Neurochem Res
DOI 10.1007/s11064-016-2067-x

ORIGINAL PAPER

Role of Estradiol in the Regulation of Prolactin Secretion During Late Pregnancy

Carlos Villegas-Gabutti[1] · Gisela E. Pennacchio[1] · Graciela A. Jahn[1] · Marta Soaje[1,2]

Received: 13 May 2016 / Revised: 6 September 2016 / Accepted: 15 September 2016

Abstract Estrogen action is necessary for evidencing the stimulatory action of mifepristone and naloxone on prolactin (PRL) secretion during late pregnancy. Our aim is to determine the mechanism mediating this facilitator action of estrogens. To investigate the hypothalamic mechanisms involved in estrogen actions in PRL secretion at the end of pregnancy, we measured the effect of pretreatment with the estrogen antagonist tamoxifen on the expression of tyrosine hydroxylase (TH), hormone receptors (ERα and β, PRs, $PRLR_{(long)}$), and μ- and κ- opioid receptors (ORs) at mRNA (by semiquantitative RT-PCR) and protein (by western blot for TH, $PRLR_{(long)}$, ERα, PRs, μ- and ORs) levels in extracts of medial basal hypothalamus (MBH) and serum PRL, E_2 and P_4 levels (by RIA) in mifepristone- and naloxone-treated rats. Tamoxifen administration partially prevented PRL release induced by the combined treatment. TH expression diminished and ERα expression increased in mifepristone-treated rats at mRNA and protein levels and tamoxifen partially prevented these changes with no effect on PRs expression. Mifepristone increased $PRLR_{(long)}$ mRNA levels; this increase was blocked by tamoxifen. Combined tamoxifen and mifepristone treatment decreased μ- and k-ORs mRNA but not protein levels. In conclusion, E_2 induces neuroadaptive mechanisms necessary to facilitate PRL release preceding delivery. Acting through ERα, E_2 modulates hypothalamic dopaminergic neurons activity, regulating TH, μ- and κ-ORs and $PRLR_{(long)}$ expression, and is necessary for evidencing the effects of P_4 withdrawal. Its presence on days 14 and 15 of pregnancy is crucial to facilitate the opioid system modulation of PRL secretion at the end of pregnancy in the rat.

Keywords Estradiol · Prolactin · Tyrosine hydroxylase · Mifepristone or RU-486 · Naloxone · Pregnancy · Rat

Introduction

Prolactin (PRL) secretion is regulated by ovarian steroids that control both its synthesis and release. Various studies have shown that progesterone (P_4) has an inhibitory control on PRL release during late pregnancy [1, 2]. Thus, the spontaneous P_4 fall observed on days 21–22 of pregnancy is accompanied with an increase in PRL. Premature withdrawal of P_4 by ovariectomy or $PGF_{2\alpha}$ treatment on days 19 or 20 of pregnancy is followed by an increase in PRL 12–24 hs later [1–4] and is mediated through a decrease in dopaminergic activity in the medial basal hypothalamus (MBH) [4]. This effect of P_4 fall seems to be dependent on estrogen action [5] since administration of the estrogen receptors (ERs) antagonist tamoxifen prevented the increase in circulating PRL [3, 6]. We also demonstrated that the blockade of the central inhibitory action of P_4 by the antiprogesterone mifepristone reduces dopaminergic tone within 8–10 h [7], which alone is not sufficient to release PRL within this time frame [3, 8], however, PRL increases 48 h after mifepristone treatment, after a decline in circulating P_4 [3]. Supporting these results, we have also found that mifepristone treatment on day 19 of pregnancy induces lactotroph activation and stimulation of PRL synthesis, but with a low rate of secretory granule exocytosis and therefore no increment

✉ Marta Soaje
msoaje@mendoza-conicet.gob.ar

[1] Laboratorio de Reproducción y Lactancia, IMBECU-CONICET, Casilla de Correo 855, 5500 Mendoza, Argentina

[2] Facultad de Ciencias Médicas, Instituto de Fisiología, Universidad Nacional de Cuyo, Mendoza, Argentina

Published online: 01 October 2016

in serum PRL [9]. However, administration of the opioid antagonist naloxone to mifepristone treated rats induces an acute release of PRL and exocytotic images of PRL granules in the lactotrophs indicative of lactotroph activation showing that at this time the opioid system inhibits PRL secretion in a mechanism depending on the lifting of the P_4 inhibitory tone, since naloxone administered alone had no effect [8, 9]. This mechanism is also active during the decline in circulating P_4 seen on day 21 of pregnancy, since the magnitude of PRL secretion in response to naloxone on this day is inversely correlated with serum P_4 concentration [8]. Furthermore, estrogen action from days 14–16 of pregnancy is crucial for activation of the opioid system as an inhibitory system for PRL secretion, because the administration of tamoxifen, on days 14 and 15, prevents the stimulatory action of mifepristone and naloxone on PRL secretion in control [6] and stressed rats [10].

Estradiol (E_2) acts at the hypothalamus-pituitary gland level to stimulate PRL synthesis and secretion by inhibiting dopamine transmission. At the pituitary gland level, E_2 controls PRL gene expression, decreases the number of dopamine D_2 receptors and increases lactotroph sensitivity to PRL releasing factors (PRFs) [11]. At hypothalamic level, E_2 inhibits tyrosine hydroxylase (TH) activity, the rate-limiting enzyme involved in dopamine synthesis in the arcuate nucleus dopaminergic neurons [11]. Although the existence of prolactin releasing factors is controversial [11, 12], E_2 increases lactotroph sensitivity to other regulators [13, 14] and modulates ER-expressing neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON), that produce two putative PRFs: TRH and oxytocin [15].

During early pregnancy in the rat, E_2 serum levels are low but begin to increase gradually at days 15–16 until reaching the peak that precedes delivery. However, despite this gradual increase, it seems clear that the elevated P_4 serum levels at the end of gestation block the stimulatory action of E_2 on PRL [1, 2, 6, 10].

Hypothalamic dopaminergic neurons express different isoforms of ER [16], progesterone receptor (PR) [16] and PRL receptor (PRLR) [17] and the hormonal environment modulates their expression [18, 19]. In particular, high ERα expression in tuberoinfundibular dopaminergic (TIDA) neurons is observed during late pregnancy [16]. Using double staining immunohistochemistry (IHC), it has been demonstrated that ER and PR are co-expressed in many hypothalamic regions [20]. PRLR expression in hypothalamic nuclei varies depending on the reproductive state, increasing in rats during pregnancy and lactation [21, 22] or in E_2 treated rats [23] compared to animals in diestrus. PRL activates its own receptor and stimulates dopamine transmission in hypothalamic hypophysotropic areas, promoting the negative feedback loop to limit its own secretion [24, 25].

Endogenous opioid peptides have dual effects on PRL release, they can suppress the activity of TIDA neurons [26–28] thereby increasing PRL secretion, [6, 29, 30] on lactating, cycling and early pregnant rats, and at the end of pregnancy they can be stimulatory or inhibitory depending on the experimental model used [8, 31, 32].

A sparse μ-opioid receptors (ORs) expression is observed in dopaminergic neurons of the arcuate nucleus, compared with the abundant expression of this receptor in the median eminence [33], suggesting a modulatory action of endogenous opioids in TIDA neurons from the arcuate nucleus, mediated by activation of μ-OR at presynaptic level [33, 34]. E_2 and P_4 regulate the expression of the μ-OR in the preoptic area and in the arcuate nucleus [35, 36] and may regulate its activation [37]. Unlike μ-OR expression, κ-opioid receptor (κ-OR) is abundant in the arcuate nucleus and in the median eminence [33] and E_2 ability to suppress the inhibition of TIDA neurons is mediated by this opioid receptor subtype [38].

The aim of the present work is to study the mechanisms whereby E_2 modulates the inhibitory action of P_4 and the opioid system on PRL secretion at the end of pregnancy, using as a model, the PRL release induced by mifepristone and naloxone administration on day 19 of pregnancy in rats. We evaluated the expression levels of TH, $PRLR_{(long)}$, ERα and β, PRs, and μ- and k- ORs in the MBH in order to establish a neurochemical correlate of E_2 actions in the regulation of PRL secretion at the end of pregnancy.

