.9 spikes/s, while the mean firing rate of the five min period immediately after prolactin administration was 8.8 ± 2.8 spikes/s (Fig. 3-7). The 1.0 µg dose of prolactin produced no significant alteration in the firing rate of vasopressin cells in virgin female rats (P = 0.7, paired t-test).
Figure 3-6 The effect of 1.0 µg of prolactin on vasopressin cell activity from a virgin rat

Ratemeter recording (in 10 s bins) of the firing rate of an identified vasopressin neuron in a urethane-anaesthetized virgin rat. Note the lack of significant change in the firing rate of the vasopressin neuron after an intracerebroventricular (i.c.v.) injection of 1.0 µg prolactin (PRL).
Figure 3-7 The effect of 1.0 µg of i.c.v. prolactin on vasopressin cell firing rate in virgin rats

Mean firing rate of SON vasopressin neurons from virgin rats before and after i.c.v. treatment with 1.0 µg of prolactin (PRL; n = 5). Each bar represents the mean (± SEM) of the 5-min period immediately before and after prolactin administration. P-value = 0.7, paired t-test.
3.1.4 Prolactin Does not Alter the Firing Rate of Neurons in the Peri-SON Vicinity

To further confirm that the prolactin effect seen is specific to oxytocin neurons, the effect of 1µg of prolactin injected i.c.v. on the firing rate of neurons in the perinuclear zone of the SON (peri-SON) in female rats was investigated. The firing rate in the peri-SON showed no alteration in response to i.c.v. prolactin injection (data not shown). The lack of alteration of the firing rate of these neurons was consistent across multiple recordings, in both virgin and lactating rats. This finding further confirms the reduction in firing rate seen is specific to oxytocin neurons in virgin rats only.

3.2 Immunohistochemistry

Double label immunohistochemistry for pSTAT5 and oxytocin was performed on brain sections collected from 34 female Sprague-Dawley rats (260–400 g), 18 lactating (day 6-8 post-partum) rats and 16 virgin rats in the dioestrous stage of their cycle. The animals were divided into four groups; vehicle treated virgin group (n = 7), prolactin treated virgin group (n = 9), vehicle treated lactating group (n = 9) and prolactin treated lactating group (n = 9). Brain sections from six animals were selected from each group for this experiment. The effect of i.c.v. injection of 1.0 µg prolactin on the activation of the transcription factor STAT5 in oxytocin cells of the SON was investigated. All animals used were treated with bromocriptine to inhibit endogenous prolactin secretion, at least four hours before perfusing the animal, to eliminate the potential confounding effects of endogenous prolactin.
3.2.1 Prolactin Increases the Phosphorylation of STAT5 in Virgin, but not Lactating, Female Rats

Fig. 3-8 shows representative images of the SON in virgin and lactating female rats. The average number of cells single labelled (for oxytocin) and the average number and percentage of double labelled (for oxytocin and pSTAT5) cells, is shown in table 3-1. The average percentage of double labelled (for oxytocin and pSTAT5) cells is shown in Fig. 3-9. In virgin female rats, the percentage of SON oxytocin cells co-labelled with pSTAT5 changed from 0.0 ± 0.0% in the vehicle treated group to 55.5 ± 17.0% in the prolactin treated group. On the other hand, SON oxytocin neurons in lactating female rats treated with vehicle, showed very high levels of pSTAT5 in oxytocin cells (70.1% ± 12.1), which was not significantly different from prolactin treated lactating female rats (85.5% ± 8.0). Two-way repeated measures ANOVA followed by Bonferroni’s post-hoc test demonstrated that the 1.0 µg dose of prolactin produced a significant elevation in the percentage of SON oxytocin cells double labelled with pSTAT5 in virgin (P < 0.05), but not lactating (P > 0.05), female rats. However, the activation of pSTAT5 in SON oxytocin cells was not significantly different between virgin and lactating rats, F-ratio = 3.2, P-value = 0.088.
Representative images of pSTAT5 in oxytocin neurons of the SON of virgin and lactating rats treated with vehicle or prolactin

