RESEARCH

TGF β 1 regulates prolactin secretion during postnatal development: gender differences

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Abstract

Serum prolactin levels gradually increase from birth to puberty in both male and female rats, with higher levels observed in female since the first days of life. The increase in lactotroph secretion was attributed to the maturation of prolactin-inhibiting and prolactin-releasing factors; however, those mechanisms could not fully explain the gender differences observed. Prolactin secretion from isolated lactotrophs, in the absence of hypothalamic control, also increases during the first weeks of life, suggesting the involvement of intra-pituitary factors. We postulate that pituitary transforming growth factor beta 1 (TGF β 1) is involved in the regulation of prolactin secretion as well as in the gender differences observed at early postnatal age. Several components of the local TGF β 1 system were evaluated during postnatal development (11, 23, and 45 days) in female and male Sprague–Dawley rats. *In vivo* assays were performed to study local TGF β 1 activation and its impact on prolactin secretion. At day 11, female pituitaries present high levels of active TGF β 1, concomitant with the highest expression of TGF β 1 target genes and the phospho-Smad3 immunostaining in lactotrophs. The steady increase in prolactin secretion inversely correlates with active TGF β 1 levels only in females. Dopamine and estradiol induce TGF β 1 activation at day 11, in both genders, but its activation induces the inhibition of prolactin secretion only in females. Our findings demonstrate that: (1) TGF β 1 activation is regulated by dopamine and estradiol; (2) the inhibitory regulation of local TGF β 1 on prolactin secretion is gender specific; and (3) this mechanism is responsible, at least partially, for the gender differences observed being relevant during postnatal development.

Key Words

- ▶ lactotrophs
- ▶ postnatal development
- ▶ TGF β 1
- ▶ prolactin
- ▶ gender differences

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Introduction

Serum prolactin levels are low during the first 2 weeks of life, in both female and male rats, and then gradually increases until puberty. The progressive maturation of the neuroendocrine network involving both prolactin-inhibiting and prolactin-releasing factors induces important changes in prolactin synthesis, storage, and release during development (Chen 1987, Becú-Villalobos *et al.* 1992). Gender differences

were described in all these processes, which results in female rats presenting higher levels of serum prolactin compared to males since the first days of life.

It is well known that dopamine is the primary hypothalamic inhibitor of prolactin synthesis and secretion (for review see Ben-Jonathan & Hnasko 2001). Nevertheless, other hypothalamic factors have been

reported to contribute to prolactin inhibition such as gamma-aminobutyric acid (GABA), somatostatin, and opiates, among others (Leong *et al.* 1983, Grattan 2015). On the other hand, although lactotrophs synthesize and secrete prolactin constitutively, several hypothalamic factors contribute to prolactin release, including serotonin, thyrotropin-releasing hormone (TRH), oxytocin, and VIP. Nonetheless, the primary stimulus of prolactin synthesis and release is exerted by estradiol (Kansra *et al.* 2005).

It was first suggested that the gradual increase observed in prolactin secretion during the first weeks of life was mainly due to a gradual decrease in the hypothalamic inhibitory activity. However, later on, it was demonstrated that the inhibitory effect exerted by hypothalamic factors increases significantly during the first postnatal weeks (Karanth *et al.* 1987). In fact, the dopaminergic input toward the pituitary, as well as the lactotroph responsiveness to dopamine, increase from birth to puberty (Nazian 1983).

Then, the maturation of this inhibitory mechanism could not explain the prolactin rise observed during the first weeks of life. So, later, the postnatal prolactin rise was associated with an increase in the efficiency of prolactin-releasing factors such as estrogen, serotonin, and opiates, among others. Additionally, *in vitro* studies performed in isolated lactotrophs revealed that the basal release of prolactin also increased with age (Karanth *et al.* 1987). By using a reverse hemolytic plaque assay in dispersed pituitary cells to detect prolactin secretion from individual lactotrophs, Chen *et al.* observed that only a half of the total lactotrophs (prolactin positive cells by ICC) are plaque-forming cells (secreting lactotrophs) during the first week of postnatal life (Chen 1987).

These results were obtained using isolated lactotrophs, in the absence of hypothalamic factors, suggesting the presence of an intra-pituitary inhibitory mechanism exercised on prolactin secretion, which decreases with age, allowing a gradual increase in the percentage of secreting lactotrophs.

