Differential changes in responses of hypothalamic and brainstem neuronal populations to prolactin during lactation in the mouse

Summary sentence: Lactation is associated with widespread induction of pSTAT5 in the mouse forebrain and brainstem due to chronic elevations in prolactin.

Keywords and Topic Category: prolactin receptor, pSTAT5, lactation

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ABSTRACT

During lactation, there are numerous functional adaptations in the maternal brain. There is evidence that the high levels of circulating prolactin present during lactation might contribute to these adaptive changes. The present study aimed to investigate levels of functional prolactin-mediated signal transduction in the brain of lactating mice, using prolactin-induced phosphorylation of signal transducer and activator of transcription 5 (pSTAT5) as a marker, and compare these to the effect of exogenous prolactin during diestrus. On day 7 of lactation, widespread induction of pSTAT5 was observed in numerous regions of the mouse forebrain and brainstem. In the medial preoptic nucleus, bed nuclei stria terminalis, paraventricular nucleus and medial amygdala of the forebrain and in the rostral periaqueductal gray, parabrachial nucleus, dorsal raphe and the raphe obscurus nucleus of the brainstem, pSTAT5 expression was markedly increased during lactation compared with the response to exogenous prolactin during diestrus. In the anteroventral periventricular nucleus, arcuate nucleus, ventromedial nucleus and dorsomedial nucleus, responses in lactation were comparable to diestrus. Conversely, in the area postrema of the brainstem, there was a reduction in response to prolactin, with a loss of pSTAT5 expression, during lactation. These differential responses following either acute or chronic elevations in prolactin were not accompanied by any changes in levels of prolactin receptor mRNA, when measured by in situ hybridization. These data are consistent with the hypothesis that prolactin might mediate widespread adaptive responses in the maternal brain.
INTRODUCTION
Levels of the anterior pituitary hormone, prolactin, and/or placental lactogen, are markedly elevated throughout pregnancy and lactation, when they play a critical role in the development and function of the mammary gland [1]. In addition to this well-established function, prolactin has a diverse range of actions in the maternal brain during pregnancy and lactation. These include the regulation of appetite and food intake [2-4], attenuation of the stress response [5-8], suppression of fertility [9, 10], stimulation of maternal neurogenesis [11, 12], expression of maternal behaviour [13-15] and, importantly, adaptation of feedback regulation of its own secretion [16]. This pleiotrophic range of actions may function to facilitate a global adaptive response preparing the maternal brain for the demands of pregnancy and lactation [17, 18]. To achieve these multiple functions, circulating prolactin must enter the brain and bind to its receptor in a variety of different hypothalamic and extrahypothalamic neurons. Transport of prolactin into the brain occurs via a saturable transport system [19] postulated to involve prolactin receptors in the choroid plexus [20]. Prolactin receptor expression in the choroid plexus is markedly increased during pregnancy and lactation [21], consistent with the hypothesis that there is enhanced access of systemic prolactin to the central nervous system in lactation.

Although there is clear evidence of high circulating prolactin and enhanced prolactin receptor expression in the choroid plexus during lactation, it is unknown whether this leads to increased prolactin action in the hypothalamus that might account for the functional adaptations in the maternal brain. Evidence for changes in prolactin receptor expression in the hypothalamus and other brain regions is equivocal. Early investigations in the rat suggested that there were increases in prolactin receptor mRNA in the brain during lactation compared to diestrus [22-24]. More recently, however, studies have suggested that the majority of changes in prolactin receptor mRNA levels are in fact restricted to the choroid plexus [21, 25], and changes in levels of prolactin receptor mRNA in the hypothalamus are more subtle. The few studies looking at prolactin receptor protein, however, show increased expression of the prolactin receptor in specific brain regions during lactation [26-28]. Whether there are functional changes in response to prolactin has not been determined. We have recently established methods for functional evaluation of prolactin-mediated signal transduction in the brain, using prolactin-induced phosphorylation of signal transducer and activator of transcription 5 (pSTAT5) as a marker [29]. The aim of the present study was to utilise this method to evaluate prolactin responses in the forebrain of lactating mice compared with diestrous mice with and without exogenous prolactin. In addition, since prolactin receptor mRNA has also been identified in the brainstem [30-32], we also evaluated functional responses to prolactin throughout the brainstem. We hypothesised that there would be enhanced functional responses to prolactin during lactation, within specific brain regions that show adaptive responses to pregnancy and lactation.