All four groups were treated with bromocriptine to control for the potential confounding effect of endogenous prolactin. Virgin female rats treated with vehicle (aCSF; A) show no pSTAT5 precipitate (stained black when present) in the nuclei of identified oxytocin cells (stained brown) in the SON. Oxytocin neurons (brown) from prolactin treated virgin females (B) show pSTAT5 precipitate (black) in a number of cell nuclei (arrow), however not all oxytocin cells were double labeled with pSTAT5 (arrowhead). Lactating female rats treated with vehicle (C) and lactating females treated with prolactin (D), both show a similar proportion of double stained pSTAT5 and oxytocin neurons (arrow) with few single labelled oxytocin neurons (arrowhead). Scale bars, 50 µm.
Table 3-1 The average counting of SON single labelled cells, double labelled cells and percentage of double labelled cells for each group

<table>
<thead>
<tr>
<th>Physiological State and Treatment</th>
<th>Single labelled oxytocin cells</th>
<th>Oxytocin and pSTAT5 double labelled cells</th>
<th>Percentage of double labelled oxytocin cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin Controls</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Virgin PRL</td>
<td>13</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td>Lactation Control</td>
<td>14</td>
<td>45</td>
<td>70</td>
</tr>
<tr>
<td>Lactating PRL</td>
<td>12</td>
<td>56</td>
<td>85</td>
</tr>
</tbody>
</table>

Figure 3-9 The effect of 1.0 µg of i.c.v. prolactin on the activation (phosphorylation) of STAT5 protein in oxytocin cells of the SON in virgin and lactating rats

Expression of pSTAT5 in SON oxytocin neurons from virgin and lactating (day 6-8 post-partum) female rats after i.c.v. treatment with 1 µl of vehicle (aCSF) or 1.0 µl (1 µg/µl) of prolactin (n = 6 per group). Each bar represents the percentage of double labelled oxytocin cells (± SEM) following treatment. F-ratio = 3.2, * P < 0.05.
Chapter 4: Discussion

4.1 Summary of Key Observations

This project has shown that i.c.v. administration of 1 µg of prolactin consistently inhibits the firing rate of SON oxytocin neurons in virgin female rats, in the dioestrous stage of their cycle, while this inhibitory influence was removed (disinhibition) in oxytocin cells of lactating rats, which showed no response to i.c.v. prolactin injection (fig. 3-3, 3-4 and 3-5).

The immunohistochemistry experiments showed that prolactin induces a significant increase in the activation of the Jak/STAT pathway, shown by a significant increase in phosphorylated STAT5 (pSTAT5) precipitate in the nuclei of SON oxytocin cells of virgin rats, compared to the vehicle-treated group. On the other hand, the prolactin- and the vehicle-treated lactating rats both showed very high levels of pSTAT5, which were at similar levels to the prolactin-treated virgin rats.

4.2 Discussion of the Electrophysiology Results

This study has confirmed previously published data documenting suppression of the firing rate of SON oxytocin neurons by i.c.v. prolactin injection [153] and has extended these observations by demonstrating disinhibition (removal of the inhibitory influence) of prolactin on oxytocin neurons during lactation. To investigate the specificity of prolactin actions, the firing rate of magnocellular vasopressin neurons, also found in the SON, was recorded during i.c.v. prolactin administration. The vasopressin neurons displayed no alteration in the firing rate following prolactin injection in virgin rats, so this was not investigated further in lactating rats. Furthermore, neurons in the peri-nuclear zone (peri-SON), which are known to project to the SON, displayed no change to their firing rate following i.c.v. prolactin injection in both virgin and lactating rats. This further confirms the specificity of the response to SON oxytocin cells. Therefore, i.c.v. injection of prolactin specifically suppresses the firing rate of oxytocin neurons of the SON only in virgin, but not in lactating, female rats.
The physiological implications of prolactin induced suppression of the firing rate of oxytocin neurons in virgin rats, in addition to the reported stimulation of oxytocin synthesis [225, 226] and secretion [227], are not clear at the present. A similar dissociation between suppression of firing and stimulation of hormone release has been reported for melanocyte stimulating hormone [228], thought to result in differential regulation of nerve terminal vs. dendritic release of the hormone. Therefore, it is possible that prolactin might be involved in the regulation of oxytocin neuronal firing patterns or regulating dendritic release of oxytocin. Dendritically released oxytocin is important as an autocrine as well as paracrine signal to coordinate the activity of oxytocin neurons, particularly during lactation and parturition. Both of these are states of hyperprolactinaemia, when oxytocin is required in large amounts at specific time, thus coordinated activation of the oxytocin neuron population is essential.