Materials and Methods

Animals

Virgin female rats, 3–4 months old (200–220 g), bred in our laboratory and originally of the Wistar strain were used. They were kept in a light (06:00–20:00 h) and temperature (22 ± 2 ºC)-controlled room; rat chow (Cargill, Argentina) and tap water were available ad libitum. Vaginal smears were taken daily; virgin rats showing two or three consecutive 4 day cycles were used. Rats were made pregnant by being caged individually with a fertile male on the night of pro-oestrus. Vaginal smears were checked for the presence of spermatozoa on the following morning and that day was considered day 0 of pregnancy. Rats from our colony normally deliver on day 22 of pregnancy. All the procedures performed on the animals were in accordance with the guidelines suggested by 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).

Drugs

Naloxone (NAL) and the antiprogesterone mifepristone (Mp) (17-hydroxy-11-[4-dimethyl-amino-phenyl]-17(-propinyl-estra-4,9-dien-3-one; RU-486) were obtained from Sigma Chemical Co, St Louis, MO, USA. Tamoxifen citrate (Tm) was provided by Gador S.A., (Buenos Aires, Argentina).

Experimental Design

Tamoxifen citrate, dissolved in 0.14 M NaCl, 0.5% (v/v) Tween 80 or vehicle was administered per os at a dose of 500 μg/kg body weight on days 14 and 15 of pregnancy. On day 19 of pregnancy, mifepristone, dissolved in sunflower seed oil, at a dose of 5 mg/kg or vehicle were administered subcutaneously at 08:00 h and naloxone, dissolved in 0.9% (w/v) NaCl was injected *i.p.* at a dose of 2 mg/kg at 17:30 h. Control animals were treated with saline. Thirty minutes after naloxone or saline administration, animals were sacrificed by decapitation and trunk blood was collected to determine PRL, E_2 and P_4 serum levels by RIA. Brains were removed and the MBH were dissected to extract total mRNA with TRIzol, in order to determine TH, ERα and ERβ, PR, $PRLR_{(long)}$, μ-and κ-opioid receptors and β-actin expression by semi-quantitative RT-PCR. After total mRNA extraction with TRIzol, proteins were isolated from phenol-ethanol supernatants, according to the manufacturer instructions. The μ-and κ-opioid receptors, ERα, PR A, PR B, $PRLR_{(long)}$ and TH protein expression were determined by Western blot.

Brain Tissue Sampling

After decapitation the brains were rapidly removed and immediately placed on an aluminum plate at 4 °C for the cutting of coronal rat brain slices (2 mm) using a rat brain matrix (RBM 4000C; ASI Instruments, Inc., Warren, MI, USA). The sections were collected onto glass microscope

Table 1 Sequences and conditions for the PCR reactions

Primer	Sequence 5′–3′	Cycles, n	Gen Bank accesion N°
TH			
Sense	CCCCACCTGGAGTATTTTGTG	25	NM_012740.3
Antisense	ATCACGGGCGGACAGTAGACC		
ERα			
Sense	AATTCTGACAATCGACGCCAG	30	NM012689
Antisense	GTGCTTCAACATTCTCCCTCCTC		
ERβ			
Sense	GCCAATCATGTGCACCAGTTCCTT	32	NM012754
	AAAGCCAAGAGAAACGGTGGGCAT		
PR A + B			
Sense	CCCACAGGAGTTTGTCAAGCTC	31	NM0228471
Antisense	TAACTTCAGACATCATTTCCGG		
PRL $R_{(long)}$			
Sense	AAAGTATCTTGTCCAGACTCGCTG	23	NM012630.1
Antisense	AGCAGTTCTTCAGACTTGCCCTT		
μOR			
Sense	ACCTGGCTCCTGGCTCAACTT	34	NM013071
Antisense	TGGACCCCTGCCTGTATTTTG		
κOR			
Sense	AGTCCCCCATCCAGATTTTCC	32	NM_011011.2
Antisense	ACGGCAATGTAAAGGTCCAC		
β-actin			
Sense	CGTCCGCCCTAGGCACCA	25	BC063166
Antisense	TTGGCCTTAGGGTTCAGAGGGG		

All reactions were carried out with the following cyclic parameters: 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, except for TH, for which the annealing temperature was 58 °C. All the reactions were terminated with a 5 min extension at 72 °C

slides and immediately frozen. A block of tissue corresponding to MBH comprising the arcuate nucleus, periventricular nucleus and median eminence was microdissected and processed for total RNA or protein extraction.

RNA Extraction and RT-PCR

Total RNA was isolated from the microdissected MBH from rats on day 19 of pregnancy with TriZol (GIBCO-BRL), according to the manufacturer´s instructions. Integrity of the isolated total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by ultraviolet light absorbance at 260 nm. Reverse transcription (RT) was carried out using 10 µg of total RNA from the MBH of each rat. RT was performed at 37 °C for 60 min using 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). Aliquots of the reverse transcription reaction were amplified with rat specific primers (Table 1). For the end point PCR amplification, oligonucleotide primers (0.5 µM each) were incubated with 5 µl of cDNA template in a 35 µl PCR reaction mixture containing 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris–HCl, pH 9, 1 µl of deoxynucleotides (1 mM each), and 1 unit of Taq polymerase (Invitrogen). Before proceeding with the semiquantitative PCR, the conditions (annealing temperatures, duration of each step and number of cycles) were established for MBH such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input RNA (see Table 1 for specific conditions of each amplification). RNA samples were assayed for DNA contamination by PCR without the prior reverse transcription. The products of each reaction were separated on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed with a digital camera. Band intensities of RT-PCR products were quantified using the NIH Image software; relative levels of mRNA were calculated as the ratio of signal intensity for the target genes relative to β-actin cDNA. The results are expressed as arbitrary OD units.

Western Blots

The TRIzol protein fractions were dissolved, separated by SDS-PAGE and electrotransferred to Hybond membranes as described previously [4]. After rinsing and blocking with BSA, 2% horse serum [4], the membranes were probed with anti-TH (monoclonal mouse anti-TH generously provided by Dr. C. Cuello, McGill University, Montreal, Canada, 1/500) [7] using horseradish peroxidase-conjugated secondary antisera (1/3,000 polyclonal goat anti-mouse, Dako Cytomation), anti-ERα (rabbit polyclonal antibody sc-7207, Santa Cruz Biotechnology Inc., Dallas TX, 1/300), anti-PR A + B which recognizes both PR isoforms with similar affinity (rabbit polyclonal antibody PR130, generated and tested in the Endocrinology and Hormone Dependent Tumors Laboratory of the National University of Litoral, Santa Fe, Argentina, 1/250) [39], anti PRLR (rabbit monoclonal [EPR7184(2)] antibody ab170935, Abcam, Cambridge, UK, 1/1000), anti-µ-OR (rabbit polyclonal antibody sc-15310, Santa Cruz Biotechnology, Inc., Dallas TX, 1/750) and anti-κ-OR (rabbit polyclonal antibody sc-9112, Santa Cruz Biotechnology, Inc., Dallas TX, 1/750) using horseradish peroxidase-conjugated secondary antisera (1/3000 polyclonal goat anti-rabbit; Dako Cytomation, CA, USA). The membranes were re-probed with anti-β-actin (1/10,000 mouse monoclonal antibody, Sigma. St. Louis, MO, USA) and horseradish peroxidase conjugated secondary antisera (1/2000 polyclonal goat antimouse immunoglobulins Dako Cytomation, CA, USA) as loading and transfer control. After three washes in PBS-T, specific receptor bands were detected by chemiluminiscence (ECL™, Amersham) using a ChemiDoc XRS+ System with Image Lab Software from Bio-Rad and then quantified by densitometry using digital image processing by the NIH Image 1.6/ppc freeware program.