MATERIALS AND METHODS
ANIMALS AND TISSUE COLLECTION
Adult female C57BL/6J mice (age 8-12 weeks, weighing 20 – 25 g) were group-housed under conditions of controlled temperature (22 °C ± 1 °C) and lighting (12-hour light, 12-hour dark cycles, with lights on at 6 am) with ad libitum access to food and water. Groups of diestrous and lactating mice were used for immunohistochemical and in situ hybridisation experiments. For diestrous groups, the estrous cycle was monitored by daily vaginal smears and, following two normal cycles, animals were killed on the morning of diestrus. For lactating groups, mice were paired with males and individually housed once pregnant. The day of parturition was counted as day 1 of lactation, and litter sizes were adjusted to 6-8 pups per litter on day 2 of lactation. Animals were killed on the morning of day 7 of lactation. Blood samples were analysed for ovine prolactin and/or mouse prolactin by radioimmunoassay as described previously[29], to evaluate overall levels of prolactin exposure in the different groups. Otago Animal Ethics Committee approved all animal experimental protocols.
**Immunohistochemistry**

For immunohistochemistry, three groups of diestrous and two groups of lactating mice were used. The diestrous groups received one of three treatments; ovine prolactin (5 mg/kg injection/ip, 45 minutes prior to perfusion, n = 5), vehicle (saline, ip, 45 minutes prior to perfusion, n = 5), or bromocriptine (100 µg injections/sc, 24 hours, 12 hours, and 2 hours prior to perfusion, n = 3). The biopotency of administered ovine prolactin was 20-50 IU/mg (Sigma). Bromocriptine-treated animals also received a vehicle injection 45 minutes prior to perfusion. Bromocriptine is a D2 dopamine receptor agonist, and is known to inhibit prolactin secretion from the pituitary gland. This group was therefore added to evaluate the effect of very low concentrations of prolactin, as vehicle-treated animals would have basal levels of prolactin, or indeed, mildly elevated prolactin due to stress of handling and injections[33]. One of the lactating groups was used without additional treatment, and pups were present until the time of euthanasia. The second lactating group was bromocriptine treated (200 µg injections/sc, 24 hours, and 16 hours prior to perfusion, n = 5) to evaluate the effect of inhibiting high levels of endogenous prolactin during lactation. Animals were anaesthetised with pentobarbital, blood collected from the right atrium, and the mice then perfused transcardially with 4 % paraformaldehyde. Brains were removed, postfixed in the same fixative for 1 hour, and cryoprotected in 30 % sucrose overnight. Two sets of 30 µm coronal sections through the forebrain and brainstem were cut on a sliding microtome. One set was used to identify phosphorylated STAT5 (pSTAT5) expression throughout the hypothalamus and brainstem by immunohistochemistry. Two further sets of brainstem sections were used to label pSTAT5 and tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH) by dual label immunohistochemistry. TH was used as a marker of catecholamine neurons, and TPH as a marker of serotonin neurons in the brainstem.

**In situ hybridisation**

For in situ hybridisation, groups of diestrous and lactating mice (n = 4-6) were killed by decapitation, trunk blood samples collected, and brains immediately removed and frozen on powdered dry ice. Two sets of serial coronal sections through the forebrain were cut at 16 µm thickness on a cryostat at -23 °C and thaw-mounted onto RNase-free aminopropyl-triethoxysilane-coated microscope slides. Sections were stored at -80 °C.

**IMMUNOHISTOCHEMISTRY**

**Single label immunohistochemistry for pSTAT5**

Immunohistochemistry for pSTAT5 was conducted as previously described [29]. Briefly, antigen retrieval was performed on all tissue prior to single label immunohistochemistry for pSTAT5. Sections were incubated in a rabbit anti-pSTAT5 primary antibody (pSTAT5 Tyr694, 1:400; Cell Signalling Technology, Beverly, MA) for 72 hours at 4 °C, followed by a 90 minute incubation in biotinylated goat antirabbit IgGs (1:200; Vector Labs, Peterborough, UK). Sections were then incubated in Vector Elite avidin-biotin-horseradish peroxidase complex (1:100) for 90 minutes. Peroxidase labelling was visualised with nickel-diaminobenzidine tetrahydrochloride (DAB) using glucose oxidase to create a black, nuclear precipitate. Counts were made by setting a fixed threshold using ImageJ (NIH, Bethesda, ME) and a nuclei counting function, and mean numbers of positive neurons per section were calculated. For each specific brain region, all pSTAT5-positive nuclei within their boundaries were counted in two sections from each animal. Counts are reported as mean number of cells per section ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA and Tukey-Kramer multiple comparison post-hoc test.

