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EFFECT OF HYPERTHYROIDISM ON CIRCULATING PROLACTIN AND HYPOTHALAMIC EXPRESSION OF TYROSINE HYDROXYLASE, PROLACTIN SIGNALING CASCADE MEMBERS AND ESTROGEN AND PROGESTERONE RECEPTORS DURING LATE PREGNANCY AND LACTATION IN THE RAT.

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Abstract

Hyperthyroidism (HyperT) compromises pregnancy and lactation, hindering suckling-induced PRL release. We studied the effect of HyperT on hypothalamic mRNA (RT-qPCR) and protein (Western blot) expression of tyrosine hydroxylase (TH), PRL receptor (PRLR) and signaling pathway members, estrogen-α (ERα) and progesterone (PR) receptors on late pregnancy (days G19, 20 and 21) and early lactation (L2) in rats. HyperT advanced pre-partum PRL release, reduced circulating PRL on L2 and increased TH mRNA (G21 and L2), p-TH, PRLR mRNA, STAT5 protein (G19 and L2), PRLR protein (G21) and CIS protein (G19). PRs mRNAs and protein decreased on G19 but afterwards PRA mRNA (G20), PRB mRNA (G21) and PRA mRNA and protein (L2) increased. ERα protein increased on G19 and decreased on G20. Thus, the altered hypothalamic PRLR, STAT5, PR and ERα expression in hyperthyroid rats may induce elevated TH expression and activation, that consequently, elevate dopaminergic tone during lactation, blunting suckling-induced PRL release and litter growth.

Highlights

- HyperT blocks suckling-induced PRL release and advances the prepartum PRL surge
- HyperT elevated hypothalamic TH mRNA and p-TH on late pregnancy and early lactation
- PRLR, STAT5, CIS, PRA and B also increased on late pregnancy and/or early lactation
- The altered PRL signaling and PRs may induce elevated TH expression and activation
- This elevates dopaminergic tone on lactation, blunting PRL release and litter growth

List of abbreviatures:

CIS: Cytokine inducible SH2-containing protein.

ERα: Estrogen receptor α.

G19/20/21: Day 19/20/21of pregnancy.

HyperT: hyperthyroidism.

L2: Day 2 of lactation.

MBH: medio basal hypothalamus.

p-TH: phosphorylated Tyrosine hydroxylase.

PR: Progesterone receptor

PRA/PRB: Progesterone receptor isoform A/B.

PRL: Prolactin.

PRLR: Prolactin receptor.

SOCS1/SOCS3: Suppressor of cytokine signaling 1/3

STAT5/STAT5b: Signal transducer activator of transcription 5/5b.

TH: Tyrosine hydroxylase.

1. Introduction

Thyroid disorders are common in childbearing age women and are involved in a variety of reproductive disorders. Female mammals with thyroid dysfunctions show cycle irregularities, sub- or infertility, abortions and stillbirth (Rosato *et al.* 1992, Poppe *et al.* 2007). Two per 1000 pregnant women have some degree of hyperthyroidism (HyperT). Most of the symptoms of HyperT are attenuated during pregnancy, but there is a marked recurrence of HyperT after delivery, which has an adverse effect on development of the offspring (Rodin 1989) Rats made hyperthyroid with high daily doses of T_4 (1 or 0.25 mg/kg/day) display changes in the cycle and in the preovulatory release of hormones (Jahn *et al.* 1995). They also display preterm birth caused by premature luteolysis, increased number of pups, defective parturition, maternal behavior and lactation failure, although lactogenesis is normal (Rosato *et al.* 1992). These rats also show an advance in the preterm prolactin (PRL) surge (Rosato *et al.* 1992, Rosato *et al.* 1998, Rosato *et al.* 2002). Rats treated with lower doses of T_4 (0.1 mg/kg/day) maintain lactation but the litters have a reduced growth rate caused by a partial blockage of the suckling induced PRL and oxytocin release and premature mammary gland involution (Varas *et al.* 2002).

PRL secretion during pregnancy and lactation is subject to complex regulation that involves ovarian steroids, placental hormones and neurotransmitter systems such as dopaminergic, adrenergic, serotonergic and opioid, the latter two exerting dual actions (Soaje & Deis 1994, Soaje & Deis 1997, Jahn *et al.* 1999, Soaje *et al.* 2004, Valdez *et al.* 2014). The main regulator of PRL secretion is dopamine (DA), produced by the tuberoinfundibular dopaminergic (TIDA) and tuberohypophysial (THDA) neurons located in the arcuate nucleus and the periventricular nucleus (Freeman *et al.* 2000). Production of DA by TIDA neurons is regulated by tyrosine hydroxylase (TH) activity, the rate limiting enzyme for DA biosynthesis (Zigmond *et al.* 1989). In turn, TH expression is induced by PRL acting through the long form of its receptor ($PRLR_{long}$) that is present in the dopaminergic neurons and the JAK2-STAT5 signaling pathway (Freeman *et al.* 2000, Grattan

2015) conforming the short-loop feedback regulation of PRL release. The transition from pregnancy to lactation is characterized by considerable changes in circulating hormone levels, mainly a fall in progesterone (P_4) and increased 17β-estradiol (E_2) which trigger the prepartum PRL peak, lactogenesis, delivery and display of maternal behavior (Jahn *et al.* 1986, Grattan *et al.* 2008). At the hypothalamic level there are also changes in the neuronal response, including desensitization of dopaminergic neurons that allows the maintenance of elevated PRL levels during lactation (Andrews & Grattan 2004). This decrease in TIDA neurons activity is evidenced at the end of gestation as a fall in TH expression and DA content (Andrews *et al.* 2001, Valdez *et al.* 2007, Grattan *et al.* 2008) which seems to be mediated through increased expression of the suppressors of cytokine signaling (SOCS) family of proteins, which limit PRL signaling, and thus activation of the short loop feedback (Starr & Hilton 1999, Anderson *et al.* 2006a, Anderson *et al.* 2006b, Grattan *et al.* 2008). These proteins are induced by PRL and E_2 and are elevated during late pregnancy and lactation in the arcuate neurons (Lee & Voogt 1999, Anderson *et al.* 2006a, Steyn *et al.* 2008). However, this decreased dopaminergic tone is not accompanied with elevated PRL levels until the prepartum PRL surge triggered by the decrease in circulating P_4, indicating a crucial role for this steroid as an inhibitor of PRL release on late pregnancy (Jahn *et al.* 1986, Andrews *et al.* 2001). In the rat, placental lactogens (PLs) and ovarian steroids are key regulators of PRL secretion during pregnancy. PLs inhibit pituitary PRL secretion by activation of TIDA neurons (short feedback loop) and induction of TH expression (Lee & Voogt 1999, Grattan *et al.* 2008). Estrogens stimulate PRL secretion, while P_4 has a dual action: stimulatory in early pregnancy, but inhibits PRL secretion on the second half of pregnancy (Jahn *et al.* 1986).

Both hypothyroidism and HyperT affect PRL secretion in virgin, pregnant and lactating rats (Rosato *et al.* 1992, Rosato *et al.* 1998, Rosato *et al.* 2002, Hapon *et al.* 2003, Hapon *et al.* 2007, Navas *et al.* 2011). Usually the effects of thyroid hormones on PRL secretion have been supposed to be mediated through their actions on TRH, that is a potent PRL stimulating factor (Freeman *et al.*

2000). However, it is also possible that some of the effects of thyroid hormones on PRL secretion may be mediated through direct actions on TIDA neurons, modulating the expression of TH, and/or receptors for PRL, steroid hormones and neurotransmitters, and the intracellular signaling of these hormones.