Hormone Determinations

Serum concentration of PRL was measured by a double-antibody radioimmunoassay (RIA) with reagents supplied by Dr A. F. Parlow from the National Hormone and Pituitary Program. PRL was radioiodinated using the chloramine T method and purified by passage through Sephadex G-75 and polyacrylamide agarose (ACA 54; LKB, Bromma, Sweden) columns. The assay sensitivity was 1 ng/ml serum and the inter- and intra-assay coefficients of variation were less than 10%. The PRL antibody does not cross-react with placental lactogen. Serum P_4 and E_2 were measured using commercial kits (DSL-3400 and DSL-4800 double-antibody radioimmunoassay from Diagnostic Systems Laboratories, Webster, TX, USA). Assay sensitivities were <70 and 10 fmol/tube, respectively, and the intra-assay coefficients of variation were <10%. All the samples were measured on the same assay in duplicate.

Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) with the statistical computer analysis system GraphPad Prism. When ANOVA revealed statistical differences, we applied the Bonferroni post hoc analysis. When variances were not homogeneous, log transformation of the data was performed. Differences between means were considered significant at the $p < 0.05$ level.

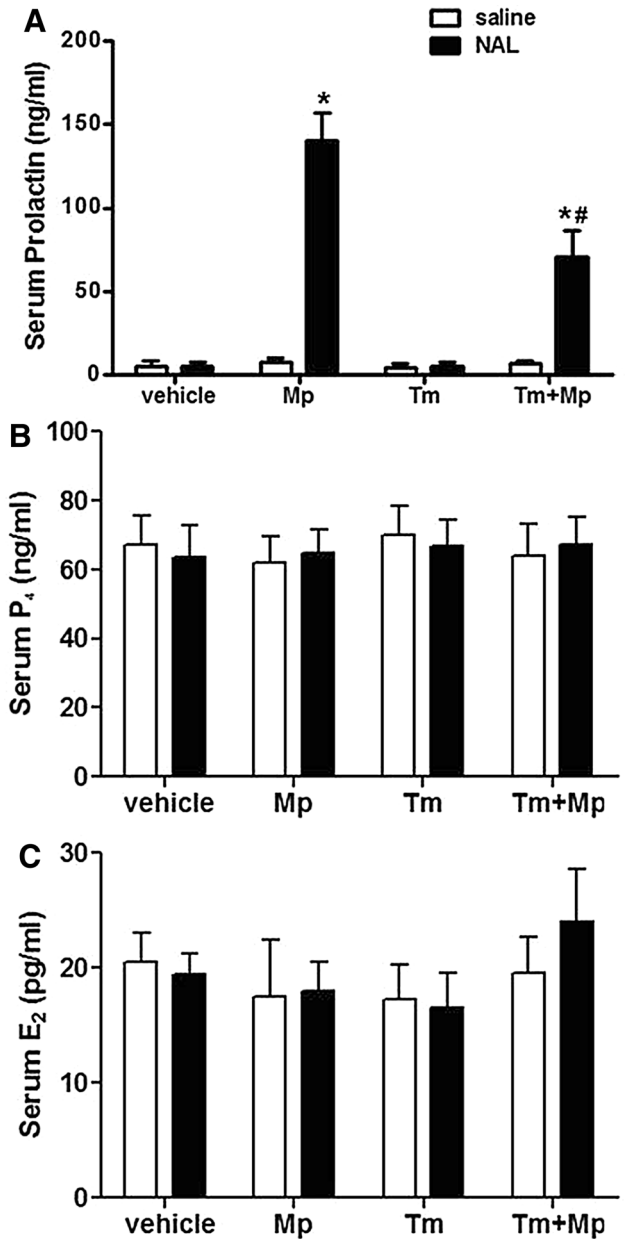

Fig. 1 Effect of mifepristone and tamoxifen treatments on circulating levels of (**a**) PRL, (**b**) P_4 and (**c**) E_2 in rats on day 19 of pregnancy treated with naloxone or vehicle. Tamoxifen (Tm; 0.5 mg/kg) was administered at 12:00 h on days 14 and 15 of pregnancy; mifepristone (Mp; 5 mg/kg) or vehicle was administered at 08:00 h and naloxone (NAL; 2 mg/kg) or saline was administered at 17:30 h on day 19. Animals were sacrificed at 18:00 h on day 19 of pregnancy. Results are means ± SEM of groups of eight animals in each experimental group. *$p < 0.001$ compared to vehicle + NAL or Mp + SAL; #$p < 0.01$ compared to Mp + NAL. Two-way ANOVA followed by Bonferroni post-hoc test

Results

Effect of Naloxone, Tamoxifen and Mifepristone Treatments on PRL Secretion and Serum P_4 and E_2 Levels

Naloxone administration on day 19 of pregnancy to mifepristone pre-treated rats induced a significant increase in serum PRL levels. In accordance with previous studies [6], administration of the ER antagonist tamoxifen on days 14 and 15 of pregnancy partially prevented the serum PRL increase induced by the combined treatment with naloxone and mifepristone (Fig. 1a).

No significant changes in P_4 (Fig. 1b) or E_2 serum levels (Fig. 1c) were observed between the different pharmacological treatments performed.

Effect of Naloxone, Tamoxifen and Mifepristone Treatments on TH Expression Levels in MBH

Since the activity of hypothalamic dopaminergic neurons maintains an inhibitory tone on PRL secretion, in all the experimental groups we evaluated TH expression levels as an indicator of dopaminergic activity. Blockade of P_4 biological activity by the antagonist mifepristone, significantly decreased MBH TH expression measured by RT-PCR (Fig. 2a) and western blot (Fig. 2b). This effect was prevented when tamoxifen was administered on days 14 and 15 of pregnancy prior to mifepristone treatment on day 19. Naloxone administration did not modify the effects of mifepristone, tamoxifen or their combination. Tamoxifen administered individually did not modify significantly TH expression levels (Fig. 2a, b). These results suggest that the decrease in dopaminergic tone induced by inhibition of P_4 action is mediated by estrogen action.

Effect of Mifepristone and Tamoxifen on ER and PR Expression Levels in the MBH

Considering the important role of sexual hormones on dopaminergic regulation of PRL secretion, first we evaluated whether the reduction in TH expression levels induced by blockade of P_4, may involve changes in ER expression levels. Interestingly, the administration of the PR antagonist mifepristone, on day 19 of pregnancy, significantly increased the relative ERα protein (Fig. 3a) and mRNA contents measured by RT-PCR (Control: 0.90 ± 0.03 vs