**Dual label immunohistochemistry for pSTAT5 and TH in the brainstem**

After nickel-DAB visualisation of pSTAT5 (conducted as described above), sections were washed in 0.1 % hydrogen peroxide and incubated in a polyclonal rabbit anti-TH primary antibody (AB151, 1:10 000; Chemicon International) for 48 hours at 4 °C, followed by a 4 hour incubation in peroxidase-labelled anti-rabbit IgGs (1: 200; Vector Labs). Peroxidase labelling was visualised with DAB (brown, cytoplasmic staining). Sections were analyzed by counting the number of labelled neurons with and without pSTAT5
using an Olympus BX51 microscope utilizing brightfield microscopy using a 60x objective. The total number of labelled neurons, and the total number of TH-labelled neurons expressing pSTAT5 were counted in two sections per animal in the nucleus tractus solitarius (NTS), and the area postrema (AP) populations.

**Dual label immunohistochemistry for pSTAT5 and TPH in the brainstem**

In a slight variation from above, sections were labelled first for TPH, followed by pSTAT5 because the antigen retrieval that was required for pSTAT5 labelling destroyed the TPH antigen. Sections were incubated in a sheep anti-TPH antibody (1:10,000, Chemicon) for 48 hours at 4 °C, followed by a 90 minute incubation in a biotinylated donkey antisheep IgGs (1:200; Jackson Immunolabs). Following TPH visualization with normal DAB (brown cytoplasmic precipitate), sections underwent antigen retrieval and were incubated in the pSTAT5 primary antibody for 72-hours at 4 °C, followed by a 90 minute incubation in biotinylated goat antirabbit IgGs. pSTAT5 labelling was visualized with nickel-enhanced DAB (black, nuclear staining). For analysis, TPH neurons were divided into three anatomically separate regions of the dorsal raphe (DRaphe), the dorsal DRaphe, the lateral DRaphe, and the caudal DRaphe. The total number of labelled neurons, and the total number of labelled neurons expressing pSTAT5 were counted in two sections per animal in each of the DRaphe regions.

In all immunohistochemistry experiments, negative controls were run, where the primary antibody was omitted. These sections were marked to allow identification, and returned to the wells they originated from to undergo the remainder of the immunohistochemistry procedure. No specific staining was observed in these sections.

**IN SITU HYBRIDISATION FOR PROLACTIN RECEPTOR mRNA**

The RNA probe was prepared and hybridisation conducted as previously reported [29]. Primers were designed to target the mRNA encoded by exons 3-5 of the Prlr gene (accession number NM 011169.3), which are translated to form the extracellular domain which are common to both short and long forms of the receptor. The forward primer sequence was 5’-GCC ATC TGC ACT TGC TTA CA-3’, and the reverse primer sequence was 5’-CGT TGC TGG CAT TTA CTG TG-3’. To confirm the specificity of the prolactin receptor probe, a series of slides were hybridised with a sense RNA probe.

To generate autoradiograms, slides were exposed to scientific imaging film (Kodak BioMax MR) for 10 days. Subsequently, slides were coated with LM-1 Hypercoat emulsion (Amersham Biosciences), placed in light-proof slide boxes containing desiccant, and stored for 5 weeks at 4 °C. Slides were developed Kodak D19, fixed with Ilford Hypan, and counterstained using Gils haematoxylin (1:4, for 10 seconds), and dehydrated in graded ethanols and dried for 1 hour at 42 °C. Sections were cleared in xylene and coverslipped with Vectamount mounting medium. Following emulsion-coating, prolactin receptor mRNA appeared as silver grains, with dense clusters of grains indicating positively-labelled cells. Quantification of prolactin receptor mRNA expression was undertaken using emulsion-coated sections. For each brain region investigated, two sections per animal were analysed. A set number of photographs were obtained per region, and two quadrants per photograph were randomly selected for analysis. Counts were made using ImageJ and a particle counting function to count silver grains. A positively labelled cell was considered to contain silver grain density over five times that of the background. Per quadrant, the mean number of cells (counterstain), the mean number of positively labelled cells (silver grains), and the mean density of silver grains per cell were counted. The mean density of silver grains was calculated as the percent area of each cell that was covered with silver grains. Data are reported as mean ± SEM.