There are several intra-pituitary peptides and growth factors involved in the paracrine and/or autocrine control of prolactin secretion. Among them, the transforming growth factor beta 1 (TGFβ1) has been proposed as a strong inhibitor of lactotroph function and as a mediator of the local inhibitory action induced by dopamine (Minami & Sarkar 1997, Sarkar *et al.* 1998). All the components of the TGFβ1 system are expressed in the pituitary gland (Sarkar *et al.* 1992, 1998, Recouvreux *et al.* 2013).

While TGFβ1 action on prolactin secretion and lactotroph proliferation has been well studied in different

pituitary cell lines and adult animals, the postnatal development of this inhibitory system, as well as its involvement in the control of lactotroph function, remain unknown. We hypothesize that the intra-pituitary TGFβ1 contributes to the inhibitory control of lactotroph function in early postnatal days. Even more, we postulate that this mechanism is involved in the gender differences observed in prolactin secretion during the first weeks of life.

The present work aimed to study the postnatal development of the pituitary TGFβ1 system in association with lactotroph function, in male and female Sprague-Dawley rats at 11, 23, and 45 postnatal days.

Materials and methods

Animals

All studies were performed in Sprague-Dawley (SD) rats of both genders. Animals were housed in a temperature-controlled room with 12 h light:12 h darkness cycle, lights on 07:00–19:00 h, and were provided with laboratory chow and tap water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (IByME-CONICET), in accordance with National Institutes of Health guidelines for animal research (8th edition 2010) and with the European Communities Council Directive of November 2010 (2010/63/UE).

Animals were killed by decapitation at 11, 23, and 45 days of age. Forty-five-day-old female rats were used in diestrus. Anterior pituitaries of different experimental groups were stored in TRIzol® reagent (Invitrogen) for posterior RNA isolation or ice-cold buffer (100 mM Tris, 10 mM CaCl₂, 1 mM MgCl₂, 1% Triton X-100 pH 7.4) containing a mix of proteases inhibitors (phenylmethylsulfonyl fluoride, tosyl-phenylalanine-chloromethyl-ketone, tert-Amyl-methyl-ether, N-carbobenzoxy-L-phenylalanine chloromethyl-ketone, and tosylamide-lysyl-chloromethyl-ketone) for posterior ELISA assays. Trunk blood was collected to measure serum prolactin levels by RIA.

In vivo experiments

TGFβ1 regulation by estradiol

Male and female 11-day-old SD rats were injected with a single dose of estradiol valerate (0.2 mg/kg sc, Progynon Depot; Bayer Schering) or castor oil (control group). *n* = 5 per

group and per gender. Animals were killed by decapitation after 3 h. Anterior pituitaries were collected in ice-cold buffer with proteases inhibitors for TGF β 1 ELISA assay. Trunk blood was collected for serum PRL determination.

TGF β 1 regulation by dopamine

Male and female 11-day-old rats ($n=5$ per treatment-group and per gender) were injected with a single dose of sulpiride (5 mg/kg ip, Vipral, Ivax Laboratories, Argentina), cabergoline (2 mg/kg ip, Beta Laboratories, Buenos Aires, Argentina), or saline (control group). Animals were killed by decapitation after 30 min. Anterior pituitaries were collected in ice-cold buffer with proteases inhibitors for TGF β 1 ELISA assay. Trunk blood was collected for serum PRL determination.

RIA

Serum prolactin levels were determined by RIA using a rat-specific primary antibody and the reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program (Dr A F Parlow, NHPP, Torrance, CA, USA). Results are expressed in nanograms per millilitre. The inter- and intra-assay coefficients of variation were 6.9% and 11.6%, respectively.

Detection of total and active TGF β 1

To quantify active or total TGF β 1 content in pituitary homogenates, an ELISA assay was performed (TGF β 1 DuoSet ELISA Kit, DY1679, R&D Systems), following the manufacturer's instructions. To assay active TGF β 1, proteins from anterior pituitary glands of different experimental groups were isolated and equal amounts of protein were loaded per well. Pituitaries from 11-day-old rats were grouped (two pituitaries=one sample) to obtain enough amount of protein. To assay total TGF β 1, samples were acidified up to pH 2.6 by adding 1 N HCl (20 min at room temperature), followed by neutralization with NaOH to pH 7.6 before being loaded. TGF β 1 content is expressed as picograms per milligram of protein. Samples used in these assays were $n=7$, 8, and 8 for 11, 23, and 45 days, respectively, for studying differences among gender and age (Fig. 2); $n=5$ per group for *in vivo* assay (Fig. 8).