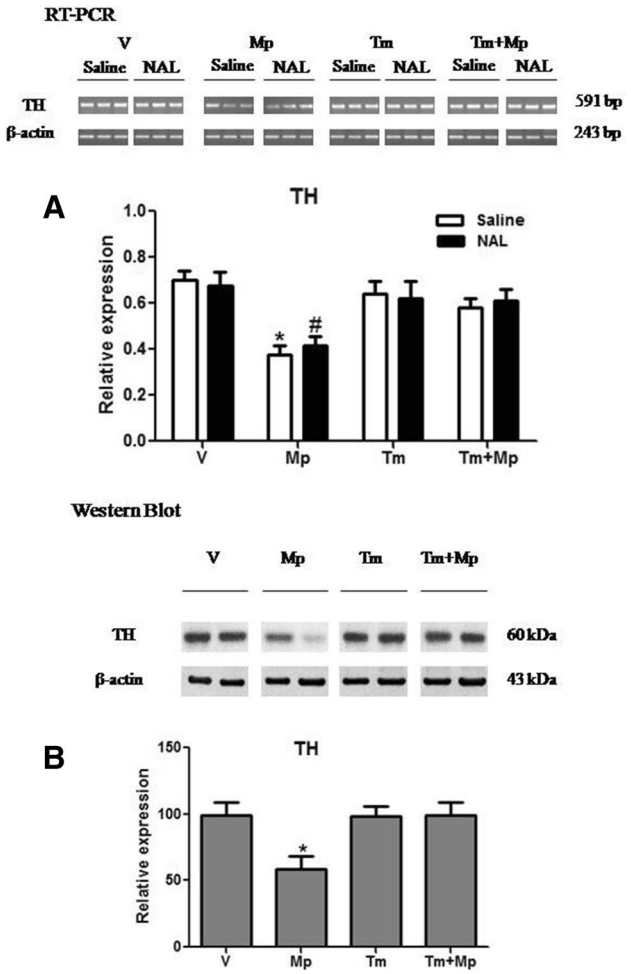

Fig. 2 Effect of mifepristone and tamoxifen treatments on TH expression in the MBH on day 19 of pregnancy. Tamoxifen (Tm; 0.5 mg/kg) was administered at 12:00 h on days 14 and 15 of pregnancy; mifepristone (Mp; 5 mg/kg) or vehicle (V) was administered at 08:00 h and naloxone (NAL; 2 mg/kg) or saline was administered at 17:30 h on day 19. Animals were sacrificed at 18:00 h on day 19 of pregnancy. **a** mRNA abundance measured by RT-PCR in rats treated with Naloxone or vehicle. *Upper panel* RT-PCR representative bands. *Lower panel* TH mRNA expression levels relative to β-actin. **b** TH protein abundance relative to β-actin after mifepristone and tamoxifen administration. *Upper panel* western blot representative bands. *Lower panel* TH protein expression relative to β-actin. Results are means ± SEM of groups of seven animals in each experimental group *$p < 0.05$, **$p < 0.01$ compared with all the other respective groups using two way ANOVA followed by Bonferroni test for multiple comparisons

Mp: 1.07 ± 0.05; $p < 0.05$) without affecting the expression of ERβ mRNA (results not shown). Moreover, tamoxifen administration prevented the increase of ERα protein expression induced by mifepristone treatment (Fig. 3a) and also lowered significantly ERα protein in control rats.

Since PR expression in the hypothalamus can be modulated by E_2, we also tested whether the recovery of TH expression levels observed in the tamoxifen treated groups involves changes in PR expression. Tamoxifen administration on days 14 and 15 of pregnancy did not induce significant changes in the relative PR A and PR B protein expression (Fig. 3b) between groups. Also, no effect in the relative total PR mRNA expression was observed suggesting that the effects of tamoxifen do not involve changes in PR expression levels in the MBH (data not shown). Also, ten hours after mifepristone administration, no changes in PR A or PR B protein expression were observed (Fig. 3b).

Effect of Tamoxifen and Mifepristone Treatments on $PRLR_{(long)}$ Expression Levels in the MBH

Taking into account that treatment with mifepristone induced an increase in the $PRLR_{(long)}$ expression levels in the anterior pituitary (unpublished results), that estrogens induce hypothalamic $PRLR_{(long)}$ expression [23] and that PRL acting through PRLR regulates TH expression [22], we evaluated the expression levels of this receptor in MBH after tamoxifen and mifepristone treatments. Treatment with the P_4 antagonist mifepristone on day 19 of pregnancy increased the relative $PRLR_{(long)}$ mRNA expression when compared with the other groups (Fig. 4a). Tamoxifen administration on days 14 and 15 of pregnancy had no effect *per se*, but prevented the effects of mifepristone (Fig. 4a). Protein PRLR levels measured by Western blot showed no significant differences among the groups, although in the control and mifepristone-treated groups the values were higher, but much more variable when compared with both tamoxifen-treated groups (Fig. 4b).

Effect of Tamoxifen and Mifepristone Treatments on μ- and κ-ORs Expression Levels in the MBH

Since ovarian steroids modulate the activity of the opioid system, and ORs and opioid peptides are expressed in the MBH, we evaluated whether tamoxifen and mifepristone treatments during gestation could modify μ- and κ-ORs expression on day 19 of pregnancy, to determine whether their actions on PRL release may be mediated through changes in OR expression. Only the combined administration of tamoxifen and mifepristone decreased the relative μ- and κ-ORs mRNA expression, measured by RT-PCR (Fig. 5a, c). However, at protein level, no changes in the expression of these receptors were observed (Fig. 5b, d).

Discussion

The present results show that the previously reported decrease in hypothalamic dopaminergic activity induced by P_4 removal [4] or blockade of its action [7] at the end of pregnancy is dependent upon estrogen action. Accordingly, the present results show that the reduction of TH expression by mifepristone [7] was prevented by the previous

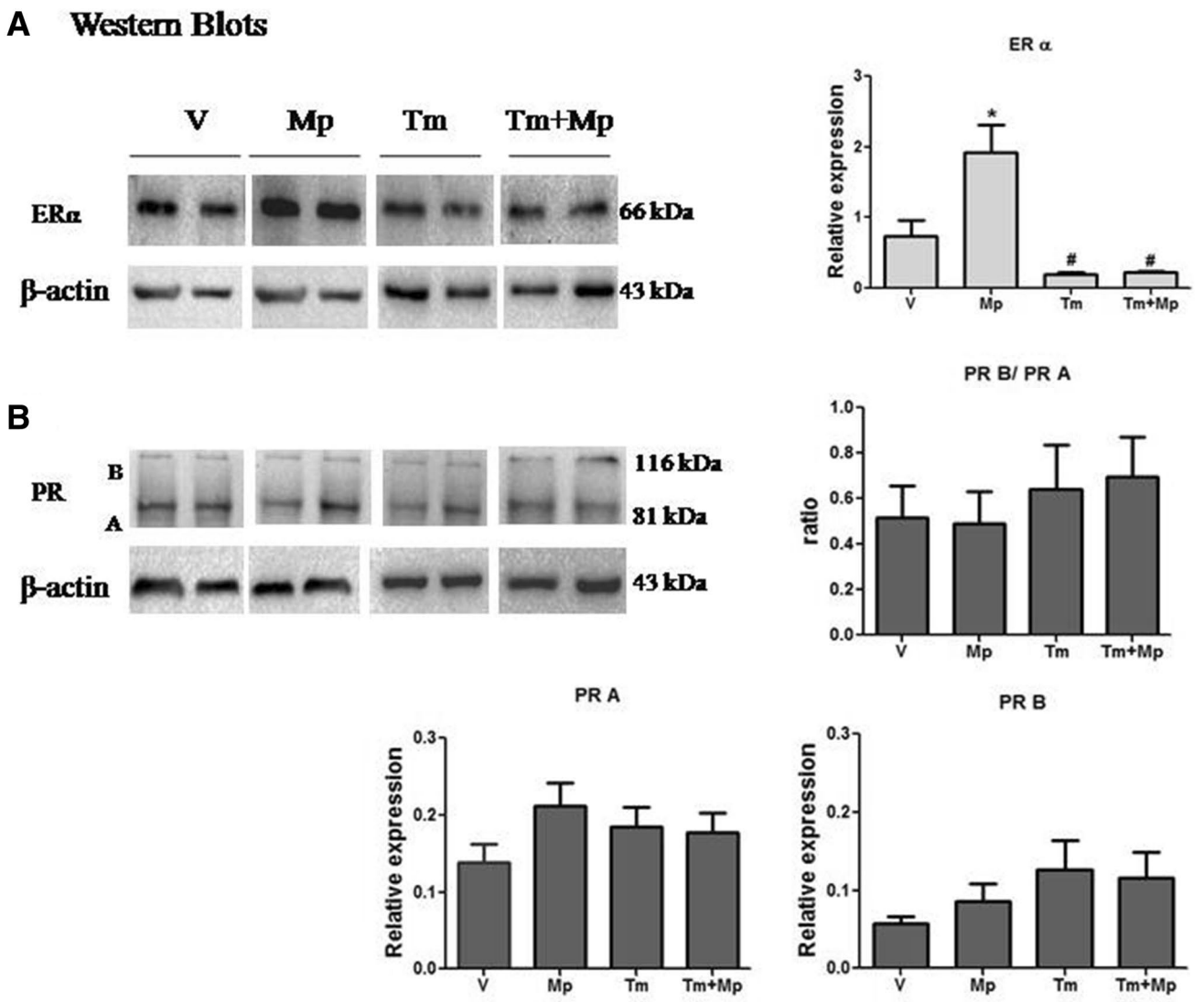

Fig. 3 Effect of mifepristone and tamoxifen treatments on MBH content of ERα and PR protein abundances relative to β-actin in rats on day 19 of pregnancy. **a** ER α: western blot representative bands (*left*) and protein expression relative to β-actin (*right*); **b** PR: western blot representative bands (*left*) and protein expression relative to β-actin or PR A and PR B isoforms and PR B/PR A ratio (*right and below*). Tamoxifen (Tm; 0.5 mg/kg) was administered at 12:00 h, on days 14 and 15 of pregnancy; mifepristone (Mp; 5 mg/kg) or vehicle (V) was administered at 08:00 h, on day 19. Animals were sacrificed at 18:00 h on day 19 of pregnancy. Results are means ± SEM of groups of 5–6 animals in each experimental group. *$p < 0.05$ compared with the other groups; #$p < 0.05$ compared with the respective control (not treated with tamoxifen) groups using two way ANOVA followed by Bonferroni test for multiple comparisons