**Nomenclature**

The nomenclature used here is based on Brain Maps: Structure of the Rat Brain [34].
RESULTS

IMMUNOHISTOCHEMISTRY FOR pSTAT5 IN THE ROSTRAL FOREBRAIN

Immunohistochemistry with the pSTAT5 antiserum resulted in a heterogeneous pattern of cell nuclei staining throughout the brain (as reported previously [29]) with highest levels of expression in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARN). All immunoreactivity was restricted to the cell nucleus. Following bromocriptine-treatment in diestrous animals, no pSTAT5 expression was observed in any of the brain regions examined. In vehicle-treated diestrous animals (with low levels of serum mouse prolactin), there were low levels of pSTAT5 expression in the rostral and mediobasal hypothalamus (Figure 1). Following acute prolactin treatment in diestrous animals, there was a significant increase in pSTAT5 expression in the AVPV, ARN, dorsomedial nucleus (DMN) and medial amygdala (MEA) (Figure 2). In lactating mice, there was significant induction of pSTAT5 expression in numerous forebrain regions, with the overall levels and expression patterns being similar to that of prolactin-treated diestrous mice (Figure 1, Figure 2). For example, in the AVPV, ARN, ventrolateral ventromedial nucleus (VMNvl) and DMN, lactating mice exhibited similar numbers of pSTAT5-expressing neurons to those observed following prolactin treatment in diestrous animals (Figure 2).

pSTAT5 staining was also observed following prolactin treatment in diestrous animals and during lactation in the subfornical organ and organum vasculosum of the lamina terminalis (OVLT). A number of areas, however, showed a pattern of expression that was specific to lactation. In the rostral forebrain, the median preoptic area (MEPO), bed nuclei stria terminalis (BST) and paraventricular nucleus (PVN) exhibited pSTAT5 expression that was significantly elevated during lactation, but unchanged in prolactin-treated diestrous animals (Figure 1; D-F and Figure 2; A). The highest level of pSTAT5 staining was observed in the medial preoptic nucleus (MPN) during lactation, with 1270 ± 304 positively labelled nuclei per section during lactation, compared to 340 ± 139 following prolactin treatment in diestrous mice (Figure 1; A-C, Figure 2; A). Similarly, in the MEA, there was significantly more pSTAT5 during lactation than in prolactin-treated diestrous animals (Figure 1; G-I, Figure 2; B).

In lactating mice, treatment with exogenous prolactin resulted in no additional expression of pSTAT5 (data not shown). Furthermore, 24 hours of bromocriptine treatment in suckling lactating mice, resulted in no pSTAT5 expression (Figure 1; D, H, and L). As reported previously, acute prolactin treatment in diestrous mice resulted in serum ovine prolactin levels of 759 ± 230 ng/ml [29]. Serum murine prolactin levels in lactating mice were 363 ± 59 ng/ml.

IN SITU HYBRIDISATION FOR PROLACTIN RECEPTOR mRNA

In order to determine whether the observed increases in pSTAT5 expression during lactation were accompanied by changes in levels of prolactin receptor mRNA, in situ hybridisation was conducted and quantified using emulsion-coated sections. Representative images of prolactin receptor mRNA silver grain labelling during diestrus and lactation day 7 are provided in Figure 3. Prolactin receptor mRNA expression was identified by the presence of clusters of silver grains over the top of cells. These clusters were highly localized to specific nuclei, and background labelling was extremely low (Figure 3F). Within a nucleus containing high numbers of prolactin receptor expressing cells, such as the mMPN or PVN (Figure 3A-D), it was possible to readily identify cells that were either positive or negative for the mRNA signal. Table 1 shows the mean number of counterstained cells in each region analyzed, the % of counterstained cells labelled for prolactin receptor and the mean density of silver grains in labelled cells. There were no significant differences between diestrous and day 7 lactating mice in the number of cells counted, percent labelled and density of silver grains in any of the regions examined (AVPV, MEPO, MPN, PVN, ARN and VMNvl) (Table 1).

IMMUNOHISTOCHEMISTRY FOR pSTAT5 IN THE BRAINSTEM

The immunohistochemistry with the pSTAT5 antiserum in the brainstem resulted in a heterogeneous pattern of cell nuclei staining throughout the brainstem. Under diestrous conditions there was very little
pSTAT5 expression in the brainstem apart from low levels of pSTAT5 expression in the periaqueductal gray (PAG) in vehicle-treated mice. There were no further increases in this region after prolactin treatment (Figure 4; A, Figure 5). In the medulla, however, prolactin treatment in diestrous mice led to significant increases in pSTAT5 expression in the area postrema (AP) and nucleus tractus solitaries (NTS) (Figure 4; K, N), with prolactin treatment leading to 214 ± 42 positively labelled nuclei in the AP, and 75 ± 8 in the NTS (Figure 5). There was no pSTAT5 expression in the brainstem of bromocriptine-treated mice.