RNA isolation and reverse transcription PCR

After the animals were killed, anterior pituitaries were collected in TRIzol® Reagent (Invitrogen) and total RNA

was isolated from the tissue following the manufacturer's protocol. Total RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and RNA purity was determined by 260:280 nm absorbance ratio. Ratios above 1.8 were considered acceptable.

RT was performed using 1 μ g of total RNA, Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, Promega) and random primers (Biodynamics, SRL, Buenos Aires, Argentina). The resulting cDNA samples were used to perform quantitative real-time PCR.

Quantitative real-time PCR

RT-qPCRs were performed using specific-designed primers and the Fast Start Universal SYBR Green Master Rox (Roche) or the EVA green qPCR mix (Solis BioDyne, Tartu, Estonia) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in duplicate and specificity of amplification was determined by melt curve analysis. Relative fold-change in target gene expression was quantified by the $2^{-\Delta\Delta CT}$ method. Two reference genes were evaluated: Cyclophilin B and the 60S ribosomal protein L38 (Rpl38) to normalize the differences of starting template among samples. Calibration curves were used to establish the efficiency of each pair of primers. Comparing the slope of the regression lines among references genes and other genes of interest, *Cyclophilin B* was selected as the most proper housekeeping gene. All primers used showed similar efficiencies (95–100%). Primer sequences used for RT-qPCR are shown in Table 1.

Double indirect immunostaining by confocal laser scanning microscopy

Pituitaries from 11- and 45-day-old male and female rats were removed immediately after killing, placed in Crioplast (Biopack, Buenos Aires, Argentina), and frozen in liquid nitrogen. Tissues were sectioned using a cryostat and slices were fixed in methanol as was previously described by Perez *et al.* (2018). The slices were blocked for 1 h in PBS-5% BSA and incubated overnight with primary antibodies (anti-T β RII, sc-17792, Santa Cruz Biotechnology, dilution 1:50; or anti-phospho-SMAD3, C25A9, Cell Signalling, dilution 1:150) in PBS-1% BSA. Then, sections were incubated with anti-PRL (NIDDK-rPRL-IC-5; Dr A F Parlow, National Hormone and Pituitary Program, Torrance, CA, USA; dilution 1:1000) for 1 h and further incubated with Alexa 488 and Alexa 594 secondary antibodies (1:1000; Invitrogen) for 1 h. Finally, sections were incubated with DAPI (Sigma-Aldrich) and

Table 1 Primers sequences for RT-qPCR.

Gene	Accession number	Forward primer sequence 5'→3'	Reverse primer sequence 5'→3'
<i>Prl</i>	NM_012629	CATCAATGACTGCCCACTTC	GAGTGCACCAACTGAGGATCA
<i>Tgfb1</i>	NM_021578	CAACAATTCCTGGCGTTACC	AGCCTGTATTCCGTCCTCT
<i>Tbr2</i>	NM_031132	CCGTGTGGAGGAAGAACGAC	CAGAGTGAAGCCGTGGTAGG
<i>Alk5</i>	NM_012775	TCCAAACCACAGAGTAGGCAC	TGGATTCCGCCAATGGAACA
<i>Tsp1</i>	NM_001013062	GTGCGCACCCTGTGGCATGA	ACCGGTCTTTGGCCTGTGGC
<i>Klf14</i>	NM_001135094	AGGCAGATTACACCCGCATC	TACGCTGGTGTGACTTGAGG
<i>Tmpai1</i>	NM_001107807	GAGAGACGACGGAGGACTGT	GGGCATAGACCTGTGGCTCC
<i>Ltbp1</i>	NM_021587	AGCACCGTCACCTCTGCTCT	ATCCTCGCAGTGGTCTCCAA
<i>Ltbp3</i>	NM_001191561	ATGGCCTCAGTGCATAGAC	AAGGAGCCTGGTGTGTTCTG
<i>CyclophilinB</i>	NM_022536	GACCCTCCGTGGCCAACGAT	GTCACTCGTCTACAGGTTCTCTC

mounted with Fluoromount (Sigma-Aldrich). Negative control slides were incubated with PBS-1% BSA without the primary antibody to validate the specificity of the immunostaining. Images were obtained using a Zeiss LSM 800 inverted confocal laser scanning microscope (Carl Zeiss) and analyzed with the software FV10-ASW 1.6 Viewer. This assay was performed using three animals (pituitaries) per age, per gender, and per first antibody to be studied. Total $n=12$. Representative images are shown.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Comparisons among gender and age were evaluated by two-way ANOVA, followed by Tukey test. Comparisons between gender or age were evaluated by Student's *t*-test or one-way ANOVA followed by Tukey test. Data were transformed when required. $P<0.05$ was considered significant.