administration of tamoxifen, indicating that E_2 action is necessary to evidence the fall in TH expression induced by P_4 action blockade. Furthermore, this fall in dopaminergic activity, evidenced by decreased DOPAC/DA ratio [7] or TH expression (present results) is not sufficient to induce PRL release, but must be present to evidence the action of PRL releasing stimuli, such as acute naloxone administration. Thus, the effects of mifepristone and tamoxifen upon naloxone-induced PRL release can be correlated with the expression of TH at protein and mRNA levels, the rate-limiting enzyme for dopamine synthesis and an important marker of dopaminergic neuron activity [7, 11]. It was demonstrated that the decrease of P_4 at the end of pregnancy is necessary for the reduction in dopaminergic neuronal activity and consequent facilitation of PRL secretion [6, 8, 40]. Although premature P_4 withdrawal, triggered by $PGF_{2\alpha}$-induced luteolysis, decreases dopaminergic activity and facilitates PRL secretion [4], the diminution of TH expression induced by mifepristone is not sufficient to induce PRL secretion at least within 10–12 h of the antiprogesterone treatment. However, concomitant naloxone treatment facilitates PRL secretion but does not modify TH expression suggesting that naloxone effects on mifepristone treated rats do not involve changes in dopamine activity, confirming previous results [7].

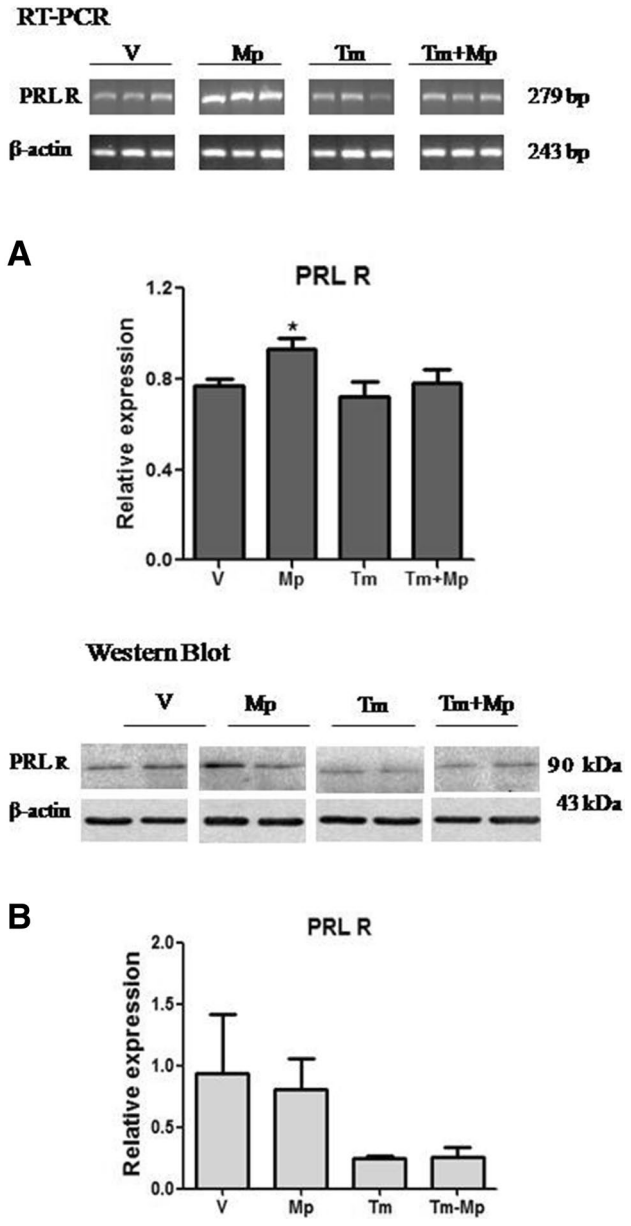

Fig. 4 Effect of mifepristone and tamoxifen treatments on MBH expression of $PRLR_{(long)}$ on day 19 of pregnancy after mifepristone and tamoxifen administration. **a** PRLR(long) mRNA abundance; *Upper panel* RT-PCR representative bands. *Lower panel* $PRLR_{(long)}$ mRNA expression levels relative to β-actin. **b** $PRLR_{(long)}$ protein abundance; *Upper panel* western blot representative bands. *Lower panel* $PRLR_{(long)}$ protein expression relative to β-actin.Tamoxifen (Tm; 0.5 mg/kg) was administered at 12:00 h, on days 14 and 15 of pregnancy; mifepristone (Mp; 5 mg/kg) or vehicle (V) was administered at 08:00 h, on day 19. Animals were sacrificed at 18:00 h on day 19 of pregnancy. Results are means ± SEM of groups of seven (for RT-PCR) or four (for western blots) animals in each experimental group. *$p < 0.05$ compared with the other groups using two way ANOVA followed by Bonferroni test for multiple comparisons

It is interesting to note that changes in PRL serum levels were not directly related to changes in E_2 and P_4 serum levels, because they were not altered by any of the pharmacological treatments, at least in the time frame used in this study. On the other hand, it seems that E_2 action may involve the participation of ERα, because we observed an increase in ERα expression in mifepristone treated animals that could mediate the decreased TH expression observed in them [7], and furthermore, tamoxifen treatment blocked the increase in ERα and also diminished its levels in control rats. It is known that E_2 diminishes TH mRNA levels in TIDA neurons [41]. No alteration in ERβ mRNA expression was observed. ERα is the main receptor subtype implicated in the neuroendocrine regulation of reproduction [42], and although both subtypes are E_2 sensitive, ERα expression in the arcuate nucleus is higher than ERβ [43]. Previously, we have demonstrated [9] that the blockade of P_4 actions by mifepristone treatment on day 19 of pregnancy induces a significant lactotroph activation. This activation can be correlated with the increased ERα expression levels in the MBH (mRNA and protein) observed in the present work as well as in the anterior pituitary [44], without changes in ERβ mRNA expression levels. These data might suggest a direct or indirect participation of E_2 in this effect, by a mechanism mediated by ERα activation.

Neuroendocrine regulation of PRL secretion changes during late pregnancy and allows the establishment of a hyperprolactinemic state during lactation. These changes allow that the short negative feedback loop of PRL secretion be refractory and induce the appearance of neuroendocrine systems like the serotonergic [45] and the opioid [8, 10], which in other experimental situations are able to stimulate PRL secretion, but at the end of pregnancy are inhibitory. Then, it can be speculated that the presence of E_2 on days 15 and 16 of pregnancy and its gradual increase up to the end of gestation is key in this adaptive process. Tamoxifen administration on day 18 of pregnancy is not capable to prevent the rise in PRL induced by mifepristone and naloxone on day 19 of pregnancy [6, 8]. However, tamoxifen administered on days 14 and 15 completely blocks mifepristone plus naloxone induced PRL secretion on day 16 [6] and partially on day 19 of pregnancy [6]. These effects of tamoxifen may be exerted through a direct action on TIDA neurons or by changes in the pre-synaptic modulation of these neurons that result in changes in TH expression.