Previous work from our laboratory has shown that (HyperT) and hypothyroidism affect the concentration of brain neuropeptides that regulate important endocrine and behavioral processes in reproduction (Ayala *et al.* 2013). It has also been described that thyroid hormones can modulate PRLR expression in some tissues (Tiong *et al.* 1992) and inhibit PRL-induced STAT5a/b nuclear translocation (Favre-Young *et al.* 2000). However, there is limited data on their effects on PRLR expression and activation in hypophysiotropic areas that regulate PRL secretion, for example on TIDA or THDA neurons. Thyroid hormones could influence PRL secretion through actions on PRL signaling pathways and TH expression in TIDA and THDA neurons, or through actions on the expression or activation of receptors for estrogens (ER) or P_4 (PR).

To explore further the mechanism whereby HyperT affects PRL secretion and impairs lactation, in the present work we have studied the effect of chronic treatments with T_4 on hypothalamic expression of TH, PRLR, members of the PRL signaling pathway (STAT5, SOCS, CIS), ER and PR during late pregnancy and early lactation in rats.

2. Materials and Methods

2.1. Animals:

Adult female Wistar rats bred in our laboratory, aged 3-4 months, weighing 200-300 g at the onset of treatment and with regular 4-day cycles were used. Rats were given free access to water and food and were kept in a light- (lights on from 06:00 h to 20:00 h) and temperature-controlled (22-24ºC) room. HyperT was induced by daily *s.c.* injections of T_4 (0.25 mg/Kg body weight, dissolved in 0.9 % NaCl alkalinized with NaOH to pH≈9). Control rats were injected with the vehicle. The treatment

was started on the day of oestrus 8 days before mating and continued until the day of sacrifice. The presence of spermatozoa in the vaginal smears the morning after caging with a fertile male on the night of pro-oestrus was considered Day 0 pregnancy. For the groups sacrificed after delivery, the daily dose of T_4 was changed to 0.1 mg/Kg body weight on day 18 of pregnancy, to assure survival of the pups. Previous work showed that the higher dose provoked 80-90 % pup mortality within 24 h postpartum and a failure of maternal behavior (Rosato *et al.* 1992, Rosato *et al.* 1998), while with the lower dose (Varas *et al.* 2002), maternal behavior was normal and the pups were able to suckle (Varas *et al.* 2002), which allowed for survival of the whole litters.

Groups of 6-10 control or HyperT rats were decapitated on days 19, 20 and 21 of pregnancy and 2 of lactation at 12:00 h. Serum was obtained from trunk blood after centrifugation at 3000 rpm for 20 min and stored at −20º C until E_2, P_4, PRL, TSH, T_3 and total T_4 determination by RIA. The brains were rapidly removed from the skull and immediately placed on an ice-cold stainless steel brain slicer (RBM 4000C; ASI Instruments, Inc., Warren, Mich., USA) for dissection, in order to obtain an approximately 2-mm coronal slice including the medio basal hypothalamus (MBH). The MBHs were dissected from the slice that was within bregma –2.12 to –4.52 mm as determined from optic chiasm and lateral hypothalamic sulci on the ventral surface of the brain, by making lateral and oblique cuts along the sides of the third ventricle. The piece of dissected tissue, that includes the PeN, arcuate nucleus and median eminence and excludes anterior hypothalamic area, ventromedial nucleus and zona incerta, was frozen on dry ice and stored at –80 ° C until processing (Soaje *et al.* 2006, Valdez *et al.* 2007). Animal maintenance and handling was performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication Nº 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986). The procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Sciences, Universidad Nacional de Cuyo, Mendoza, Argentina (Protocol approval Nº 17/2012).

164 **2.2. Hormone determinations**

165 PRL and TSH were measured in all the groups by double antibody radioimmunoassay, using
166 materials provided by Dr. AF Parlow and the NHPP (National Hormone and Pituitary Program,
167 Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radio-iodinated using the
168 Chloramine T method and purified by passage through Sephadex G75. The results were expressed
169 in terms of rat PRL RP-3 or TSH RP-3, standard preparations. Assay sensitivities were less than 0.5
170 ng/ml serum and the intra-assay coefficient of variation were less than 10%. All the samples were
171 measured in the same assay in duplicate.

172 Serum T_3, T_4, P_4 and E_2 were measured using commercial kits (Coat-a-Count total T_3, total T_4, and
173 Progesterone kits, Siemens, USA and DSL-4800 estradiol kits, Beckman-Coulter, USA). Assay
174 sensitivities were 7 ng/dl for T_3, 0.25 µg/dl for T_4, less than 0.02 ng/ml for P_4 and 2.2 pg/ml for
175 estradiol, and the intra-assay coefficients of variation were <10% for all RIAs. All the samples were
176 measured in the same assay in duplicate.

177 **2.3. Real Time PCR.**

178 MBH samples were homogenized in 0.5 ml of TRIzol (GIBCO-BRL) and total RNA isolated
179 according to the manufacturer's instructions. Total RNA concentrations were determined
180 spectrophotometrically, integrity of the RNA was examined by 1% agarose gel electrophoresis.
181 First strand cDNA synthesis from 2.5 µg RNA per sample was performed using Moloney murine
182 leukemia virus retrotranscriptase and random hexamer primers (Invitrogen/Life Technologies,
183 Buenos Aires, Argentina) in a 20 µl reaction mixture. Real-time quantification was monitored by
184 measuring the increase in fluorescence caused by the binding of EvaGreen dye (Biotium) to double-
185 strand DNA at the end of each amplification cycle. The cDNAs were amplified with rat-specific
186 primers for TH, $PRLR_{long}$, ERα, total PR and PRB, STAT5b and SOCS1, 3 and CIS in the
187 conditions described in Supplementary Table 1. Samples were run in duplicate. Simultaneously,
188 each PCR run included a no-template control and a sample without reverse transcriptase. Real-time

PCR was performed with a Corbett Rotor Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd (Sydney, Australia) in a final volume of 20 µl. The reaction mixture consisted of 2 µL of 10xPCR Buffer, 1 µL of 50 mM $MgCl_2$, 0.4 µL of 10 mM dNTP Mix (Invitrogen), 1 µL of 20x Eva Green (Biotium), 0.25 µL of 5 U/µL Taq DNA Polymeræse (Invitrogen) 0,1 µL of each 2.5 mM primer (forward and reverse primers) and 10 µL of diluted cDNA. The PCR reactions were initiated with 5 min. incubation at 95ºC, followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s. To select the reference gene, we estimated the expression stability of four candidate reference genes, β-Actin, S16, GAPDH and HPRT1 using the freely available online software BestKeeper version 1 [http://gene-quantification.com/bestkeeper.html]. This approach allowed us to select S16 as the reference gene since it showed the lowest coefficient between days and treatment groups compared to β-Actin, GAPDH and HPRT1. Relative levels of mRNA were normalized to S16 reference gene. Cycle threshold (CT) versus input concentration was plotted and efficiencies for each primer pair calculated using the equation $E=10^{-1/s}-1$ where s is the slope. Melt curve analysis (60 °C–95 °C in 0.2 °C increments) was performed at the end of the amplification and some samples were subjected to 1.5% agarose gel electrophoresis to examine product purity and verify correct size for the PCR product. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

2.4. Western blots

For total TH, ERα and PR proteins, the TRIzol protein fractions were prepared according to the manufacturer instructions from the pellets remaining from RNA preparation and quantified using the Bradford method. Aliquots of the dissolved fractions containing 8 (for TH) and 20 µg (for the rest) proteins were separated by 12.5 % SDS-PAGE and electrotransferred to Hybond membranes as previously described (Bonafede *et al.* 2011). Samples from control and HyperT from the same day were processed and run simultaneously. The membranes were probed with anti-PR A+B (1/500 rabbit polyclonal PR130, generated and tested in the Endocrinology and Hormone Dependent

Tumors Laboratory of the National University of Litoral, Santa Fe, Argentina), and anti-ERα (1/500 rabbit polyclonal antibody sc-7207, Santa Cruz Biotechnology Inc Dallas TX.) using horseradish peroxidase-conjugated secondary antisera (1/3,000 polyclonal goat anti-rabbit; Dako Cytomation, Calif., USA), anti-TH (monoclonal mouse anti-TH generously provided by Dr. C. Cuello, McGill University, Montreal, Canada, 1/500) using horseradish peroxidase-conjugated secondary antisera (1/3,000 polyclonal goat anti-mouse – Dako Cytomation) and chemiluminescence reagent to detect specific PR, ERα and TH bands that were quantified by densitometry using NIH Image 1.6/ppc freeware program. The membranes were stripped and reprobed with anti-α-tubulin (1/12,000 mouse monoclonal antibody, Sigma, St. Louis, Mo., USA) and horseradish peroxidase-conjugated secondary antisera (1/3,000 polyclonal goat anti-mouse immunoglobulins, Dako Cytomation) as loading and transfer control.