Results

Serum prolactin levels were measured at different postnatal days: day 11, day 23, and day 45 in male and female rats ($n=24$, 8, and 20, respectively, for each gender). According to previous results (Becú-Villalobos *et al.* 1992, Díaz-Torga *et al.* 1994), prolactin levels are low at day 11 and gradually increase until 45-days-old in both female and male rats. As previously described, prolactin levels remain lower in males during postnatal development (Fig. 1A). Not only serum levels but also PRL synthesis (mRNA, assayed by RT-qPCR) steeply increases with age, always maintaining higher values in females (Fig. 1B, see scale).

Active and total pituitary TGF β 1

Pituitary content of active TGF β 1 and total TGF β 1 (active+latent TGF β 1) was measured by a specific ELISA.

Total TGF β 1 protein expression was found significantly increased at day 11 and decreased thereafter (Fig. 2A) without gender differences. However, when active TGF β 1 levels were assayed, the profile observed was gender-specific (Fig. 2B). Higher active cytokine levels are found in females at day 11 and they decrease afterwards. In contrast, male pituitaries express higher active TGF β 1 levels at day 45. Then, the bioactive cytokine in the pituitary is significantly higher in females than in males at day 11, but this profile is reversed at day 45 (Fig. 2B).

To evaluate whether this gender-specific profile in active TGF β 1 levels was also observed in the biological activity of

Figure 1

(A) Serum prolactin levels during postnatal development. Serum prolactin levels were measured by RIA. Data were analyzed by two-way ANOVA (age $P<0.0001$, gender $P<0.05$) followed by Tukey test. $*P<0.05$. $**P<0.01$ and $****P<0.0001$ vs 45-day-old females. (B) mRNA levels of PRL. Pituitary expression of prolactin was measured by RT-qPCR. Data were analyzed by two-way ANOVA (age and gender, interaction $P<0.0001$) followed by Tukey test. $**P<0.01$ and $****P<0.0001$ vs 45-day-old animals (within the same gender). $n=5-9$ per group.

Figure 2

(A) Pituitary content of total TGF β 1. The cytokine levels were assayed by ELISA. Data were analyzed by two-way ANOVA (age $P < 0.0001$) followed by Tukey test. **** $P < 0.0001$ vs 45-day-old females. $n = 7-8$ per group. (B) Pituitary content of active TGF β 1. The cytokine levels were assayed by ELISA. Data were analyzed by two-way ANOVA (interaction $P < 0.05$) followed by Tukey test. * $P < 0.0001$ vs 45-day-old females. Gender differences at days 11 and 45 were analyzed by Student's t -test. $P < 0.05$. $n = 7-8$ per group. (C) Pituitary expression of *Klf-14*. mRNA levels were assayed by RT-qPCR. Data were analyzed by two-way ANOVA (interaction and age $P < 0.01$), followed by Tukey test. *** $P < 0.001$ vs 45-day-old females. Gender differences at days 11 and 45 were analyzed by Student's t -test. $P < 0.05$. $n = 5-9$ per group. (D) Pituitary expression of *Tmpai-1*. mRNA levels were assayed by RT-qPCR. Data were analyzed by two-way ANOVA (interaction and age $P < 0.01$), followed by Tukey test. * $P < 0.05$ and *** $P < 0.001$ vs 45-day-old females. $n = 5-9$ per group.

the cytokine, we measured the expression of two TGF β 1-target genes: the androgen-induced trans-membrane protein, *Tmpai* (Brunschwig *et al.* 2003, Levy & Hill 2005), and the Krüppel-like factor 14 (*Klf14*) (Truty *et al.* 2009). The expression pattern of both target genes correlates with pituitary levels of active TGF β 1. mRNA levels of *Tmpai* and *Klf-14* were found significantly higher in 11-day-old female rats compared to males, and then this gender difference was inverted at 45 days old (Fig. 2C and D).