Prolactin suppression of oxytocin cell firing in virgin rats might not have a major physiological role, as prolactin levels are normally low in non-pregnant and non-lactating animals. However, if oxytocin cells respond to prolactin in a similar manner during pregnancy as in virgin rats, i.e. suppressed by prolactin, the inhibitory influence of prolactin on oxytocin cell activity might be important to stop premature activation of oxytocin neurons that might lead to preterm delivery, which is suboptimal for the survival of the offspring. On the other hand, during parturition and lactation, oxytocin is required in large amounts to stimulate the process of birth and milk ejection, respectively. As prolactin is elevated towards the end of pregnancy and during lactation, having an inhibitory influence on oxytocin firing and release during this time of reproduction would be physiologically disadvantageous. This is supported by our finding of prolactin disinhibition of oxytocin cell activity during lactation, which would allow other stimulatory inputs, such as the suckling stimulus, to enhance oxytocin release upon demand.

In addition to possible prolactin actions on oxytocin neurons, there is extensive evidence in the literature for a role of oxytocin in the regulation of prolactin secretion. Oxytocin stimulates prolactin secretion both in vitro from hypothalamic explants [229-232] and in vivo from the pituitary gland [233]. Conversely, oxytocin antagonists can block prolactin secretion under certain physiological conditions [230, 234, 235]. It has also been suggested that oxytocin may play a major role in stimulating the prolactin surges of early pregnancy [236, 237], and the pro-oestrous prolactin surge in the female rat [230].
Therefore, PRL-Rs on oxytocin neurons might be involved in feedback regulation of prolactin secretion via oxytocin. The predominant effect of prolactin on oxytocin cell firing in virgin females appears to be inhibitory, which could be interpreted as negative feedback suppression of a potent prolactin releasing factor. However, both stimulatory [238] and inhibitory [158, 239] actions 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.

The changes seen to the prolactin-oxytocin feedback loop are broadly similar to those seen in the prolactin-DA feedback loop. In virgin rats, prolactin stimulate DA release from TIDA neurons, which in turn inhibits prolactin synthesis. Thus prolactin ultimately suppresses its own release via this negative feedback loop also. During pregnancy and lactation, however, dopaminergic neurons do not respond to prolactin, thus, removing the inhibitory input of DA on prolactin secretion and allowing a state of hyperprolactinemia[154]. Hence, it may be concluded that oxytocin neurons may undergo adaptive changes to prevent the inhibitory influence of prolactin during, or prior to, lactation which would render oxytocin neurons more responsive to stimulatory inputs during pregnancy and lactation.

The mechanism by which oxytocin neurons respond to prolactin in virgin rats, and how this mechanism alters during lactation is not clear. Several studies have described different effects of peptides on the electrical activity of MNCs that are dependent on the route of administration, as well as the physiological conditions of the animals. For instance, i.c.v. injection of somatostatin increases SON oxytocin MNCs firing rate, while when retrodialyzed directly onto the SON, somatostatin decreases oxytocin cell activity [240]. However, large molecules such as prolactin cannot be given by microdialysis administration directly onto SON neurons. In addition, i.c.v. prolactin administration more accurately reflects normal physiology, where prolactin is likely transported from the blood into the cerebral ventricles, and therefore the brain, via PRL-Rs in the choroid plexus of the cerebral ventricles.
As prolactin is administered i.c.v. into the lateral ventricles, the actions on oxytocin cells might be direct, by activating PRL-R on oxytocin cells, or indirect, via activating afferent pathways that project to the oxytocin MNCs of the SON. However, the SON oxytocin neurons do express the PRL-R, suggesting that at least some of the actions of prolactin on oxytocin cells are likely to be direct. In addition, the i.c.v. injection may mimic the effects of prolactin entering the brain affecting prolactin-sensitive afferents to the SON oxytocin neurons. The actions of prolactin released locally within the SON might be different to those that result from i.c.v. administration.