E_2 induces PR expression in the hypothalamus [46]. The PR gene contains E_2 response elements that promote PR mRNA transcription [46]. There are two PR isoforms, the long form (PR B ~116 kDa) and the truncated form (PR A ~81 kDa), which differ in their ability to activate different target genes within a cell and the PR B/PR A ratio in terms of mRNA and protein varies between different brain areas and under different hormonal conditions [47]. However, tamoxifen administration on days 14 and 15 did not change the isoforms A or B nor their ratio. These data indicate that tamoxifen prevents mifepristone action on TH expression

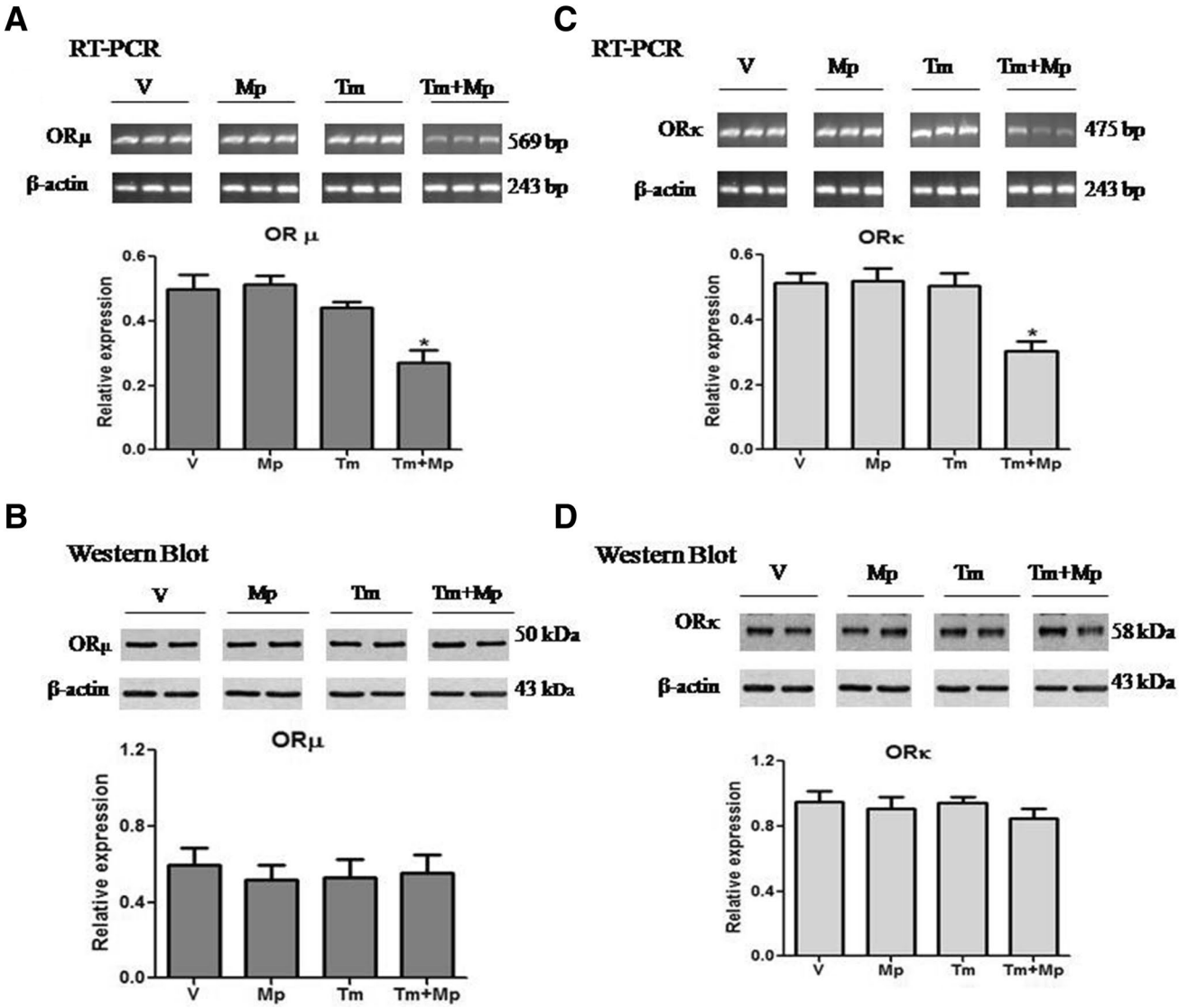

Fig. 5 Effect of mifepristone and tamoxifen treatments on mRNA expression (**a, c**) and protein (**b, d**) of μ- (ORμ, **a, b**) and κ- (ORκ, **c, d**) opioid receptors abundance relative to β-actin in rats on day 19 of pregnancy in MBH. **a, c** *Upper panel* RT-PCR representative bands; *Lower panel* mRNA expression levels relative to β-actin. **b, d** *Upper panel* western blot representative bands; *Lower panel* protein expression levels relative to β-actin. Tamoxifen (Tm; 0.5 mg/kg) was administered at 12:00 h, on days 14 and 15 of pregnancy; mifepristone (Mp; 5 mg/kg) or vehicle (V) was administered at 08.00 h, on day 19. Animals were decapitated at 18:00 h on day 19. Results are means ± SEM of groups of seven animals in each experimental group.*$p < 0.05$ compared with the other groups using two way ANOVA followed by Bonferroni test was used for multiple comparisons

without modifying the levels of PR expression. It has been shown that mifepristone administration to parturient rats did not induce changes in both hypothalamic PR isoforms protein expression [48] and P_4 fall induced by $PGF_{2\alpha}$ had no effect on the expression of both PR isoforms at protein or mRNA levels [4]. Based on this evidence and considering that mifepristone induced an increase in the ERα expression, our results suggest that estrogenic inhibition of hypothalamic dopaminergic activity may be exerted via a mechanism involving changes in ER expression levels without affecting PR expression. Furthermore, since it has been described that estrogen induces the expression of its cognate receptors [49, 50] it is quite possible that the reduced ER_{α} expression observed in the tamoxifen treated rats may be a consequence of the blunting of the estrogen action upon the expression of its receptors.

The two PRLR isoforms (long and short) are expressed in the arcuate nucleus [21]. The long isoform is involved in the short negative feedback loop, in which PRL controls its own secretion increasing TH expression levels and dopaminergic activity [21, 24, 25]. During late pregnancy and lactation, an increase in $PRLR_{(long)}$ expression has been described in

the arcuate nucleus [21, 22, 51] in opposition to the inhibition of $PRLR_{(long)}$ expression in the preoptic area observed in ovariectomized animals that received a prolonged pregnancy-like regimen of P_4 and E_2 [52]. Our results suggest that the blockade of P_4 action by mifepristone treatment significantly increases PRLR mRNA, through an estrogenic action once P_4 action has been removed. Indeed, E_2 treatment induces $PRLR_{(long)}$ expression in hypothalamic dopaminergic neurons [23] and treatment with tamoxifen prevents the effect of mifepristone (present results). The pattern of changes observed in ERα and PRLR expression after mifepristone or $PGF_{2\alpha}$ [4] suggest that the elevated P_4 levels at the end of pregnancy prevent E_2 stimulation of ER and PRLR expression. Interestingly, $PRLR_{(long)}$ expression in MBH and in the anterior pituitary increases simultaneously [44] suggesting that the PRL short negative feedback loop may be still functional at the end of gestation resulting in a residual sensitivity to PRL [9]. However, the increase in PRLR mRNA induced by mifepristone was not translated to a corresponding increase in the PRLR protein levels, most probably because the time elapsed (10 h) after mifepristone administration was not sufficient to evidence changes in the expression of the protein.