As observed in Fig. 3A and C, serum PRL levels inversely correlated with values obtained for pituitary active TGF β 1 only in females. In contrast, a direct correlation between these two variables was observed in males (Fig. 3B and D). On the other hand, no correlation between serum prolactin and total TGF β 1 was observed, neither in females nor in males (data not shown).

Pituitary expression of other components of the TGF β 1 system

Once synthesized, TGF β 1 is secreted to the extracellular matrix (ECM) coupled to a latent TGF β binding protein (LTBP). LTBPs are important mediators of TGF β 1 secretion, storage in the ECM, and appropriate activation (Rifkin

Figure 3

Serum prolactin levels and pituitary active TGF β 1 during postnatal development in females (A) and males (B). Prolactin levels were measured by RIA and TGF β 1 content was assayed by ELISA. (C) Inverse correlation between prolactin and TGF β 1 in females. Data were analyzed with a correlation test. $P = 0.0395$. $n = 7, 6$, and 7 samples for 11-, 23-, and 45-day-old rats, respectively. (D) Direct correlation between prolactin and TGF β 1 in males. Data were analyzed with a correlation test. $P = 0.0165$. $n = 8$ samples/age.

2005). In the ECM, TGF β 1 remains inactive, as a reservoir, and must undergo a tightly regulated activation process by which TGF β 1 is released from its latent proteins. This activation process enables the cytokine to be biologically active (Annes *et al.* 2003, Yoshinaga *et al.* 2008).

We have previously described that the four LTBP isoforms (LTBP-1, -2, -3, and -4) are expressed in the pituitary gland of adult rats and mice (Recouvreux *et al.* 2013, 2016), LTBP-1 and LTBP-3 being the two isoforms with the highest expression. At the time, there is no data available on pituitary LTBPs expression at early postnatal days.

We found that pituitary expression of *Ltbp-1* is higher at postnatal day 11 in both female and male rats and markedly decreases with age (Fig. 4A). On the contrary, pituitary *Ltbp-3* expression remains relatively constant during postnatal life (Fig. 4B), being higher in females than in males at day 45.

Several proteases have been suggested as TGF β 1 activators, which are tissue specific. Among them, thrombospondin 1 (TSP-1), kallikrein 1, and matrix metalloproteinases (MMP9 and MMP2) are known activators in several tissues (Ribeiro *et al.* 1999, Paez-Pereda *et al.* 2005). TSP1 has been previously suggested as a local activator of pituitary TGF β 1 (Pastorcic *et al.* 1995, Recouvreux *et al.* 2012, Faraoni *et al.* 2017). We found high expression of pituitary *Tsp1* mRNA in postnatal day 11 in both genders. *Tsp1* levels significantly decreased with age in females (Fig. 4C). On the contrary, *Tsp1* decreased at postnatal day 23 in males, keeping those values at day 45,

Figure 4

(A) Pituitary expression of *Ltbp-1*. mRNA levels were measured by RT-qPCR. Data were analyzed by two-way ANOVA (age $P < 0.0001$), followed by Tukey test. **** $P < 0.0001$ vs 45-day-old females. $n = 5-7$ per group. (B) Pituitary expression of *Ltbp-3*. mRNA levels were measured by RT-qPCR. Data were analyzed by two-way ANOVA (age $P < 0.0001$ and gender $P < 0.05$), followed by Tukey test. * $P < 0.05$. $n = 5-7$ per group. (C) Pituitary expression of *Tsp-1*. mRNA levels were measured by RT-qPCR. Data were analyzed by two-way ANOVA (interaction $P < 0.01$ and age $P < 0.0001$, 0.0001), followed by Tukey test. **** $P < 0.0001$ vs 45-day-old females. * $P < 0.05$. $n = 5-7$ per group.

being significantly higher than females at this age, as observed with active TGF β 1 (Figs 2B and 4C).

After activation, TGF β 1 binds to the receptor type 2 (T β RII), which recruits and activates a receptor type 1 (T β RI; ALK5), forming hetero-tetrameric complexes in the presence of the dimeric ligand (reviewed by Wrana 2013). Signalling is initiated by direct phosphorylation of Smad2 and Smad3 proteins which, after binding to Smad4, migrate to the nucleus to modulate the transcription of target genes (Shi & Massague 2003).

When the mRNA expression of TGF β 1 receptors was assayed by RT-qPCR, we found a high expression of *T β RII* in pituitaries from 11-day-old female and male rats that decreased with age. This decrease was more pronounced in males, and then a gender difference appears in postnatal days 23 and 45, where lower *T β RII* expression was observed in males compared to females (Fig. 5A).