Considering that prolactin actions on oxytocin neurons are probably mediated by directly activating the PRL-Rs on oxytocin cells, one might expect down-regulation of PRL-Rs to be a candidate mechanism for oxytocin cells to stop being inhibited by prolactin during lactation. However, we have data suggesting that there is no down-regulation in PRL-R expression on oxytocin neurons, rather it actually increases, during lactation [153, 241]. Hence, it seems most likely that signalling pathways downstream of the PRL-R, which mediate prolactin actions in oxytocin neurons, adapt during lactation to stop the inhibitory influence of prolactin on oxytocin cell firing. Investigating these signalling pathways is essential to understanding the mechanisms of action of prolactin in regulating oxytocin neurons.
4.3 Discussion of Signal Transduction Experiments

The PRL-R is a single membrane-bound protein that belongs to the type 1 cytokine receptor family [52], of which there are three key isoforms described in rats; the short, intermediate, and long forms [59]. The long form (PRL-RL) is the most studied and best characterized in the literature. PRL-RL has full functional capacity, i.e. is capable of activating all the signalling pathways that prolactin can induce. Among the signalling pathways that prolactin may induce are the Jak/STAT and ERK1/2 MAPK pathways. The major pathway that mediates prolactin action and is known to be required for some actions of prolactin in the brain is the Jak/STAT signal transduction pathway. The Jak/STAT5 pathway has been shown to be the major pathway mediating prolactin effects in TIDA neurons of the arcuate nucleus in virgin rats. In addition, inhibition of the Jak/STAT5 pathway in TIDA neurons during lactation prevents the stimulatory actions of prolactin on DA release from TIDA neurons. Therefore, the Jak/STAT5 signalling pathway is a prime candidate that might mediate prolactin effects on oxytocin neurons in a similar manner to TIDA neurons.

The immunohistochemistry results show that prolactin induces a significant increase in the activation of the Jak/STAT pathway, leading to a significant increase of phosphorylated STAT5 (pSTAT5) precipitate in the nuclei of SON oxytocin cells in virgin rats, compared to the vehicle-treated virgin rats which showed no pSTAT5 precipitate. Prolactin levels in virgin rats are quite low, thus, it was expected to find a very low pSTAT5 signal in oxytocin cells of the vehicle-treated virgin rats. However, the complete absence of pSTAT5 signal observed in brain sections from the vehicle-treated virgin rats, is most likely due to the administration of bromocriptine, which inhibits endogenous prolactin secretion. On the other hand, and rather unexpectedly, brain sections from the vehicle-treated lactating rats showed very high levels of pSTAT5 in the nuclei of SON oxytocin neurons, which was at similar levels to the pSTAT5 expression in the oxytocin cells of the prolactin-treated virgin and lactating females.
In lactating rats, prolactin levels are very high and thus there will be a dramatic increase in STAT5 activation, compared to virgin animals. If the half-life of pSTAT5 is more than four hours, some pSTAT5 will still be detectable in the nuclei of oxytocin cells in female rats treated with bromocriptine four hours earlier, as in the present protocol. Therefore, the elevated pSTAT5 levels in oxytocin cells of lactating rats seen in the current experiments might be explained by increased activation of STAT5 because of the increased endogenous levels of prolactin during lactation coupled with inadequate time to allow pSTAT5 levels to fall to baseline following bromocriptine injection, which inhibits endogenous prolactin.

Studies on the half-life of pSTAT1 protein, which belongs to the same family as pSTAT5, indicate that the half-life of pSTAT1 is less than one hour. In the presence of protease inhibitors to prevent rapid degradation of the protein, the half-life of STAT1 was still less than two hours. After four hours of activating STAT1 there was no detectable pSTAT1 in the nucleus [242]. If this proves to also be correct for pSTAT5, it will suggest that ligands other than pituitary prolactin might act on oxytocin cells of lactating rats to stimulate the Jak/STAT5 pathway, which may, or may not, be acting on the PRL-R. This could indicate activation of STAT5 via prolactin produced locally in the SON, which is not under the control of DA, and therefore would not be reduced by bromocriptine. In addition, pSTAT5 can be activated by alternative pathways, most likely involving the closely related cytokine growth hormone, or other ligands of the cytokine family that could also stimulate pSTAT5.

