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Activin-inhibitory action on lactotrophs is decreased in lactotroph hyperplasia

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Abstract

Among all the hormone-secreting pituitary tumours, prolactinomas are the most frequently found in the clinic. Since dopamine is the primary inhibitor of lactotroph function, dopamine agonists represent the first-line therapy. However, a subset of patients exhibits resistance to these drugs, and therefore, alternative treatments are desired. As activins inhibit prolactin gene expression through the inhibition of *Pit-1* involving the p38MAPK pathway, in the present work, we studied the local activin system as an alternative inhibitory system for lactotroph hyperplasia treatment. We used two different mouse models of prolactinoma: transgenic mice with overexpression of the human chorionic gonadotropin β-subunit (hCGβ) and mice lacking dopamine receptor type 2. In both models, females, but not males, develop lactotroph hyperplasia from the fourth month of life. We found reduced expression of pituitary activin subunits and activin receptors in hyperplastic pituitaries from both models compared with wild-type counterparts. Consequently, hyperplastic pituitaries presented a reduced activin-inhibitory action on prolactin secretion. Additionally, while female wild-type lactotrophs presented high levels of phospho-p38MAPK, it was lost in prolactinomas, concomitant with decreased activin expression, increased *Pit-1* expression and tumour development. In contrast, male pituitaries express higher mRNA levels of activin subunits βA and βB, which would suggest a stronger activin inhibitory function on lactotrophs, protecting this sex from tumour development, despite genotype. The present results highlight the importance of the activin inhibitory action on lactotroph function and place the local activin system as a new target for the treatment of dopamine agonist-resistant prolactinomas.

Key Words

- prolactinomas
- activins
- lactotrophs
- prolactin

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Introduction

Pituitary tumours account from 10 to 15% of all intracranial neoplasms with a relatively high prevalence, accounting for 1 case per 1000 (Mete *et al.* 2018). These tumours are usually benign and can present slow growth rates over many years. However, besides their benign features, pituitary tumours may cause significant clinical problems due to the 'mass effects' (headache and visual failure from optic chiasm compression) and hormone hypersecretion from functioning tumours (Kovacs *et al.* 2001, Farrell 2006).

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Prolactinomas, which derive from hypertrophy and hyperplasia of lactotroph cells, are the pituitary tumours with the highest incidence in the clinic (Ciccarelli *et al.* 2005). Sexual differences in the clinical manifestations, prevalence, tumour size, and behaviour have been widely described (Mindermann & Wilson 1994, Gillam *et al.* 2006). In women, the hyperprolactinemia promotes alterations in the menstrual cycle that lead to an early clinical evaluation and, therefore, microprolactinomas are more frequent in this gender at the time of diagnosis (Fainstein Day *et al.* 2010). In contrast, in men, clinical manifestations due to hyperprolactinemia, such as sexual dysfunction or decreased libido, are underestimated and leads to a delay in diagnosis. Thus, in general, men attend a medical consultation with symptoms related to mass effects, due to the presence of macroprolactinomas (Colao *et al.* 2003, Wong *et al.* 2015*a*).

The primary goals in the treatment of prolactinomas are to normalize prolactin levels in order to restore fertility and sexual function, to reduce the tumour mass to potentially relieve the visual defects and headaches and to preserve the residual pituitary function (Liu & Couldwell 2004, Gillam *et al.* 2006, Schlechte 2007).

As the primary inhibitory control of prolactin synthesis and release is mediated by dopamine, the standard treatment for patients with prolactinoma is with dopamine agonists. Even though most prolactinomas are successfully treated with these agonists (Kars *et al.* 2010, Colao & Savastano 2011, Wong *et al.* 2015*b*), a reasonable percentage of patients do not respond to this therapy, even at high doses of dopamine agonists (Vroonen *et al.* 2012). To date, there is no alternative treatment for dopamine agonist-resistant prolactinomas (DARPs). Radiotherapy becomes the next therapeutic option, but with limited efficiency (Molitch 2014), and then, trans-sphenoidal surgery is indicated (Primeau *et al.* 2012, Smith *et al.* 2015). However, even after surgery, aggressive prolactinomas often recur. Thus, the management of DARPs remains a challenge, and new therapeutic approaches for the inhibition of exacerbated lactotroph function are necessary (Maiter 2019).