For PRLR, STAT5, CIS and p-TH Western blots MBHs were homogenized in 250 ul of homogenization buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 10 mM benzamidine, 10 mM NaF, 5 mM sodium pyrophosphate, 20 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM p-nitrophenylphosphate, and aprotinin, leupeptin, and pepstatin at 2 mg/l) in an ice bath. The homogenates were centrifuged at 10,000 g for 30 min and the supernatants separated and frozen in several aliquots at −20 °C until used. Proteins were quantified using the Bradford method. Aliquots containing 20 µg proteins from the dissolved fractions were separated by SDS-PAGE and electrotransferred to Hybond membranes as previously described (Bonafede *et al.* 2011). After rinsing and blocking with BSA 2% the membranes were probed with anti-p-TH (ser-40) (1/500 rabbit polyclonal antibody sc-135715, Santa Cruz Biotechnology Inc.), anti-CIS (H-80): (1/300 rabbit polyclonal antibody sc-15344 Santa Cruz Biotechnology Inc.), STAT5 (c-17) (1/500 rabbit polyclonal antibody sc-835 Santa Cruz Biotechnology Inc.), anti-PRLR (rabbit monoclonal antibody EPR7184(2) AbCam) and anti-α-tubulin (1/12,000 mouse monoclonal antibody, Sigma, St. Louis, Mo., USA) used as a loading and transfer control.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism, and one or two way ANOVA followed by the Bonferroni post hoc test to compare any two individual means. When variances were not homogeneous log transformation of the data was performed. For comparison of two means only, Student's *t* test was used. Differences between means were considered significant at the $P < 0.05$ level.

3. Results.

3.1. Effects of HyperT on circulating hormone levels on late pregnancy.

Elevated circulating, T_3 and T_4 levels and low TSH levels (Supplementary Table 2) in HyperT rats confirmed the effectiveness of the treatments. The fall in circulating P_4 observed on day 21 of pregnancy (G21) in controls was advanced to G20 in the HyperT rats (Fig. 1B). Concomitantly, PRL was significantly increased on G21 midday compared with controls and with the previous day in the HyperT rats (Fig. 1A). These results confirm our previous results (Rosato *et al.* 1992, Rosato *et al.* 1998), where we show that circulating PRL starts to increase on the afternoon of G20 and continues elevated until midday of G21, decreasing afterwards (Anderson *et al.* 2006a). In the control group circulating estradiol levels were similar on the three days while in the HyperT rats, circulating E_2 was significantly lower than controls on G19 and G21, with no differences on G20 (Fig. 1C). There were no significant differences in the weight of control and HyperT rats (not shown).

3.2. Effects of HyperT on MBH TH expression on late pregnancy.

To investigate the hypothalamic mechanism by which HyperT modifies the pattern of PRL secretion between days 19 and 21 of pregnancy, we measured the expression of TH mRNA by qPCR and total TH and p-TH proteins by Western blot. In control rats, TH mRNA values

diminished from G19 to G21 (Fig. 2 A). HyperT did not modify the mRNA content of TH on G19 nor G20, but, interestingly, on G21 TH content remained similar to G20, at values that were significantly higher than controls (Fig. 2A). TH protein also decreased progressively between G19 and 21 in controls. HyperT decreased significantly TH protein at G19 and G20 (Fig. 2B), but at G21 TH expression was significantly increased compared with the previous day (G20), reaching values similar to controls. In euthyroid rats, p-TH declined from G20 to G21 (Fig. 2C). HyperT increased significantly p-TH on G19, but values fell on G20, one day earlier than controls, and remained low on G21 with values similar to controls (Fig. 2C). Thus, the changes in p-TH in each group were parallel to the decrease in circulating P_4 (Fig. 1B).

3.3. Effects of HyperT on MBH expression of PRLR, STAT5b and members of the SOCS-CIS family on late pregnancy.

To determine the hypothalamic mechanisms by which HyperT induced changes in TH expression and activation and thereby on circulating PRL on late pregnancy, we measured the expression of $PRLR_{long}$, STAT5b and members of the SOCS-CIS family mRNA by qPCR and PRLR, STAT5 and CIS proteins by Western blot.

In control rats, the mRNA contents of $PRLR_{long}$, STAT5b SOCS3, SOCS1 and CIS (Fig. 3A) showed similar patterns of variations with high values in G19 that fell markedly on G20 and G21. HyperT rats showed a similar pattern with high values on G19 that decreased sharply afterwards. However, in HyperT rats PRLR mRNA was significantly increased on G19 (Fig. 3A).

In both groups the protein levels of STAT5 decreased from G19 to G20 and remained lower than G19 thereafter (Fig. 3B), in parallel with changes in PRLR mRNA. Interestingly, in the HyperT group STAT5 protein levels were significantly higher than controls in G19, fell markedly on G20 and increased on G21 (Fig. 3B). CIS protein levels increased in controls from G19 to G20 and tended to decrease afterwards to levels that were not different from G19, while in the HyperT group

CIS was significantly higher at G19 compared with controls and declined afterwards to values similar to controls (Fig. 3B). We attempted to measure SOCS proteins by Western blot but were not able to detect them.

3.4. Effects of HyperT on MBH expression of ER and PR on late pregnancy.

Since P_4 and E_2 have leading roles in the regulation of PRL, we measured the expression in MBH of ERα and PR mRNA by real time PCR and of the proteins by Western blot. To calculate the levels of PRA mRNA we used the method proposed by (Hayashi *et al.* 2012).

The mRNA content of total PR, PRA and PRB behaved similarly, decreasing abruptly from G19 to G20 in the control group (Fig. 4A), while in the HyperT group the mRNA content of total PR and both isoforms were significantly decreased in G19 compared with the controls (Fig. 4A); total PR and PRA were significantly higher on G20, while PRB mRNA was significantly increased in G21 compared with the controls (Fig. 4A). We were unable to detect PRB protein, but PRA was readily detectable, and in control rats decreased significantly from G19 to G21. In HyperT rats PRA protein was significantly lower on G19 and G20 compared with controls (Fig. 4B); however, it tended to increase from G20 to G21, reaching values that were not different from controls (Fig. 4B).

The level of ERα mRNA in control groups decreased from G19 to G20 (Fig. 4A). In HyperT rats the mRNA level of ERα tended to be lower than controls in G19 but, following the same pattern as controls, also decreased afterwards to values that were not different from controls (Fig. 4A). In contrast with mRNA values, the expression of ERα protein in control rats was similar on all the days studied (Fig. 4 B). In HyperT rats, ERα protein abundance was significantly higher than controls on G19, fell to values significantly lower than control in G20 but on G21 the values were similar to controls, (Fig. 4 B).

3.5. Effects of HyperT on circulating hormone levels and MBH expression of TH, PRLR, STAT5b, members of the SOCS-CIS family, ER and PR on early lactation (L2).