There were notable changes in both the pattern of expression and the abundance of pSTAT5 in the brainstem during lactation from that observed in diestrous mice. In the midbrain, there was significant expression of positively-labelled nuclei in the parabrachial nucleus (PBN), with an increase from zero in diestrous groups to 225 ± 68 pSTAT5-positive nuclei during lactation (Figure 4; B-C, Figure 5). In the rostral PAG, there were higher levels of pSTAT5 expression during lactation, with significant increases compared to prolactin-treated diestrous animals observed (from 169 ± 97 positively-labelled pSTAT5 nuclei following prolactin-treatment in diestrous animals, to 545 ± 124 in lactation) (Figure 4; A-C, Figure 5). In the pons, there was significant expression of positively-labelled nuclei in the dorsal raphe (DRaphe) during lactation compared with very low levels of expression in all diestrous groups (Figure 4; B-C, Figure 5). In the medulla, pSTAT5 expression in the raphe obscurus nucleus (ROb) was only observed during lactation (Figure 4; G-I, Figure 5). In the NTS and AP of the medulla however, there were significant decreases in pSTAT5 expression during lactation, compared to following prolactin treatment in diestrous animals (Figure 4; J-O, Figure 5). In the AP, no significant pSTAT5 expression was observed during lactation (Figure 4; L, Figure 5).

A number of the prolactin-responsive neurons in the brainstem were identified to be serotonergic (Figure 6) and catecholaminergic (Figure 7) neurons. In the DRaphe, a small sub-population of serotonergic (TPH) neurons was found to express pSTAT5 during lactation; 11 ± 3 %, 5 ± 2 %, 12 ± 2 % in the rostral, lateral and caudal DRaphe respectively (Figure 6; A-E). A larger sub-population of catecholaminergic neurons in the AP were found to express pSTAT5 following prolactin-treatment in diestrous animals (37 ± 12 % of TH neurons) (Figure 7; D-G). Interestingly, these catecholaminergic neurons became unresponsive to prolactin during lactation (Figure 7; G). In the catecholaminergic populations found in the NTS and locus coeruleus, no significant expression of pSTAT5 was observed during diestrous or lactation.

**DISCUSSION**

We have previously reported that phosphorylated STAT5 can be used as a reliable marker of prolactin receptor activation in the diestrous mouse [29]. Using that approach, we have shown here that during day 7 of lactation there is widespread induction of pSTAT5 expression in numerous regions in the mouse forebrain and brainstem. There were significantly different patterns of prolactin-induced activation of STAT5 in neurons in these regions during lactation compared to that observed in response to exogenous prolactin during diestrus. In the MPN, BST, PVN, and MEA of the forebrain and in the rostral PAG, PBN, DRaphe and ROb of the brainstem, responses to prolactin were markedly increased during lactation, with more cells expressing pSTAT5, while in the AVPV, ARN, VMNvl and DMN, responses in lactation were comparable to diestrus. Conversely, in the AP of the brainstem, there was a reduction in the number of cells responding to prolactin during lactation. These differential responses were not accompanied by any changes in levels of prolactin receptor mRNA. The induction of pSTAT5 expression seen during both diestrus and lactation was specifically mediated by prolactin, because suppression of endogenous prolactin secretion by bromocriptine treatment, even in the presence of the suckling stimulus, resulted in the absence of any pSTAT5 staining. Furthermore, during lactation, the widespread distribution of pSTAT5 caused by the elevated levels of endogenous prolactin was maximal, with additional prolactin leading to no further increase in pSTAT5 expression. Thus, we were able to compare,
through changes in the total number of cells expressing pSTAT5, overall pattern of responses to prolactin in the presence of high prolactin levels from exogenous (diestrus) or endogenous (lactation) sources.