Pituitary *Alk-5* expression did not undergo remarkable changes during postnatal development. However, it was found higher in 45-day-old female pituitaries compared to age-matched males (Fig. 5B).

T β RII and p-Smad3 expression by immunostaining using confocal laser scanning microscopy

Figure 6 shows T β RII expression in 11- and 45-day-old female and male pituitaries. Double staining with PRL

Figure 5

Pituitary expression of TGF β 1 receptors measured by RT-qPCR. (A) *T β RII*. Data were analyzed by two-way ANOVA (interaction $P < 0.05$, age and gender $P < 0.0001$), followed by Tukey test. *** $P < 0.001$ vs 45-day-old females. $P < 0.05$. $n = 5-7$ per group. (B) *Alk-5*. Data were analyzed by two-way ANOVA (interaction $P < 0.01$ and age $P < 0.001$), followed by Tukey test. * $P < 0.05$ vs 45-day-old females. $n = 5-7$ per group.

(merge) indicates that lactotrophs express this receptor in all the groups analyzed.

When TGF β 1 binds to T β RII, this receptor transactivates the T β RI, which phosphorylates Smad2 and Smad3 proteins. We next assayed phospho-Smad3 (p-Smad3) expression in 11- and 45-day-old male and female pituitaries. Double staining with PRL (merge) indicates that lactotrophs express p-Smad3. As Fig. 7 shows, a strong p-Smad3 expression is observed in 11-day-old females. This expression was higher than that found in 45-day-old females and males.

Gender differences in active TGFβ1 regulation by dopamine and estradiol at early postnatal age

We have previously demonstrated that pituitary TGF β 1 activity is regulated by dopamine and estradiol. We demonstrated that dopamine and estradiol also regulate pituitary TGF β 1 activation (Recouvreux *et al.* 2011, 2013). Although this regulation is well studied in adult rats and mice, little is known about the involvement of dopamine and estradiol in the regulation of pituitary TGF β 1 system at early postnatal days.

The impact of dopamine and estradiol on pituitary TGF β 1 activation was studied *in vivo* in 11-day-old

Figure 6

TGF β 1 receptor type II (T β RII) expression in pituitaries from 11- and 45-day-old female and male rats. Identification of T β RII (red) and prolactin (green) by double indirect immunofluorescence. Arrows show lactotrophs expressing T β RII. (*) T β RII expression in non-lactotroph cells. Nuclei were immunostained with DAPI (blue). Scale bar = 20 μ m.

female and male rats. As Fig. 8 shows, both estradiol and cabergoline (Drd2 agonist) increased pituitary levels of TGF β 1 in 11-day-old rats (both genders). In females, the activation of the cytokine was concomitant with a decrease in serum prolactin levels. In contrast, the increased pituitary levels of active TGF β 1 had no impact on serum prolactin in males (Fig. 8A, B, E and F). On the other hand, although sulpiride (Drd2 antagonist) did not alter active TGF β 1 expression in the pituitary, it induced the expected increase in prolactin levels in both female and male rats (Fig. 8C and D).

Discussion

In the present work, we demonstrated that pituitary TGF β 1 system is involved in the control of prolactin secretion during early postnatal life, as well as in the gender differences observed. Only in females, prolactin levels negatively correlate with pituitary active TGF β 1.

Moreover, dopamine and estradiol stimulate pituitary TGF β 1 activation at postnatal day 11 in both genders, nevertheless, the impact of increased active TGF β 1 levels on prolactin inhibition was observed only in females.

It is well known that serum prolactin increases from birth to puberty in both genders, but prolactin levels remain higher in females. However, the gradual and continuous increase in prolactin secretion as well as the gender differences previously described could not be explained neither by the decrease in hypothalamic inhibitory impact nor by the increase observed in serum estradiol levels.

The inhibitory hypothalamic impact, including dopaminergic tone and lactotroph responsiveness to dopamine, also increases during the first weeks of life in both genders (Nazian 1983). Moreover, prolactin levels remain higher in females even though the dopamine-inhibitory action on lactotroph function is higher in females since the first weeks of postnatal life (Becú-Villalobos *et al.* 1992). Then, the gradual increase in

Figure 7

p-Smad3 expression in pituitaries from 11- and 45-day-old female and male rats. Identification of p-Smad3 (red) and prolactin (green) by double indirect immunofluorescence. Nuclei were immunostained with DAPI (blue). Scale bar = 20 μ m.

prolactin secretion and the gender differences found cannot be fully explained by the maturation of the dopamine inhibitory regulation.