Rats made hyperthyroid with the dose of 0.25 mg/Kg and allowed to deliver showed impaired maternal behavior and the pups were unable to obtain adequate amounts of milk from their mother, which caused a mortality of 80-90 % within 24 h confirming previous results (Rosato *et al.* 1998). To be able to study the effect of HyperT on lactation we lowered the dose to 0.1 mg/kg from day 18 onwards, a dose that maintains the hyperthyroid state (Supplementary Table 2). This treatment regimen advanced delivery by approximately 11 h, most control rats delivered on the afternoon (at 18.25 h ± 90 min of day 22, while HyperT rats delivered in the morning (at 07.50 h ± 130 min of day 22; mean ± SD, $p < 0.0001$, Mann Whitney test). HyperT rats also had a significantly increased number of pups (Controls 10.4 ± 0.4 vs. HyperT 12.9 ± 0.5, $p < 0.01$, Student's *t* test) but allowed for normal maternal behavior and milk production sufficient for pup survival, confirming previous results (Rosato *et al.* 1998, Varas *et al.* 2002).

T_3 and T_4 values on L2 in the HyperT rats were lower than values of the pregnancy groups, reflecting the lower dose administered to these rats from G18 onwards; however, they were still significantly higher than controls and TSH was significantly lower, indicating that this treatment regimen was also capable of inducing an hyperthyroid state (Supplementary Table 2). Circulating PRL levels were significantly lower in L2 HyperT rats compared with controls, while circulating E_2 values were not affected by HyperT (Fig. 1).

In controls, MBH TH mRNA and protein contents and p-TH on L2 were similar to the values observed on G21 (Fig. 2). In HyperT rats, TH mRNA and p-TH levels were significantly increased, while total TH protein values were similar to controls on L2 (Fig. 2).

In control rats, mRNAs for PRLR, STAT5b, SOCS1, SOCS3 and CIS were significantly increased when compared with G21, while STAT5 protein was significantly diminished and CIS protein content was similar to G21 ($p < 0.05$, ANOVA and Bonferroni post-hoc test, Fig. 3). On L2 HyperT

increased significantly PRLR mRNA and STAT5 protein levels, while there were no significant differences on SOCS1, SOCS3 and CIS mRNAs and on CIS protein (Fig. 3).

Total PR and PRA mRNAs decreased significantly in control rats between G21 and L2, while PRB mRNA PRA protein, ERα mRNA and protein did not change ($p < 0.05$, one-way ANOVA and Bonferroni post-hoc test, Fig. 4). On L2, HyperT increased significantly total and PRA mRNA content, without significant effects on the expression of PRB and ERα mRNAs (Fig. 4). HyperT increased significantly PRA protein level, while there were no significant differences on ERα protein (Fig. 4).

4. Discussion

We have previously shown that HyperT has deleterious effects on lactation, through a partial blockade in suckling induced PRL release that leads to impaired milk production and release and stunted litter growth (Varas *et al.* 2002). The present results confirm our previous findings of low circulating PRL in HyperT rats during established lactation (days 7 and 14) (Varas *et al.* 2002) and extend these results to early lactation (L2). Our findings also confirm the advancement in luteolysis and prepartum PRL release previously described (Rosato *et al.* 1992, Navas *et al.* 2011).

Hypothalamic TH and p-TH expression are good indicators of hypophysiotropic dopaminergic activity, and show variations that correlate inversely with circulating PRL. In the present work we explored the mRNA and protein abundance and the phosphorylation state of the TH enzyme as a marker of its activity. Acting through the JAK2/STAT5 signaling pathway, PRL induces TH expression and phosphorylation of serine 40 (Grattan *et al.* 2008). Phosphorylation confers on TH a greater affinity for its tetrahydrobiopterin cofactor, resulting in an increased rate of dopamine synthesis (Kumer & Vrana 1996), thus limiting PRL secretion through the short feedback loop mechanism (Ben-Jonathan *et al.* 1980, Grattan *et al.* 2001, Grattan *et al.* 2008, Brown *et al.* 2012, Romano *et al.* 2013, Grattan 2015). Ovarian steroids, in particular P_4 also can modulate TH

expression and activation through phosphorylation, acting directly upon the expression of TH or indirectly through modulation of the expression of PRLR and members of the SOCS family, the latter molecules being the main inhibitors of PRL signaling that counteract the activation of this pathway (Arbogast & Voogt 1993, Arbogast & Voogt 1996, Jensik & Arbogast 2011). Although P_4 acutely inhibits DA synthesis and release (and stimulates PRL secretion) through inactivation of TH through an increase in its dephosphorylation (Arbogast 2010), it also stimulates TH mRNA and protein synthesis, so that long term exposure to elevated P_4 increases DA synthesis resulting in inhibition of pituitary PRL release (Jensik & Arbogast 2011). In accordance with previous results (Wang *et al.* 1993, Arbogast & Voogt 1996, Fliestra & Voogt 1997, Li *et al.* 1999, Andrews *et al.* 2001, Valdez *et al.* 2007, Feher *et al.* 2010, Romano *et al.* 2013), in control rats TH mRNA, protein and phosphorylated form decreased gradually in the transition from late pregnancy to lactation, reflecting the establishment of the peripartum damping of the short loop feedback necessary for the maintenance of hyperprolactinemia during lactation (Wang *et al.* 1993). Furthermore, p-TH levels decreased significantly from G20 to G21, in parallel with the fall in circulating P_4, a fall that would promote TH inactivation and the subsequent increase in circulating PRL that is observed on the afternoon of G21 (Rosato *et al.* 1992, Valdez *et al.* 2007, Grattan *et al.* 2008).

It has been described that circulating levels of T_4/T_3 modulate brain TH activity by altering kinetic properties of the enzyme, which in turn influence catecholaminergic activity (Zimmermann *et al.* 2001, Chaube & Joy 2003). The changes produced by HyperT in circulating PRL can be explained fairly accurately by the changes in the expression of TH and p-TH. Thus, in parallel with the changes in P_4 levels, a significant fall in p-TH was observed between G19 and G20, one day earlier than in controls. This fall may be responsible for the increased circulating PRL previously found on the afternoon of G20 that is still detectable on G21 ((Rosato *et al.* 1992) and present results), along with the decrease in total TH protein observed on this day, while on G19, the elevated p-TH may compensate the low total TH levels maintaining the low circulating PRL levels characteristic of this

388 day of pregnancy. The elevated levels of p-TH in HyperT rats on G19 may suggest an elevated
389 basal hypothalamic TH activity compared with controls. On the other hand, on early lactation (L2),
390 the elevated p-TH and TH mRNA levels are indicative of maintenance of TH synthesis and
391 activation, that in turn, suggest an elevated dopaminergic tone that will impair PRL release in
392 response to suckling. Thus, in HyperT rats the normal postpartum attenuation of the short loop
393 regulation of PRL secretion seems to be considerably hindered.
394 At the end of pregnancy in control rats the fall in PRLR and STAT5 mRNAs between G19 and 20
395 may contribute to the attenuation of the shortloop feedback mechanism, making the cells less
396 responsive to PRL and/or PLs. This process is necessary for the desensitization of hypothalamic
397 neuroendocrine dopaminergic neurons to elevated PRL levels, that allows a normal transition to
398 lactational hyperprolactinemia. Although there was a slight increase on L2, the values continued to
399 be much lower than on G19, maintaining a decreased responsiveness to PRL during lactation. Thus,
400 the fall in TH mRNA and protein and in p-TH between G19 and L2 in control rats may be a
401 consequence of the decreased PRLR and STAT5 expression. In turn, the decrease in the mRNAs of
402 the members of the SOCS family may also reflect the physiological low responsiveness to PRL,
403 since they are target genes for PRL but also attenuate the inhibition of PRL signaling produced by
404 the decreased PRLR and STAT5 (Anderson *et al.* 2006a, Anderson *et al.* 2006b). At the end of
405 pregnancy, PRL has a diminished ability to activate STAT5b (Anderson *et al.* 2006a), however, in
406 HyperT rats the PRL signaling pathway seems to be maintained in a more active state, since
407 $PRLR_{long}$ mRNA and STAT5 protein were significantly increased in G19 and L2. Furthermore, the
408 lack of change in the mRNAs of SOCS1, SOCS3 and CIS in HyperT rats on L2, in the presence of
409 increased PRLR mRNA and STAT5 protein may contribute to the failure to attenuate PRL
410 signaling in early lactation. Thus, the brain is no exception to the effect of thyroid status on the
411 expression of PRLR, as seen in other organs such as liver, kidney, adrenal, prostate, mammary
412 gland and ovary (Tiong *et al.* 1992).