Signaling through the prolactin receptor, when measured by changes in the pattern of pSTAT5 protein labeling, is affected by lactation in a region-specific manner. In many regions, including the AVPV, ARN, VMNvl and DMN, there was no significant difference between lactation-induced pSTAT5 and that induced by exogenous prolactin treatment during diestrus. This suggests that endogenous prolactin was acting on neurons in those regions, with no change in overall responses to prolactin during lactation. In the MPN, BST and MEA, however, responses to prolactin appeared to be greatly enhanced during lactation. Although prolactin did not induce significant pSTAT5 expression in the MPN and BST, and only a small increase in the MEA during diestrus, significantly higher levels of pSTAT5 expression were present during lactation. Most notable was the MPN, where lactation led to a 3-fold greater increase in the number of cells expressing pSTAT5 than that induced by prolactin during diestrus. It does not appear that a change in prolactin receptor mRNA expression underlies this change in the pattern of prolactin-responses, with levels of mRNA expression during lactation resembling that seen in diestrus, and no additional regions showing expression during lactation. There were no changes in the number of cells expressing prolactin receptor mRNA, or in the transcript abundance per cell in any of the brain regions examined. The absence of change in receptor expression parallels that seen in the lactating rat, where no differences have been detected in hypothalamic and forebrain mRNA expression of both the long and the short forms of the prolactin receptor when measured by real-time PCR and in situ hybridisation [21, 35]. It seems unlikely that acutely higher levels of prolactin during lactation could account for the lactation-induced increase in pSTAT5 expression, because peripheral treatment with exogenous prolactin in diestrous animals in fact resulted in higher levels of serum prolactin than observed during lactation (although, conceivably, there is a different biopotency of the exogenous ovine prolactin and endogenous mouse prolactin). Similarly, it also seems unlikely that an increase in transport of prolactin into the brain could account for the increased response, because the lactation-induced changes in pSTAT5 induction were highly localised and did not occur equally in all brain regions. Thus, it seems likely that post-transcriptional regulation of prolactin receptor expression, or increased responsiveness through signal transduction pathways, must be occurring during lactation in a region-specific manner. Potential mechanisms might include changes in the expression of suppressors of cytokine signalling (SOCS), which act as endogenous regulators of the JAK/STAT pathway [36].

The elevated endogenous, prolactin-induced pSTAT5 expression in the MPN suggests prolactin action is enhanced in this region during lactation, probably contributing to the well known function of this region in mediating maternal behaviour [7, 14, 15, 37]. As prolactin receptor mRNA expression is unaltered during lactation, the downstream change in pSTAT5 signalling during lactation provides a mechanism by which the MPN can become more responsive to prolactin. Similarly, the BST and MEA may be involved in maternal responsiveness [38].

The most noticeable change in hypothalamic pSTAT5 expression during lactation was observed in the MEPO and the PVN. In these neurons, strong pSTAT5 expression was observed during lactation, while prolactin-treatment during diestrus failed to induce any pSTAT5 expression. The reason for this increase in the number of cells expressing pSTAT5 in lactation is not known, with prolactin receptor mRNA expression observed in both of these regions during diestrus. One possible explanation for the lack of pSTAT5 expression during diestrus could be a switch in prolactin receptor expression, from primarily short form during diestrus, to long form during lactation. The short form of the receptor lacks the intracellular domains required for activation of the JAK/STAT pathway [39, 40]. As the in situ hybridisation conducted here does not distinguish the two receptor forms, it is possible that the short form is overexpressed during diestrus, accounting for much of the signal detected. However, in situ hybridisation studies conducted in the rat suggest that levels of the long form do not change between diestrous and lactating animals in these nuclei [35]. This suggests that the change in pSTAT5 expression
observed here is not due to a shift in receptor expression but rather to post-transcriptional changes occurring during lactation which alter prolactin signalling. In support of this, is the finding that immunohistochemical labelling of the prolactin receptor is only observed in the PVN during lactation [26].

The functional relevance of altered prolactin signalling in the MEPO and PVN during lactation remains unexplored. The MEPO is known to be involved in the regulation of sleep and arousal [41, 42] and in thermoregulation [43, 44], and there are certainly changes in these functions during pregnancy and lactation. For example, during pregnancy in rats, there is suppression of the fever response [45], and changes in sleep patterns [46], and prolactin may be involved in mediating these changes. Additionally, neurons that express estrogen receptor α and project to GnRH neurons are found in the MEPO [47], suggesting this region may also play a role in mediating estrogen feedback. Prolactin effects here could contribute to the well known suppression of fertility during lactation. In the PVN, prolactin can regulate the activity of oxytocin neurons [48], with greater prolactin receptor expression observed in these neurons during pregnancy and lactation [26]. Prolactin has also been shown to increase oxytocin mRNA expression in the hypothalamus during lactation in rats [49]. Prolactin may also act in the PVN [50] during lactation to down-regulate the responsiveness of the HPA axis to a variety of stresses [51-53]. This role of prolactin has been demonstrated in lactating rats, where the attenuated stress response is reversed by disrupting prolactin signalling by injecting anti-sense prolactin receptor into the brain [7].