In the present work, a significant reduction in μ-and κ-ORs mRNA levels was observed after tamoxifen and mifepristone treatments. It is interesting to point out that the decreased ORs mRNA expression is not reflected in the receptor protein expression. This lack of correlation may be due to a different synchronization between mRNA expression and synthesis of their respective proteins. Although it is generally accepted that pregnancy and parturition are characterized by increased hypothalamic levels of β-endorphin, Met-enkephalin and the μ-ORs, controversial results have been observed due to different experimental conditions, including brain areas studied and techniques used for protein determination [53, 54]. Moreover, it has been shown that in late pregnancy, μ- and κ-ORs are involved in regulation of PRL secretion, through a mechanism that directly or indirectly involves changes in dopaminergic activity [55, 56]. Opioid neurons expressing β-endorphin and enkephalins, also express ER [57, 58] and ovarian steroids can regulate the expression of both peptides [59, 60]. However, there is some discrepancy regarding the effect of sex steroids on the opioid system, which may depend not only on their presence or absence but also on the concentrations and duration of exposure [61]. Indeed, E_2 and P_4 treatment to ovariectomized rats can alter the μ-OR density in the hypothalamus. In most cases, E_2 treatment by itself does not affect μ-OR density but in combination with P_4, μ-OR density decreases in the hypothalamus [62, 63] and increases in the arcuate nucleus [64]. On the other hand, it has been shown that E_2 can stimulate endogenous opioid peptides release inducing internalization of the μ-OR, while P_4 blocks E_2 action [36]. The effect of E_2 is mediated by ERα activation [65] because tamoxifen inhibits μ-OR internalization by reducing endogenous opioid peptides release [65]. Thus, the blockade of E_2 and P_4 effects by tamoxifen plus mifepristone administration, may modify endogenous opioid peptides synthesis and release, and thus, regulate the expression of their receptors. Also, it is important to consider that besides dopaminergic neurons, other neurons localized in MBH, shuch as POMC and kisspeptin-neurokinin B-dynorphin (KNDY) neurons express μ-and κ-ORs and are regulated by ovarian steroids [37, 66]. Thus, mifepristone and/or tamoxifen may be acting upon other neurons expressing opioid receptors, that may be related or not to the regulation of PRL secretion.

In conclusion, we demonstrated that E_2 plays an important role in the neuroadaptive mechanisms necessary to facilitate the PRL release that precedes delivery. The results obtained in this study suggest that E_2 acting through ERα modulates the activity of the hypothalamic dopaminergic neurons, regulating TH, μ- and κ-ORs and $PRLR_{(long)}$ expression, and its presence on days 14 and 15 of pregnancy is crucial to facilitate the opioid system modulation on PRL secretion at the end of pregnancy in the rat. Apparently, E_2 action is necessary in order to evidence the effects of P_4 withdrawal on TH, ER and $PRLR_{(long)}$ expression.

Acknowledgments The authors are grateful to Mr. J. Rosales for his skilful technical assistance.

Funding This work has been supported by PIP 2012–2014 00863-CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas) and Proyecto 06/J458, SeCTyP, Universidad Nacional de Cuyo. CVG, GEP, GAJ and MS are members of CONICET.

Compliance with Ethical Standards

Conflict of Interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Vermouth NT, Deis RP (1972) Prolactin release induced by prostaglandin F2 in pregnant rats. Nat New Biol 238:248–250
2. Vermouth NT, Deis RP (1974) Prolactin release and lactogenesis after ovariectomy in pregnant rats: effect of ovarian hormones. J Endocrinol 63:13–20
3. Jahn GA, Rastrilla AM, Deis RP (1993) Correlation of growth hormone secretion during pregnancy with circulating prolactin in rats. J Reprod Fertil 98:327–333
4. Bonafede MM, Valdez SR, Arbocco FC, Pennacchio GE, Soaje M, Jahn GA (2011) Effect of progesterone withdrawal on hypothalamic mechanisms related to prolactin release in late pregnant rats. Neuroendocrinology 94:148–157
5. Grattan DR, Averill RL (1990) Effect of ovarian steroids on a nocturnal surge of prolactin secretion that precedes parturition in the rat. Endocrinology 126:1199–1205
6. Soaje M, Deis RP (1997) Opioidergic regulation of prolactin secretion during pregnancy: role of ovarian hormones. J Endocrinol 155:99–106

7. Soaje M, Valdez S, Bregonzio C, Penissi A, Deis RP (2006) Dopaminergic mechanisms involved in prolactin release after mifepristone and naloxone treatment during late pregnancy in the rat. Neuroendocrinology 84:58–67
8. Soaje M, Deis RP (1994) A modulatory role of endogenous opioids on prolactin secretion at the end of pregnancy in the rat. J Endocrinol 140:97–102
9. Gabutti CV, Ezquer M, Deis R, Maldonado C, Soaje M (2009) Pituitary changes involved in prolactin secretion induced by mifepristone and naloxone during late pregnancy. Neuroendocrinology 89:200–209
10. Valdez SR, Pennacchio GE, Gamboa DF, de Di Nasso EG, Bregonzio C, Soaje M (2014) Opioid modulation of prolactin secretion induced by stress during late pregnancy. Role of ovarian steroids. Pharmacol Rep 66:386–393
11. Freeman ME, Kanyicska B, Lerant A, Nagy G (2000) Prolactin: structure, function, and regulation of secretion. Physiol Rev 80:1523–1631
12. Crowley WR (2015) Neuroendocrine regulation of lactation and milk production. Compr Physiol 5:255–291
13. Raymond V, Beaulieu M, Labrie F, Boissier J (1978) Potent antidopaminergic activity of estradiol at the pituitary level on prolactin release. Science 200:1173–1175
14. West B, Dannies PS (1980) Effects of estradiol on prolactin production and dihydroergocryptine-induced inhibition of prolactin production in primary cultures of rat pituitary cells. Endocrinology 106:1108–1113
15. Suzuki S, Handa RJ (2005) Estrogen receptor-beta, but not estrogen receptor-alpha, is expressed in prolactin neurons of the female rat paraventricular and supraoptic nuclei: comparison with other neuropeptides. J Comp Neurol 484:28–42
16. Steyn FJ, Anderson GM, Grattan DR (2007) Expression of ovarian steroid hormone receptors in tuberoinfundibular dopaminergic neurones during pregnancy and lactation. J Neuroendocrinol 19:788–793
17. Kokay IC, Grattan DR (2005) Expression of mRNA for prolactin receptor (long form) in dopamine and pro-opiomelanocortin neurones in the arcuate nucleus of non-pregnant and lactating rats. J Neuroendocrinol 17:827–835
18. Hou Y, Yang SP, Voogt JL (2003) Changes in estrogen receptor-alpha expression in hypothalamic dopaminergic neurons during proestrous prolactin surge. Endocr 20:131–138
19. Lonstein JS, Blaustein JD (2004) Immunocytochemical investigation of nuclear progestin receptor expression within dopaminergic neurones of the female rat brain. J Neuroendocrinol 16:534–543
20. Greco B, Allegretto EA, Tetel MJ, Blaustein JD (2001) Coexpression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology 142:5172–5181
21. Pi XJ, Grattan DR (1999) Increased expression of both short and long forms of prolactin receptor mRNA in hypothalamic nuclei of lactating rats. J Mol Endocrinol 23:13–22
22. Augustine RA, Kokay IC, Andrews ZB, Ladyman SR, Grattan DR (2003) Quantitation of prolactin receptor mRNA in the maternal rat brain during pregnancy and lactation. J Mol Endocrinol 31:221–232
23. Pi X, Zhang B, Li J, Voogt JL (2003) Promoter usage and estrogen regulation of prolactin receptor gene in the brain of the female rat. Neuroendocrinology 77:187–197
24. Grattan DR (2001) The actions of prolactin in the brain during pregnancy and lactation. Prog Brain Res 133:153–171
25. Hentschel K, Fleckenstein AE, Toney TW, Lawson DM, Moore KE, Lookingland KJ (2000) Prolactin regulation of tuberoinfundibular dopaminergic neurons: immunoneutralization studies. Brain Res 852:28–36
26. Callahan P, Baumann MH, Rabii J (1996) Inhibition of tuberoinfundibular dopaminergic neural activity during suckling: involvement of mu and kappa opiate receptor subtypes. J Neuroendocrinol 8:771–776
27. Manzanares J, Lookingland KJ, Moore KE (1991) Kappa opioid receptor-mediated regulation of dopaminergic neurons in the rat brain. J Pharmacol Exp Ther 256:500–505
28. Soaje M, Bregonzio C, Caron RW, Deis RP (2004) Neurotransmitters involved in the opioid regulation of prolactin secretion at the end of pregnancy in rats. Neuroendocrinology 80:11–20
29. Kapoor R, Willoughby JO (1991) Activation of mu opioid receptors in the pre-optic-anterior hypothalamus releases prolactin in the conscious male rat. Neuropeptides 19:231–236
30. Krulich L, Koenig JI, Conway S, McCann SM, Mayfield MA (1986) Opioid kappa receptors and the secretion of prolactin (PRL) and growth hormone (GH) in the rat. I. Effects of opioid kappa receptor agonists bremazocine and U-50,488 on secretion of PRL and GH: comparison with morphine. Neuroendocrinology 42:75–81
31. Sagrillo CA, Voogt JL (1991) Endogenous opioids mediate the nocturnal prolactin surge in the pregnant rat. Endocrinology 129:925–930
32. Andrews ZB, Grattan DR (2002) Opioid control of prolactin secretion in late pregnant rats is mediated by tuberoinfundibular dopamine neurons. Neurosci Lett 328:60–64
33. Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. J Comp Neurol 350:412–438
34. Mansour A, Fox CA, Thompson RC, Akil H, Watson SJ (1994) mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. Brain Res 643:245–265
35. Hammer RP Jr, Zhou L, Cheung S (1994) Gonadal steroid hormones and hypothalamic opioid circuitry. Horm Behav 28:431–437
36. Sinchak K, Micevych PE (2001) Progesterone blockade of estrogen activation of mu-opioid receptors regulates reproductive behavior. J Neurosci 21:5723–5729
37. Bouret S, Prevot V, Croix D, Jegou S, Vaudry H, Stefano GB, Beauvillain JC, Mitchell V (1999) Mu-opioid receptor mRNA expression in proopiomelanocortin neurons of the rat arcuate nucleus. Brain Res Mol Brain Res 70:155–158
38. Wagner EJ, Manzanares J, Moore KE, Lookingland KJ (1994) Neurochemical evidence that estrogen-induced suppression of kappa-opioid-receptor-mediated regulation of tuberoinfundibular dopaminergic neurons is prolactin-independent. Neuroendocrinology 59:197–201
39. Campo Verde Arbocco F, Sasso CV, Nasif DL, Hapon MB, Jahn GA (2015) Effect of hypothyroidism on the expression of nuclear receptors and their co-regulators in mammary gland during lactation in the rat. Mol Cell Endocrinol 412:26–35
40. Andrews ZB (2005) Neuroendocrine regulation of prolactin secretion during late pregnancy: easing the transition into lactation. J Neuroendocrinol 17:466–473
41. Arbogast LA, Voogt JL (1993) Progesterone reverses the estradiol-induced decrease in tyrosine hydroxylase mRNA levels in the arcuate nucleus. Neuroendocrinology 58:501–510
42. McEwen BS, Alves SE (1999) Estrogen actions in the central nervous system. Endocr Rev 20:279–307
43. Osterlund M, Kuiper GG, Gustafsson JA, Hurd YL (1998) Differential distribution and regulation of estrogen receptor-alpha and -beta mRNA within the female rat brain. Brain Res Mol Brain Res 54:175–180
44. Villegas-Gabutti C (2014) Regulación de la secreción de prolactina al final de la preñez en la rata: rol del sistema opioide. Tesis Doctoral, Universidad Nacional de Cuyo