The ovarian steroids E_2 and P_4, are arguably one of the most important regulators of PRL synthesis, secretion and action in several different physiological states, acting at pituitary and hypothalamic levels. TIDA neurons express receptors for both (ERα and PR), therefore the steroids can directly modulate TH expression and PRL signaling (Steyn *et al.* 2007, Anderson *et al.* 2008, Steyn *et al.* 2008), and have been shown to regulate the expression of their cognate receptors as well as of PRLR in several brain areas (Pi *et al.* 2003). The fall in PRA protein and mRNA for total, A and B PR isoforms from G19 to L2 may be one factor down regulating TH expression and activation in the control, euthyroid rats. This fall was advanced in HyperT rats, which, along with the early fall in circulating P_4 may participate in the mechanism that induces the premature increase in PRL secretion seen in the present work on G21 and, since P_4 has been shown to inhibit estrogen induction of PR expression, may be responsible for the subsequent slight increases in the mRNAs of PRA on G20 and PRB on G21. In contrast, the increased PRA protein seen in HyperT rats on L2, may contribute to maintain the elevated TH expression and activation during lactation, repressing PRL secretion. P_4 is able to activate the TH promoter acting through both PR isoforms, although PRB is much more effective (Jensik & Arbogast 2011). Unfortunately we could not detect PRB protein and thus are unable to ascertain whether it changes in parallel with PRA.

Other authors found stable levels of ERα mRNA (Wagner & Morrell 1995, Mann & Babb 2005) during pregnancy and early postpartum; our results may confirm these findings, with the exception of the high levels of ERα mRNA found on G19. However, the previous works did not study this particular day of pregnancy, which may differ from previous or subsequent days in ER mRNA expression. We did find constant levels of ERα protein in controls between G19 and L2, that are in accord with the findings of Steyn et al. (Steyn *et al.* 2007), who found similar values of ER immunoractivity in TH+ hypothalamic neurons between G19 and G21, with a significant diminution only on day 5 of lactation. In contrast, in euthyroid rats we found a steady decrease in PR mRNA and protein expression between G19 and L2, confirming previous results (Mann & Babb

2005). Steyn et al. (Steyn *et al.* 2007) also found decreased PR and ER expression in TH+ cells between G21 and early lactation. The patterns of PR and ERα expression were disrupted by HyperT, in particular the protein levels, which along with the changes in circulating P_4 and E_2, may account for some of the changes observed in PRL signaling pathway and TH expression.

The changes in ERα and PR expression patterns observed in the HyperT rats may have been caused by a combination of the hormonal changes observed at the times studied, along with possible direct actions of thyroid hormones at hypothalamic dopaminergic level. Thus, the fall in PRs mRNAs and PRA protein observed on G19 in HyperT rats may be a consequence of the low circulating E_2 observed on this day, since estrogens induce expression of PR in TH+ arcuate neurons (Steyn *et al.* 2007). The elevated ERα protein levels observed in HyperT rats may have not been able to compensate for the low circulating E_2. The combination of increased ERα and decreased PRA at the protein level in HyperT rats on G19 may account for the increased expression of $PRLR_{long}$ mRNA and CIS proteins, since it has been shown that E_2 stimulate their expression and P_4 blocks the stimulatory action of E_2 (Pi *et al.* 2003, Steyn *et al.* 2008).

The different patterns of expression of ERα and PRA between mRNA and protein along the days studied may be due to changes with time of the regulatory mechanisms acting at posttranscriptional levels that may modulate translation, processing or degradation of the protein (Jacobsen & Horwitz 2012). In turn, HyperT may also have modified these mechanisms, since PRA and ERα proteins showed patterns markedly different from controls. We have also found co-expression of thyroid hormone receptors (TRs) and TH in hypothalamic neurons (unpublished results), suggesting that at least some of the actions on TH expression, receptors and PRL signaling pathway members may be exerted directly by thyroid hormones on TH+ cells. Furthermore, TRs interact with steroid hormone receptors at various levels (Freyschuss *et al.* 1994, Vasudevan *et al.* 2002, Fujimoto *et al.* 2004), modifying their expression and actions. In particular, TRs activation has been shown to increase ER

levels in liver and in a pituitary cell line (Freyschuss *et al.* 1994, Fujimoto *et al.* 2004), and thus may account for the increased ERα protein levels found in HyperT rats on G19.

In previous works from our laboratory we described that both hyper- and hypothyroidism affect the content of the neuropeptide NEI, which is involved in cognitive and behavioral responses and in neuroendocrine function, in discrete brain areas in female and male rats (Ayala *et al.* 2011, Ayala *et al.* 2013). We described differential effects during the estrous cycle where NEI content was affected by the circulating levels of ovarian steroids (Ayala *et al.* 2013). More recently we reported that altered thyroid status affects the interaction between TH+ neurons and fibers and NEI+ neurons in a specific hypothalamic dopaminergic population of neurons, the A13 group of the incertohypothalamic area (Ayala *et al.* 2015). These are further evidences of the impact of thyroid disturbances in specific brain areas related to neuroendocrine function and its regulation.

The $PRLR_{(long)}$ and steroid hormone receptors are also expressed by non-TH neurons in the ARC, including KNDy neurons (Kokay *et al.* 2011) and POMC neurons (Cave *et al.* 2001, Kokay & Grattan 2005), and thus, the observed changes could be the summation of the effects on TH neurons and other populations (POMC or KNDy neurons, etc.) residing in the MBH. However, the most prevalent neurons in this area are dopaminergic TH+ neurons that also express abundantly PRLR, ERs and PRs (Kokay & Grattan 2005, Steyn *et al.* 2007). Furthermore, in Cave et al., 2001 it is shown that the TH+ neurons are much more responsive to PRL than other PRLR expressing population, such as POMC neurons. Thus, it is quite probable that the changes observed in the expression of the members of the PRL signaling pathway take place in the TH+ neurons, especially on L2 HyperT rats, when we observed elevated STAT5 protein and elevated PRLR mRNA, and that these variations are responsible for the changes observed in TH expression and activation. We cannot exclude the possibility that HyperT induces changes in the expression of PRLR, members of PRL signaling pathways and steroid hormone receptors in other MBH cell populations, that may affect the overall expression of these genes.

In conclusion, the changes induced by HyperT in TH expression and activation may explain the concomitant changes in circulating PRL, such as the premature prepartum increase and the low levels observed in early lactation. Our findings also may show that the short loop negative feedback mechanism is constitutively more active in HyperT than in euthyroid rats (Fig. 5)**,** compromising its physiological attenuation at the end of pregnancy and during lactation and accounting for the deficit in lactation of HyperT mothers previously described (Rosato *et al.* 1992, Varas *et al.* 2002). The changes observed in the present work at different time points in PRLR, PR and ER expression at hypothalamic level and the consequent increase in STAT5, contribute to maintain elevated levels of TH mRNA and p-TH, thus maintaining an increased dopaminergic tone during lactation and thereby blunting the suckling induced PRL release (Varas *et al.* 2002).