In addition to the rostral forebrain, we show here that there are also populations of prolactin-responsive neurons in the female mouse brainstem. A few previous studies have reported detecting prolactin receptor mRNA [31, 54] in the brainstem of rats and rabbits. In mice, the Allan Mouse Brain Atlas [55] has identified expression of mRNA for the long form of the prolactin receptor in the brainstem of male mice. There have, however, been no reports of protein expression nor any insight into the functional role of prolactin in this brain region. Prolactin-induced pSTAT5 expression was observed in diestrous mice in the PAG, NTS and AP. During lactation, however, very different patterns of prolactin-induced pSTAT5 in the brainstem emerged in comparison to diestrous mice, with a number of regions becoming prolactin-responsive during lactation. Increased numbers of cells expressing pSTAT5 during lactation, compared to diestrus, were observed in the NTS, ROb, PAG, PBN and DRaphe. In contrast to all other regions examined, lactation led to a decrease in pSTAT5 expression in the AP compared to that induced by prolactin during diestrus. These data suggest that, unlike all other areas examined, this nucleus becomes unresponsive to prolactin during lactation, possibly involving receptor down regulation or activation of SOCS proteins. Despite the presence of prolactin receptor in the AP [31], the functional significance of this remains unknown. In the present study, we found that approximately half of the neurons that were responsive to prolactin during diestrus were catecholamine neurons. Interestingly, pSTAT5 expression was also observed in the subfornical organ and OVLT, which together with the AP form the sensory circumventricular regions of the brain [56]. These regions contain neuronal cell bodies that are found outside of the blood brain barrier, suggesting that there are groups of prolactin-responsive cells that are capable of rapidly conveying information regarding blood prolactin levels to the brain. These neurons are known to respond to a range of circulating factors, including leptin, angiotensin, ghrelin, cholecystokinin and amylin [57, 58].

Although these studies show that prolactin and lactation can lead to pSTAT5 expression in numerous brainstem regions, the only function so far attributed to prolactin in the brainstem is an increase in lordosis reproductive behaviour in rats [59]. However, there are a number of functions that prolactin influences, that are also known to be mediated in part, by the brainstem. For example, a number of brainstem region project directly to the rostral preoptic GnRH neurons in mice, including the serotonin neurons of the DRaphe, the noradrenergic neurons of the NTS, and unidentified neurons of the NTS and PAG [60]. Potentially, prolactin could act on some of these neurons during lactation to mediate the suppression of GnRH neuronal activity [61-64], although in contrast to serotonergic neurons in the
DRaphe, noradrenergic neurons of the NTS did not express pSTAT5 under any conditions. The PAG was also found to be prolactin responsive in these studies, and lesion studies have shown that this region is involved in maternal behaviour, with site specific lesions demonstrating distinct roles of the caudal and rostral portions of the PAG [65, 66]. As prolactin is known to be involved in initiating maternal behaviour in the brain [14], the PAG provides another region by which prolactin could do this.

In conclusion, prolactin-responsive neurons show a widespread distribution throughout the mouse forebrain and brainstem. During lactation, there were differential patterns of prolactin-induced responses in the brain, with some neuronal populations showing markedly more cells expressing pSTAT5 than seen in response to exogenous prolactin during diestrus. Many of the regions showing altered responses are also known to show functional changes during pregnancy and lactation. These data are consistent with the hypothesis that prolactin might mediate widespread adaptive responses in the maternal brain [17].

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REFERENCES

**Figure Legends**

**Figure 1.** Representative sections showing pSTAT5 immunohistochemistry in the MPN (A-C), PVN (D-F), and MEA (G-I) in diestrous mice with and without exogenous prolactin treatment, and in lactating mice with and without bromocriptine treatment. pSTAT5 labelling seen as black nuclear staining. A, E, I: Diestrous mice treated...

Figure 2. pSTAT5 expression in the rostral forebrain following vehicle and prolactin treatment in diestrous mice and in day 7 of lactation. A: pSTAT5 expression at the level of the rostral hypothalamus. B: pSTAT5 expression at the level of the mediobasal hypothalamus. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 3. Representative emulsion-coated sections and autoradiograms (inset images) from in situ hybridisation showing mouse prolactin receptor mRNA in the MPN and PVN during in diestrous and lactating (day 7) mice. In the autoradiograms, regions with dark labelling are positive or prolactin receptor mRNA. In the autoradiograms, nuclei are counterstained purple with Gills hematoxylin, and prolactin receptor mRNA is labelled with black silver grains. A, C: prolactin receptor mRNA during diestrus. B, D: prolactin receptor mRNA during day 7 of lactation. Black arrows illustrate 2 representative cells that do not show prolactin receptor mRNA labelling, while red arrows highlight 2 cells that show positive prolactin receptor mRNA labelling. E: control section through the MPN hybridised with a sense probe. F: low power image at the level of the ARH, showing high levels of prolactin receptor mRNA expression localised specifically in the ARH, with low levels of background expression outside the nucleus.