STAT5 is a transcription factor that mediates gene transcription. Whether prolactin-induced STAT5 activation leads to the transcription of oxytocin hormone genes or other genes, is not clear. Nevertheless, prolactin has been shown to increase oxytocin secretion both in vivo into the portal circulation, and in vitro from hypothalamic explants [213], which when coupled with our finding of prolactin-induced STAT5 activation may suggest a possible role of pSTAT5 in regulating oxytocin synthesis. Since pSTAT5 is a transcription factor, it is unlikely that it mediates the almost instantaneous inhibitory response to prolactin in the firing rate of oxytocin neurons of virgin rats. Nevertheless, pSTAT5 might be involved in the long term changes that occur in oxytocin cells of lactating rats that cause them to respond differently to prolactin, perhaps increasing oxytocin synthesis to meet the increased secretory demands of lactation.
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 studies showing rapid actions of prolactin on excitable cells. In vitro experiments produced evidence that prolactin induces rapid changes in the excitability of arcuate nucleus neurons [243] and ventromedial hypothalamic neurons [244-246]. The mechanism of prolactin action to bring about these rapid effects is not fully understood but is unlikely to involve activation of the Jak/STAT pathway, as this pathway results in changes in gene transcription, which alters cell function over a much longer time course. Nevertheless, prolactin-induced Jak2 activation, can also lead to phosphorylation, i.e. activation, of a range of ion channels [247] and intracellular protein kinases, including ERK1/2 MAPK, and PKC [248].

Prolactin administration to Chinese hamster ovary cells transfected with the PRL-RL, results in a rapid increase of intracellular Ca\(^{2+}\) [249] through a voltage-insensitive Ca\(^{2+}\) channel, leading to influx of extracellular Ca\(^{2+}\), as well as mobilization of intracellular Ca\(^{2+}\) stores [250, 251]. In addition, prolactin can induce membrane hyperpolarization, through direct activation of Ca\(^{2+}\)-dependent potassium (K\(^{+}\)) channels [104, 106, 250]. It is possible that this response also involves prolactin-induced activation of phosphoinositol-3 kinase and the production of a range of inositol phosphate molecules in the cytoplasm [252]. Similar prolactin-induced increases in intracellular Ca\(^{2+}\) are also seen in glial cell lines [253, 254] and GABAergic neurons [255] that endogenously express the PRL-R. Hence, there is evidence for both rapid stimulatory and inhibitory actions of prolactin on neuron activity, and it is plausible that either response could be induced, perhaps depending on the physiological state of the animal.
The ERK1/2 MAPK pathway is another signalling pathway mediating prolactin intracellular signal transduction [95-102], which was also shown to regulate ion channels in a manner that might render cells unresponsive to inhibitory stimuli. The ERK1/2 MAPK pathway is mainly associated with the short form of the PRL-R (PRL-RS), and the expression of PRL-RS was shown to be increased in the SON during lactation [241]. Therefore, investigating this pathway using both electrophysiological as well as immunohistochemical techniques is the next logical step to expand upon this project and to enhance our understanding of adaptations of prolactin regulation of oxytocin cell activity during reproduction. During preparation for this project, I have collected enough brain sections that another lab member will use to measure ERK1/2 MAPK pathway activation in both virgin and lactating rats, to determine whether this response is also altered during lactation.

4.4 Future Work

As mentioned above, prolactin activates the ERK1/2 MAPK, in addition to the Jak/STAT pathway. To investigate the involvement of the ERK1/2 MAPK pathway in regulating the activity of oxytocin neurons, I would like to carry out double-labelled fluorescent immunohistochemistry to detect any difference in the activation of this pathway in oxytocin neurons of virgin versus lactating rats. If a difference is detected, a MAPK pathway inhibitor would then be used during recording of oxytocin cell firing from both virgin and lactating rats, to investigate any effects this might have on prolactin-induced changes in oxytocin cell firing rate. As the MAPK pathway is closely attached to the short PRL-R isoform, activation of the MAPK pathway is likely to be increased during lactation since the expression of the short PRL-R isoform is increased in this group [241].
I would also like to further investigate the elevated levels of pSTAT5 seen in oxytocin cells of the vehicle treated lactating rats. As mentioned earlier, the high pSTAT5 levels might be a result of the increased activation of the Jak/STAT pathway due to the hyperprolactinaemic nature of lactation. In addition to this, the time course (4 hours) from inhibiting endogenous prolactin by bromocriptine and perfusing the animal, might not have been long enough to allow pSTAT5 levels to go back to baseline. I would like to administer two bromocriptine injections to lactating (and virgin) female rats at 12 and 4 hours before perfusion. The first injection (12 hours prior to perfusion) would be expected to suppress endogenous prolactin, and therefore, inhibit prolactin induced pSTAT5 activation, which will allow enough time for pSTAT5 to decrease to baseline. The second injection, at 4 hours before perfusion is to maintain the low prolactin levels. As with the present study, I would then administer prolactin or vehicle i.c.v. into the lateral ventricle of these lactating rats. If pSTAT5 remains elevated in the vehicle treated group, it will provide evidence to suggest that ligand(s) other than pituitary prolactin is acting on oxytocin cells of lactating rats to stimulate the Jak/STAT pathway, which may, or may not, be acting on the PRL-R. Potential candidates are brain prolactin or alternative pathways, most likely involving the closely related cytokine growth hormone or other ligands of the cytokine family that could also induce phosphorylation of STAT5, which are not under the control of DA, and therefore bromocriptine.