It was proposed that the continuous increase in prolactin levels is related to an increase in the efficiency of prolactin-releasing factors such as estradiol, serotonin, and opiates among others, but considering estradiol as the primary stimulus (Karanth *et al.* 1987). However, serum estradiol levels alone neither explain the steady increase in prolactin levels after birth nor the gender differences observed. After several contradictory reports in the literature of the 80s and 90s about postnatal serum estradiol levels, in 2012, Walker *et al.* described that serum estradiol concentration is similar in developing female and male rats. Estradiol levels gradually increase after birth, presenting the highest concentration on postnatal day 15 and then undergo a decrease as the animals mature, while prolactin levels remain increasing after postnatal day

15 to puberty. The gender differences in estradiol levels appear after 45 days of life (Walker *et al.* 2012).

Then, the complete mechanism involved in both the control of prolactin secretion during the first weeks of postnatal life as well as the gender differences observed is still unsolved.

The lactotroph population, the synthesis of prolactin, and the amount of prolactin secreted from individual lactotrophs increase from birth to puberty, in both female and male pituitaries. However, Chen *et al.* demonstrated that, after birth, only a percentage of lactotrophs are secreting cells (Chen 1987). These results were obtained using a reverse hemolytic plaque assay for detection and measurement of PRL secretion from individual cells in conjunction with PRL by immunocytochemistry. Authors demonstrated that the percentage of plaque-forming cells (secreting lactotrophs) is about 5% of the total anterior pituitary cell population at postnatal day 5 in both

Figure 8

Gender differences in the regulation of active TGF β 1 and PRL secretion by dopamine and estradiol *in vivo*. (A, C, and E) Pituitary active TGF β 1 content measured by ELISA in pituitary extracts. (B, D, and F) Serum PRL levels measured by RIA. DA regulation: 11-day-old male and female rats were injected with the Drd2 agonist, cabergoline (A and B, caberg, 2 mg/kg, i.p.), or Drd2 antagonist, sulpiride (C and D, sulp, 10 mg/kg, i.p) or saline (control, i.p.), and killed after 30 min. E2 regulation: 11-day-old male and female rats were injected with estradiol valerate (E2 3 h, 0.2 mg/kg, s.c.) or castor oil (control, s.c.) and killed after 3 h. Data were analyzed by Student's *t*-test within the same gender (control vs treatment). **P* < 0.05. *n* = 5 per group.

male and females and that the fraction of pituitary cells staining for PRL (lactotrophs) is twice that of cells forming plaques. The proportion of secreting lactotrophs gradually increases, in a gender-specific rate, until reaching the adult values of about 37% in males and 54% in females at proestrus (Chen 1987). These results were obtained in dispersed pituitary cells, avoiding hypothalamic influence, and suggest the presence of an intra-pituitary factor involved in the inhibition of prolactin secretion (Chen 1987).

Substantial evidence supports the relevance of the TGF β 1 inhibitory action on lactotroph function as well as its involvement in prolactinoma development. colleagues mRNA and protein expression in the pituitary gland and the inhibitory effect of TGF β 1 on prolactin secretion and lactotroph proliferation (Sarkar *et al.* 1992). Few years later, TGF β 1 and T β RII expression were found expressed in human pituitaries and human pituitary adenomas (Fujiwara *et al.* 1995, Jin *et al.* 1997).

Interestingly, TGF β 1 activity is reduced in prolactinomas, concomitant with the increase observed in prolactin secretion and lactotroph proliferation (Pastorcic *et al.* 1995, Recouvreux *et al.* 2013). It was postulated that this decreased cytokine activity is involved in prolactinoma development. Indeed, we have previously demonstrated, using animal models of prolactinomas, that the pharmacological recovery of local TGF β 1 activity reverts hyperprolactinemia and tumour development (Recouvreux *et al.* 2012, 2016, Faraoni *et al.* 2017).