These findings indicate that HyperT has non-negligible effects at hypothalamic levels that in turn, compromise lactational performance and the development of the newborn. Although hyperthyroidism in pregnant women is not frequent, its consequences when not treated adequately are often severe. The results of the present study may contribute to the management of clinical hyperthyroidism in the puerperium when the mothers wish to nurse their infant.

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Legends to Figures:

Figure 1: Effect of hyperthyroidism (HyperT, 0.25-0.1 mg/Kg/day *s.c.* T_4, black bars), on circulating (A) PRL, (B) P_4 or (C) E_2 concentrations in female rats during days 19, 20, and 21 of pregnancy (G19, G20, G21) and day 2 of lactation (L2) measured by RIA. Control rats (gray bars) were injected with saline. See Materials and Methods section for further details. Results are means ± SEM of groups of 8–10 animals in each experimental group.

*$p < 0.05$ comparing the different groups within the same time point (day). Different superscript letters represent significant differences at $p < 0.05$ between the different days of pregnancy or lactation within the same experimental group, using two-way ANOVA followed by Bonferroni post hoc test.

Figure 2: Effect of hyperthyroidism (HyperT, 0.25-0.1 mg/Kg/day *s.c.* T_4, black bars), on MBH content of tyrosine hydroxylase (TH) in female rats on days 19, 20 and 21 of pregnancy (G19, G20, G21) and day 2 of lactation (L2). Control rats (gray bars) were injected with saline. mRNA levels were measured by real time RT-PCR on samples of MBH total RNA and protein levels by Western blot. The graphs may have different scales. See Materials and Methods section for further details. (A) TH mRNA expression; (B) Total TH protein expression; (C) p-TH protein expression. Each column represents mean ± SEM of groups of 6-8 rats.

*$p < 0.05$ comparing the different groups within the same time point (day). Different superscript letters represent significant differences at $p < 0.05$ between the different days of pregnancy or lactation within the same experimental group, using two-way ANOVA followed by Bonferroni post hoc test.

Figure 3: Effect of hyperthyroidism (HyperT, 0.25- 0.1 mg/Kg/day *s.c.* T_4, black bars), on MBH content of $PRLR_{Long}$; STAT5b; CIS, SOCS1 and SOCS3 mRNAs (A) and STAT5 and CIS proteins (B) in female rats on days 19, 20 and 21 of pregnancy (G19, G20, G21) and day 2 of lactation (L2). Control rats (gray bars) were injected with saline. mRNA levels were measured by real time RT-

PCR on samples of MBH total RNA and protein levels by Western blot. The graphs may have different scales. See Materials and Methods section for further details. Each column represents mean ± SEM of groups of 6-8 rats.

*p < 0.05 comparing the different groups within the same time point (day). Different superscript letters represent significant differences at p < 0.05 between the different days of pregnancy or lactation within the same experimental group, using two-way ANOVA followed by Bonferroni post hoc test.

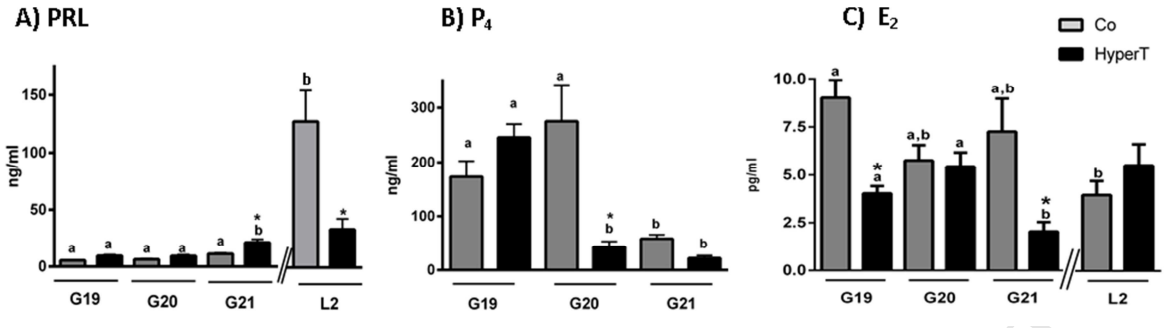
Figure 4: Effect of hyperthyroidism (HyperT, 0.25 mg/Kg/day *s.c.* T_4, black bars), on MBH content of total PR, PRA, PRB and ERα mRNA (A) and PRA and ERα protein (B) in female rats on days 19, 20 and 21 of pregnancy (G19, G20, G21) and day 2 of lactation (L2). Control rats (gray bars) were injected with saline. mRNA levels were measured by real time RT-PCR on samples of MBH total RNA and protein levels by Western blot. The graphs may have different scales. See Materials and Methods section for further details. Each column represents mean ± SEM of groups of 6-8 rats.

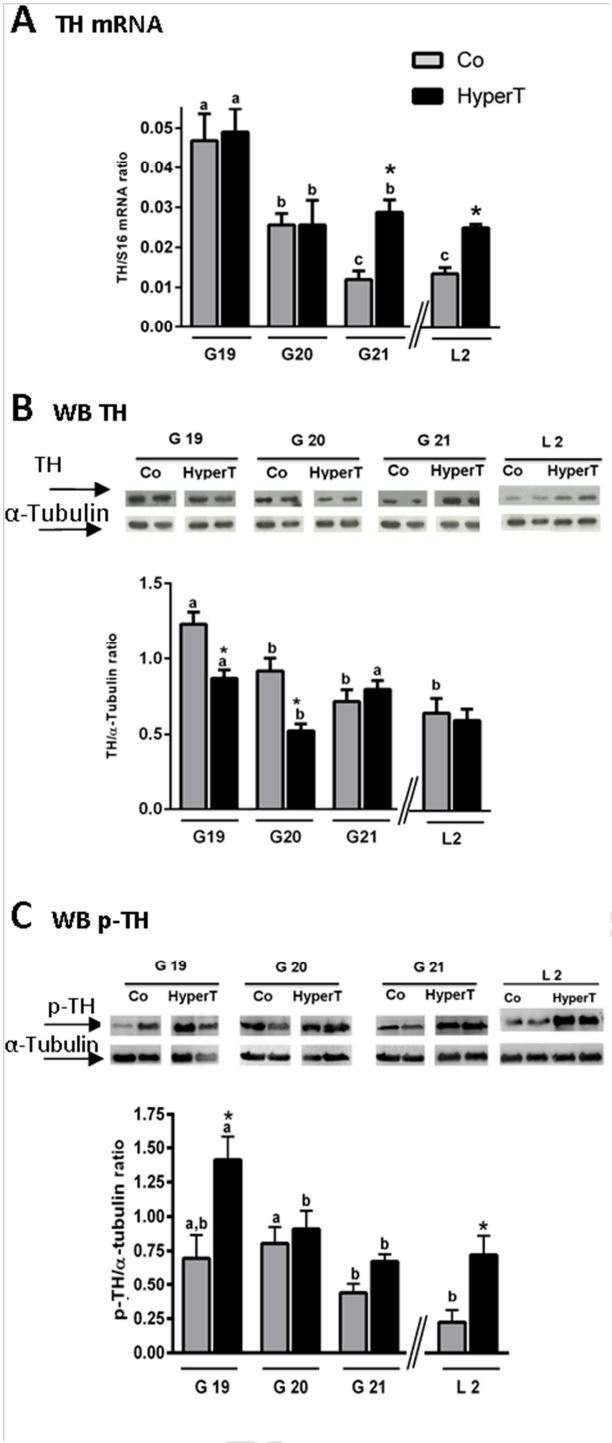
*p < 0.05 comparing the different groups within the same time point (day). Different superscript letters represent significant differences at p < 0.05 between different days of pregnancy or lactations of the same experimental group.