Furthermore, we have only examined virgin and lactating animals, and it is possible that responses to prolactin may change during pregnancy. Thus, I would like to perform the electrophysiology and immunohistochemistry protocols for pregnant rats at early, mid and late pregnancy, to determine the effects of prolactin on oxytocin cell activity during these times and also to establish when does disinhibition occurs. This could possibly be initially investigated by using a pregnancy model with hormone replacement. Once a difference is detected, this could be expanded into a full experiment looking at pregnant rats at different time point of pregnancy. In addition, I would like to use different doses of prolactin (both lower and higher than 1 µg) to establish the sensitivity of the response measured. These experiments will help further enhance our understanding of the interaction between prolactin and oxytocin.
4.5 Summary

Prolactin and oxytocin are essential reproductive hormones, and have 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 [256, 257], including the induction and maintenance of maternal behaviour, 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 may be further understood by defining the interaction between prolactin and oxytocin neurons.

Lactogenic hormones, mainly prolactin and placental lactogen, are the only hormones known to be elevated throughout pregnancy and lactation. Thus, prolactin may serve as a signal to the maternal brain, indicating the reproductive state, and in so doing, drives a range of neuronal and neuroendocrine adaptations to pregnancy, lactation and motherhood. This role in reproduction would add to the role of prolactin as the key hormone inducing milk secretion during lactation, which is the core feature that defines us as mammals [154]. My data suggests that among the signals that prolactin sends to the brain during reproduction, prolactin seems to signal to oxytocin neurons to drive coordinated adaptive responses to modulate the function of oxytocin neurons to match their activity with the changing physiological demands during various reproductive stages, most notably during pregnancy and lactation.

Finally, it is essential to determine how Jak/STAT signals are integrated with signals from other pathways downstream of the PRL-R, such as those originating from ERK1/2 MAPK pathway. An integrated picture will be required to more completely understand the molecular basis of prolactin action within oxytocin neurons, as well as other cells.
Bibliography


Appendix 1: List of Solution

1/ Blocking solution
0.3% Triton X-100 300 µl
0.25% BSA (Bovine Serum Albumin) 250 mg
added to 0.05 M TBS 100 ml

2/ Cryoprotectant:
0.1 M PB 500 ml
   NaCl 9 g
   Sucrose 300 g
   Polyvinylpurrolidone (PVP-40) 10 g
   Ethylene glycol 300 ml
Dissolved NaCl, sucrose and PVP im PB, then added ethylene glycol. The final volume was brought to 1000 ml with dH2O.

3/ Peroxidase Blocking solution:
   TBS 11.4 ml
   Methanol 8 ml
   H2O2 (30%) 600 µl
4/ Phosphate-buffered Solution (PB)

Sodium phosphate dibasic (Na₂HPO₄·2H₂O; MW = 177.99)
44.5 g

MQ H₂O
500 ml

Sodium phosphate monobasic (NaH₂PO₄·H₂O; MW = 137.99)
34.5 g

MQ H₂O
500 ml

0.2 PB:

Added 160 ml of sodium phosphate dibasic (Na₂HPO₄·2H₂O) solution to 40 ml of sodium phosphate monobasic (NaH₂PO₄·H₂O) solution, plus 300 MQ H₂O.

pH = 7.3

5/ 0.05 M TBS (Tri-buffered Saline)

Tris-HCl (MW = 157.6)
6.06 g

Tris-Base (MW = 121.1)
1.39 g

NaCl
8.7 g

dH₂O
1000 ml

pH = 7.6

6/ 0.01 M Tris-HCl (for antigen retrieval)

Tris-HCl
0.157 g

dH₂O
100 ml

pH = 10
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### Appendix 2: List of Antibodies

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