Additionally, gender differences have been described in the pituitary TGF β 1 activity. Studies performed in two animal models of prolactinoma (mice lacking dopamine receptor type 2 and mice overexpressing the β subunit of chorionic gonadotrophin) demonstrated that only females develop prolactinomas, concomitant with a weakened TGF β 1 system (Recouvreux *et al.* 2013, 2017, Faraoni *et al.* 2017). Interestingly, males present a stronger pituitary TGF β 1 system compared to females, in both mouse models, and this gender does not develop prolactinoma, even in the absence of dopaminergic control (Recouvreux *et al.* 2012, 2013).

All the previously mentioned results corroborate the impact of the intra-pituitary TGF β 1 inhibition on lactotroph function, and even more, confirm that this regulation is gender specific.

We here described that total pituitary TGF β 1 content presents the highest levels at day 11 and gradually decreases with age, without gender differences. The pituitary expression of *LTBP1* and *T β RII* was also found increased at day 11, and it also decreased with age in female and males. However, the levels of pituitary cytokine activity followed a gender-specific profile. Female pituitaries presented the highest active TGF β 1 levels at day 11, and then it decreased with age. The cytokine biological activity, measured by target genes expression, correlated with active TGF β 1 levels. Moreover, the strongest p-Smad3 immunostaining was observed in pituitaries from 11-day-old females and specifically in lactotrophs. Interestingly, the steady decrease in the TGF β 1 activity observed in females inversely correlates with the gradual increase observed in serum prolactin levels in this gender. In contrast, in male pituitaries, cytokine levels were similar at days 11 and 23 but then increased at 45 days of age. Neither active nor total TGF β 1 levels explain the prolactin secretion in males. Moreover, by an *in vivo* assay, we demonstrated that dopamine as well as estradiol induce an increase in the pituitary cytokine activity in both genders; however, those treatments impacted on prolactin secretion only in females.

Although no gender differences were observed in total TGFβ1 pituitary expression during postnatal development, active TGFβ1 levels followed a gender-specific profile. Female pituitaries express higher levels of active cytokine than males at day 11, but that relationship is reversed at day 45, when the highest levels of active TGFβ1 were found in males. This suggests an age and gender-specific activity of local TGFβ1 activators.

TSP1 is one of the most characterized physiologic activators of TGFβ1 *in vitro* and *in vivo* (Schultz-Cherry *et al.* 1994). We have previously demonstrated that an *in vivo* treatment with TSP1 analogues improve pituitary levels of active TGFβ1, decreasing hyperprolactinemia in female rats carrying prolactinomas (Recouvreux *et al.* 2012). In the present work, we found increased pituitary TSP1 mRNA expression at day 11 in both genders, and then *Tsp1* gradually decreased with age only in females, in direct correlation with the cytokine activity found in this gender. On the other hand, the highest expression of *Tsp1* in male pituitaries was observed at day 45, and this fact could explain the higher levels of active TGFβ1 found in this gender at this time. However, the *Tsp1* expression observed in 11-day-old pituitaries, without gender differences, does not explain the higher TGFβ1 biological activity observed in females at this age. In the same way, the lower cytokine activity observed in 11-day-old males could not be associated either with decreased latent cytokine or with impediments in the process of TGFβ1 activation, as cabergoline and estradiol were able to induce TGFβ1 activation in 11-day-old pituitaries from both genders.

The complete mechanism involved in pituitary TGFβ1 activation and the gender differences observed at early postnatal life could not be elucidated with the present results and deserve future studies.

We have previously postulated, by studying the pituitary TGFβ1 system in adult mice and rats, that the TGFβ1 system is a target of the estradiol-dopamine interaction in the regulation of PRL secretion and lactotroph proliferation. Moreover, we suggested that the gender differences observed in the pituitary TGFβ1 system could explain the gender differences found in the control of lactotroph function (Recouvreux *et al.* 2011, 2017, Faraoni *et al.* 2017). The present results show, once more, the relevance of this intra-pituitary mechanism involved in the inhibitory regulation of prolactin secretion. This inhibitory control exercised by TGFβ1 is female-specific and is responsible for the gender differences observed in the control of lactotroph function, even at early postnatal age.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A A M, P A P, S G, and G D T performed conception and design of research. A A M, P A P, M A C, E Y F, and F P performed experiments. A A M, P A P, S G, J P P, and G D T analyzed the data. A A M, P A P, J P P, S G, and G D T interpreted the results of the experiments. A A M and P A P prepared figures. A A M and G D T drafted the manuscript. P A P, F P, M A C, E Y F, J P P, and S G edited and revised the manuscript. All authors approved the final version of the manuscript.

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