Figure 5: Model of the proposed changes in the regulation of PRL secretion at the end of pregnancy and early lactation in euthyroid and HyperT rats. Panel A: representative scheme of circulating hormones (PRL and P_4); Panel B: representative changes in hypothalamic dopaminergic activity and the short loop feedback (SLF). In control euthyroid rats, during late pregnancy (G19) SLF is active, with high expression of the members of the PRLR/JAK/STAT5 signaling pathway (which change in parallel with the SLF, not shown in the figure) that maintain high TH activity and expression and low PRL levels. In addition, high levels of P_4 contribute to maintain elevated TH expression and activity. In euthyroid conditions, as delivery approaches, the short loop negative feedback mechanism begins to attenuate, as shown by the decreases in the expression of TH, PRLR

and STAT5, but elevated P_4 maintains p-TH at high levels and low circulating PRL. When P_4 falls in G21, p-TH decreases and initiates the prepartum PRL surge. In the postpartum, P_4 and PRs are low and the short loop feedback continues to attenuate (with low PRLR and STAT5 expression and increases in SOCS and CIS mRNAs), enabling high PRL release induced by suckling.

In HyperT rats, the short loop negative feedback mechanism seems to be constitutively more active than in euthyroid rats, evidenced by high PRLR, STAT5 and p-TH on G19 and in the postpartum. However, the premature luteolysis and fall in P_4 induces an advancement in the fall in total TH, p-TH and in the PRL surge. However, on G21 and after delivery, the short loop negative feedback mechanism seems to be less attenuated, as evidenced by the increases in PRLR protein on G21 and PRLR mRNA, and STAT5 protein on L2, without significant changes in SOCS and CIS mRNAs, that in turn results in elevated p-TH and TH mRNA and, as a consequence low serum PRL levels.