45. Jahn GA, Deis RP (1988) Effect of serotonin antagonists on prolactin and progesterone secretion in rats: evidence that the stimulatory and inhibitory actions of serotonin on prolactin release may be mediated through different receptors. J Endocrinol 117:415–422
46. Scott RE, Wu-Peng XS, Pfaff DW (2002) Regulation and expression of progesterone receptor mRNA isoforms A and B in the male and female rat hypothalamus and pituitary following oestrogen treatment. J Neuroendocrinol 14:175–183
47. Guerra-Araiza C, Villamar-Cruz O, Gonzalez-Arenas A, Chavira R, Camacho-Arroyo I (2003) Changes in progesterone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments. J Neuroendocrinol 15:984–990
48. Carrillo-Martinez GE, Gomora-Arrati P, Gonzalez-Arenas A, Morimoto S, Camacho-Arroyo I, Gonzalez-Flores O (2011) Role of progesterone receptors during postpartum estrus in rats. Horm Behav 59:37–43
49. Mann PE, Babb JA (2005) Neural steroid hormone receptor gene expression in pregnant rats. Brain Res Mol Brain Res 142:39–46
50. Liu X, Shi H (2015) Regulation of estrogen receptor alpha expression in the hypothalamus by sex steroids: implication in the regulation of energy homeostasis. Int J Endocrinol 2015:949085
51. Pi X, Grattan DR (1999) Expression of prolactin receptor mRNA is increased in the preoptic area of lactating rats. Endocr 11:91–98
52. Bridges RS, Hays LE (2005) Steroid-induced alterations in mRNA expression of the long form of the prolactin receptor in the medial preoptic area of female rats: effects of exposure to a pregnancy-like regimen of progesterone and estradiol. Brain Res Mol Brain Res 140:10–16
53. Dondi D, Maggi R, Panerai AE, Piva F, Limonta P (1991) Hypothalamic opiatergic tone during pregnancy, parturition and lactation in the rat. Neuroendocrinology 53:460–466
54. Petraglia F, Baraldi M, Giarre G, Facchinetti F, Santi M, Volpe A, Genazzani AR (1985) Opioid peptides of the pituitary and hypothalamus: changes in pregnant and lactating rats. J Endocrinol 105:239–245
55. Andrews ZB, Grattan DR (2003) Opioid receptor subtypes involved in the regulation of prolactin secretion during pregnancy and lactation. J Neuroendocrinol 15:227–236
56. Soaje M, Deis RP (2004) Involvement of opioid receptor subtypes in both stimulatory and inhibitory effects of the opioid peptides on prolactin secretion during pregnancy. Cell Mol Neurobiol 24:193–204
57. Morrell JI, McGinty JF, Pfaff DW (1985) A subset of beta-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol. Neuroendocrinology 41:417–426
58. Akesson TR, Micevych PE (1991) Endogenous opioid-immunoreactive neurons of the ventromedial hypothalamic nucleus concentrate estrogen in male and female rats. J Neurosci Res 28:359–366
59. Romano GJ, Harlan RE, Shivers BD, Howells RD, Pfaff DW (1988) Estrogen increases proenkephalin messenger ribonucleic acid levels in the ventromedial hypothalamus of the rat. Mol Endocrinol 2:1320–1328
60. Priest CA, Roberts JL (2000) Estrogen and tamoxifen differentially regulate beta-endorphin and cFos expression and neuronal colocalization in the arcuate nucleus of the rat. Neuroendocrinology 72:293–305
61. Acosta-Martinez M, Etgen AM (2002) Estrogen modulation of mu-opioid receptor-stimulated [35S]-GTP-gamma-S binding in female rat brain visualized by in vitro autoradiography. Neuroendocrinology 76:235–242
62. Martini L, Dondi D, Limonta P, Maggi R, Piva F (1989) Modulation by sex steroids of brain opioid receptors: implications for the control of gonadotropins and prolactin secretion. J Steroid Biochem 33:673–681
63. Weiland NG, Wise PM (1990) Aging progressively decreases the densities and alters the diurnal rhythms of alpha 1-adrenergic receptors in selected hypothalamic regions. Endocrinology 126:2392–2397
64. Petersen SL, LaFlamme KD (1997) Progesterone increases levels of mu-opioid receptor mRNA in the preoptic area and arcuate nucleus of ovariectomized, estradiol-treated female rats. Brain Res Mol Brain Res 52:32–37
65. Micevych PE, Rissman EF, Gustafsson JA, Sinchak K (2003) Estrogen receptor-alpha is required for estrogen-induced mu-opioid receptor internalization. J Neurosci Res 71:802–810
66. Weems PW, Witty CF, Amstalden M, Coolen LM, Goodman RL, Lehman MN (2016) kappa-opioid receptor is colocalized in GnRH and KNDy cells in the female ovine and rat brain. Endocrinology 157:2367–2379