Figure 4. Representative sections showing pSTAT5 immunohistochemistry in the brainstem of vehicle (A, D, G, J, M) and prolactin-treated (B, E, H, K, N) diestrous mice and of lactating (day 7) mice (C, F, I, L, O). A-C: pSTAT5 labelling seen as black nuclei staining. pSTAT5 expression is shown in the periaqueductal grey (PAG). D-F: pSTAT5 expression is shown in the parabrachial nucleus (PBN). G-I: pSTAT5 expression is shown in the raphe obscuras (ROb). J-L: pSTAT5 expression is shown in the area postrema (AP). M-O: pSTAT5 expression is shown in the nucleus of the solitary tract (NTS).

Figure 5. Mean ± SEM pSTAT5 expression in the brainstem following vehicle and prolactin treatment in diestrous mice and in day 7 of lactation. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 6. Representative sections showing pSTAT5 and TPH immunohistochemistry in the dorsal DRaphe (A-C), lateral DRaphe (D-F), and caudal DRaphe (G-I) in prolactin-treated diestrous and lactating (day 7) mice. pSTAT5 labelling exists as black nuclei staining and TPH as brown cytoplasmic staining Immunostaining for TPH was used as a marker of serotonin neurons. A, D, G: Diestrous mice treated with prolactin. B, C, E, F, H, I: Mice on day 7 of lactation. J: Mean ± SEM pSTAT5 expression in the DRaphe divisions following vehicle and prolactin treatment in diestrous mice and in day 7 of lactation. ** p < 0.01, *** p < 0.001.

Figure 7. Representative sections showing pSTAT5 and TH immunohistochemistry in the NTS (A-C) and AP (D-F) in prolactin-treated diestrous and lactating (day 7) mice. pSTAT5 labelling exists as black nuclei staining and TH as brown cytoplasmic staining Immunostaining for TH was used as a marker of catecholamine neurons. A, C, D, F: Diestrous mice treated with prolactin. B, E: Mice on day 7 of lactation. G: Mean ± SEM pSTAT5 expression in the AP following vehicle and prolactin treatment in diestrous mice and in day 7 of lactation. ** p < 0.01, *** p < 0.001.
Table 1. Prolactin receptor mRNA expression in the female mouse forebrain during diestrus and lactation day 7.

<table>
<thead>
<tr>
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<th>Diestrus</th>
<th>Lactation</th>
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<tbody>
<tr>
<td>AVPV</td>
<td>Number of cells</td>
<td>72.8 ± 9.3 (n=5)</td>
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<td>% of cells labelled</td>
<td>89.8 ± 2.4</td>
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<td>Silver grain density (% area/cell labelled)</td>
<td>16.2 ± 1.1</td>
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<td>MEPO</td>
<td>Number of cells</td>
<td>89.4 ± 1.8 (n=5)</td>
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<td></td>
<td>% of cells labelled</td>
<td>85.7 ± 4.9</td>
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<td></td>
<td>Silver grain density (% area/cell labelled)</td>
<td>14.1 ± 1.6</td>
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<tr>
<td>MPN</td>
<td>Number of cells</td>
<td>61.8 ± 3.1 (n=4)</td>
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<td></td>
<td>% of cells labelled</td>
<td>81.8 ± 6.9</td>
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<td></td>
<td>Silver grain density (% area/cell labelled)</td>
<td>14.6 ± 1.8</td>
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<tr>
<td>PVN</td>
<td>Number of cells</td>
<td>66.2 ± 3.5 (n=5)</td>
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<td></td>
<td>% of cells labelled</td>
<td>72.0 ± 8.7</td>
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<td>Silver grain density (% area/cell labelled)</td>
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<td>ARN</td>
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<td></td>
<td>% of cells labelled</td>
<td>80.8 ± 3.1</td>
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<td>Silver grain density (% area/cell labelled)</td>
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<tr>
<td>VMNvl</td>
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<td></td>
<td>% of cells labelled</td>
<td>82.8 ± 4.0</td>
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<tr>
<td></td>
<td>Silver grain density (% area/cell labelled)</td>
<td>18.6 ± 2.3</td>
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</tbody>
</table>

Data show the mean number of counter-stained cells per photos, the mean percent of cells positive for prolactin receptor mRNA, and the mean percent of prolactin receptor mRNA expression covering each labelled. Cells were counted as positive for expressing prolactin receptor mRNA if the amount of silver grain expression was 5-fold greater than background.
Figure 3
Figure 5

Number of pSTAT5-labelled nuclei per section

- Diestrus + Vehicle (n = 5)
- Diestrus + Prolactin (n = 5)
- Lactation (n = 6)

Brainstem Region:
- NTS
- AP
- ROB
- rPAG
- cPAG
- PBN
- DRaphe