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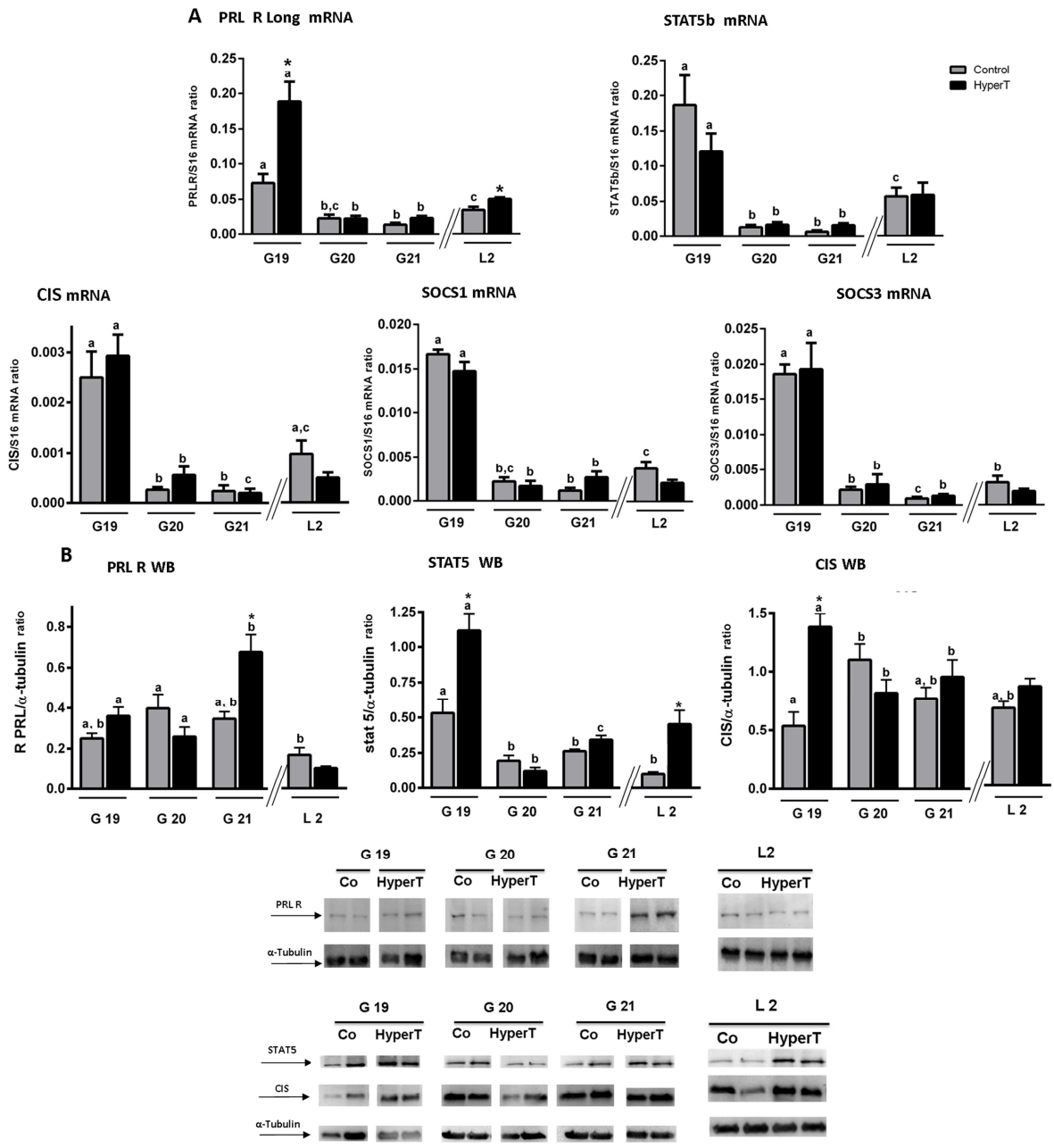
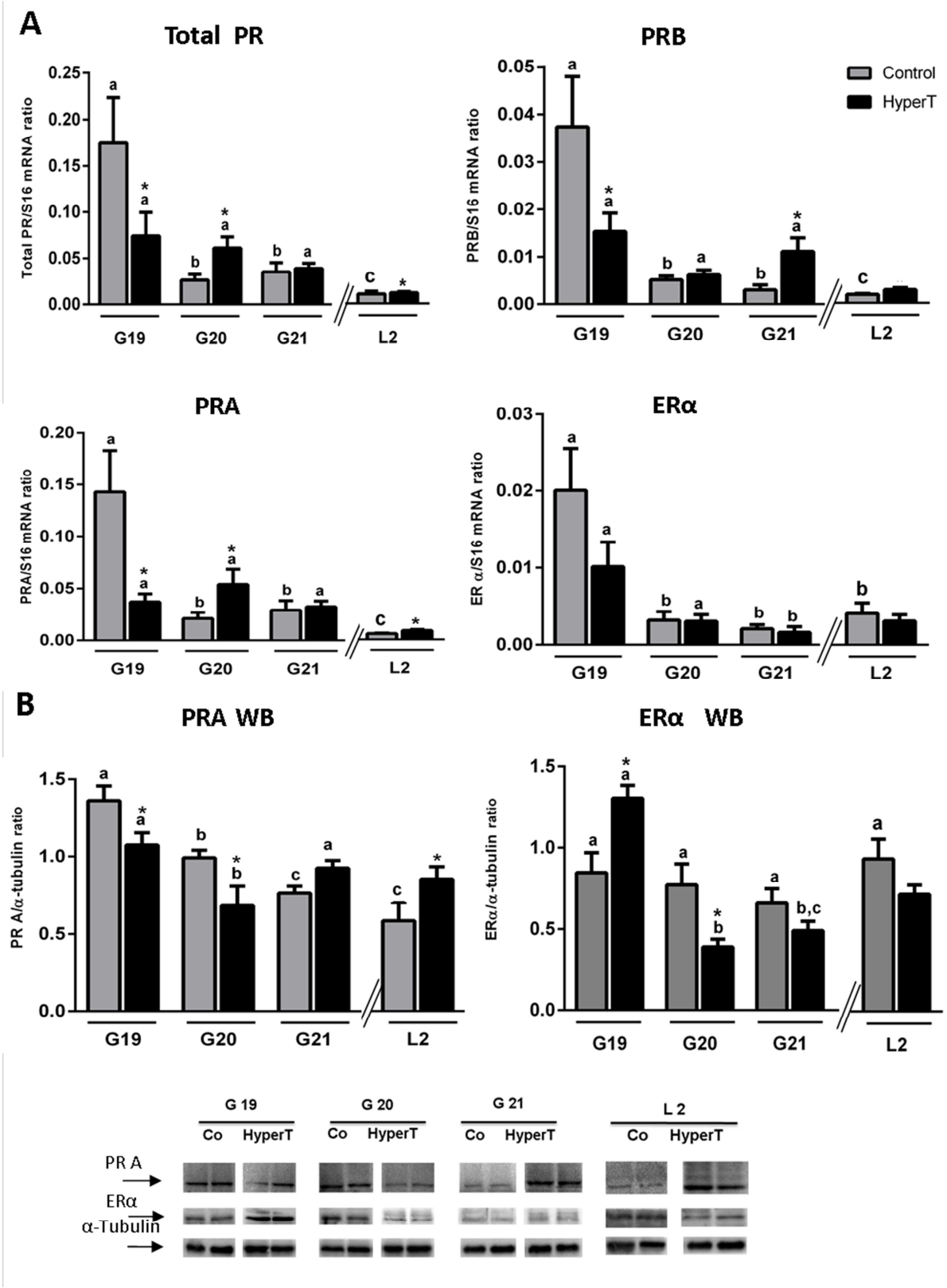
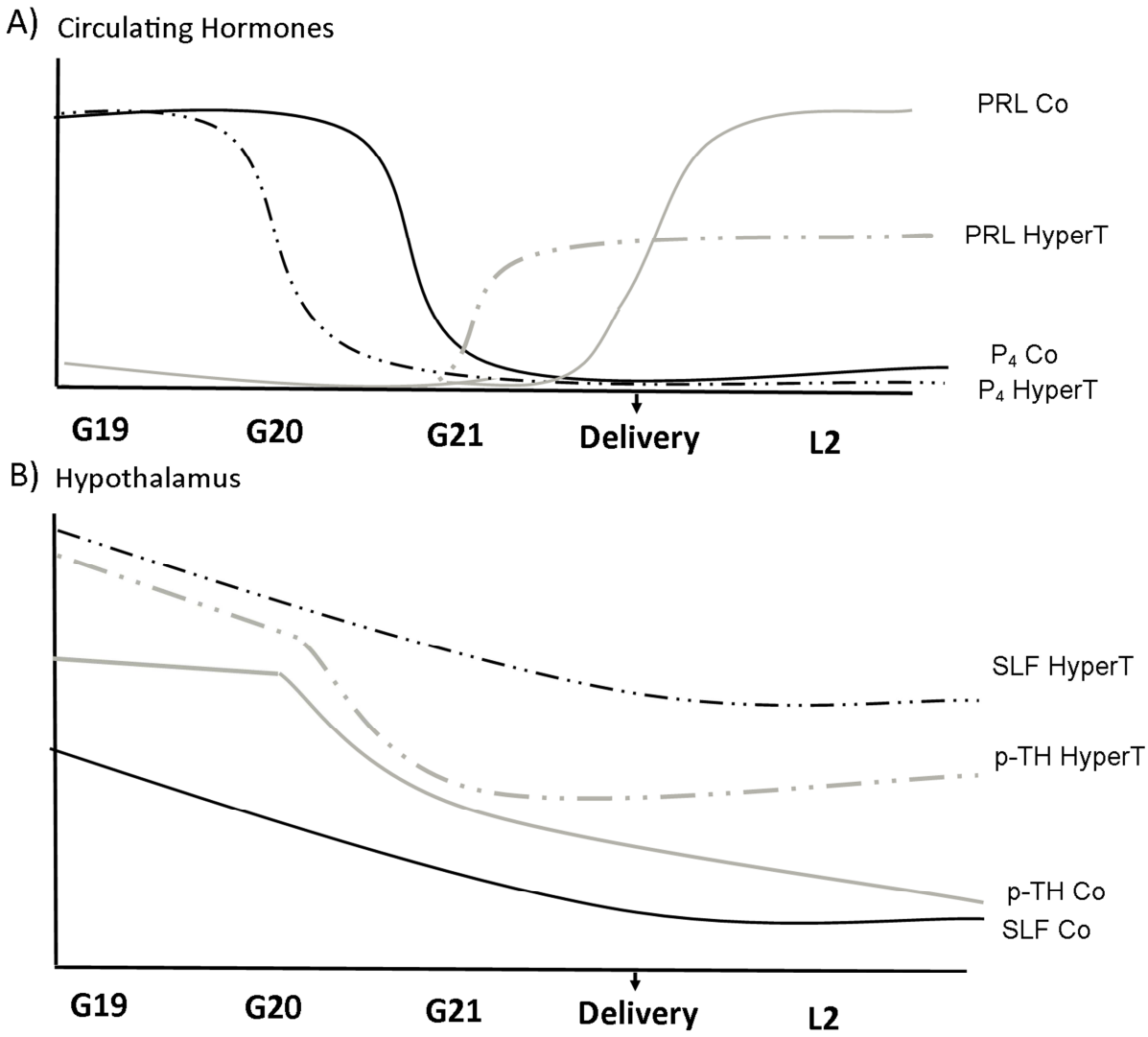
1.5
C WB p-TH
p-TH
α-Tubulin
p-TH/α-tubulin ratio
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75


A
PRL R Long mRNA
PRLR/S16 mRNA ratio
STAT5b mRNA
STAT5b/S16 mRNA ratio
Control
HyperT
CIS mRNA
CIS/S16 mRNA ratio
SOCS1 mRNA
SOCS1/S16 mRNA ratio
SOCS3 mRNA
SOCS3/S16 mRNA ratio
G19
G20
G21
L2
B
PRL R WB
R PRL/α-tubulin ratio
STAT5 WB
stat 5/α-tubulin ratio
CIS WB
CIS/α-tubulin ratio
G 19
G 20
G 21
L 2
Co
HyperT
PRL R
α-Tubulin
STAT5
CIS


A
Total PR
Total PR/S16 mRNA ratio
PRB
PRB/S16 mRNA ratio
Control
HyperT
PRA
PRA/S16 mRNA ratio
ERα
ER α/S16 mRNA ratio
G19
G20
G21
L2
B
PRA WB
PR A/α-tubulin ratio
ERα WB
ERα/α-tubulin ratio
G 19
G 20
G 21
L 2
Co
HyperT
PR A
ERα
α-Tubulin


A) Circulating Hormones
PRL Co
PRL HyperT
P4 Co
P4 HyperT
G19
G20
G21
Delivery
L2
B) Hypothalamus
SLF HyperT
p-TH HyperT
p-TH Co
SLF Co
G19
G20
G21
Delivery
L2

Highlights

- HyperT blocks suckling-induced PRL release and advances the prepartum PRL surge
- HyperT elevated hypothalamic TOH mRNA and p-TOH on late pregnancy and early lactation
- PRLR, STAT5, CIS, PRA and B also increased on late pregnancy and/or early lactation
- The altered PRL signaling and PRs may induce elevated TOH expression and activation
- This elevates dopaminergic tone on lactation, blunting PRL release and litter growth