Among the growth factors participating in the intra-pituitary regulation, activins have been proposed as potent inhibitors of lactotroph proliferation and prolactin secretion (Lacerte *et al.* 2004, de Guise *et al.* 2006, Lebrun 2009).

Activins and inhibins are dimeric protein hormones, members of the TGFβ family, first described for their stimulatory (activin) and inhibitory (inhibin) effects on follicle-stimulating hormone (FSH) production and secretion by gonadotroph cells (Keogh *et al.* 1976, Vale *et al.* 1986). Activins are homodimers of beta (β) subunits. In the pituitary, the isoforms most commonly synthesized are Activin A (βA-βA) and Activin B (βB-βB), which act as autocrine/paracrine molecules. Pituitary activin function is antagonized by inhibins, betaglycan and follistatins (Thompson *et al.* 2005). Inhibins are heterodimers of an α-specific subunit with a βA-subunit (Inhibin A) or a βB-subunit (Inhibin B). Although they are locally synthesized, the gonads represent the primary source of inhibins, and therefore, they mostly regulate pituitary activin function as endocrine factors (Makanji *et al.* 2014).

Betaglycan acts as a local functional inhibitor of activin binding and signalling (Lewis *et al.* 2000, Wiater *et al.* 2009). Acting as a co-receptor, it promotes a specific interaction between inhibins and the activin type II receptor preventing the recruitment of the activin type I receptor and the initiation of the intracellular activin signalling (Lewis *et al.* 2000, Bilezikjian *et al.* 2012).

Another critical player in the local control of activin action is follistatin (FST). FST has been characterized as a specific activin-binding protein that can bind activins in the extracellular compartment, with high affinity, neutralizing its effects (Gregory & Kaiser 2004). FST present two primary isoforms: FST288 and FST315, which originated from alternatively splicing (Shimasaki *et al.* 1988, Sugino *et al.* 1997, Lerch *et al.* 2007). While both isoforms bind activins with high affinity, FST288 also has a high affinity for heparan-sulfate proteoglycans of the cell surface, and mostly prevent the potential autocrine and paracrine action of activins. In contrast, FST315 has no affinity for heparan-sulfate proteoglycans and is, predominantly, a circulating isoform (Welt *et al.* 2002, Schneyer *et al.* 2004).

It has been described that disturbances in activins functionality can lead to the development of endocrine and neuroendocrine tumours (Risbridger *et al.* 2001, Namwanje & Brown 2016). On the other hand, an aberrant FST expression has been observed in a variety of solid tumours, including gonadal, gastric and hepatocellular carcinoma (Shi *et al.* 2016). However, to date, the participation of activins and inhibins in prolactinoma development is still not fully elucidated (Davis *et al.* 2001, Farrell 2006).

Since activins are involved in the inhibition of lactotroph function, in the present work, we investigated alterations in the activin-inhibin system in lactotroph hyperplasia development in two different animal experimental models of prolactinoma.

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Materials and methods

Animals

hCGβ+ transgenic mice, with FVB/N background, overexpress the human chorionic gonadotropin β-subunit (hCGβ) under the control of human ubiquitin C promoter. Generation and genotyping have been previously described (Rulli *et al.* 2002). These mice were used at six months at which time the pituitaries from hCGβ+ females were hyperplastic (Rulli *et al.* 2002, Faraoni *et al.* 2017).

Drd2ko (KO) mice, (official strain designation B6.129S2-$^{Drd2tm1Low/J}$ by the Induced Mutant Resource at The Jackson Laboratory), were generated by targeted mutagenesis of the Drd2 gene in embryonic stem cells, as previously described (Kelly *et al.* 1997, Asa *et al.* 1999). Both isoforms of Drd2 (D2L and D2S) are non-functional in mutant mice. Drd2$^{+/+}$ (WT), heterozygous, and Drd2$^{-/-}$ (Drd2ko, KO) mice were identified by PCR of genomic DNA as described previously (Díaz-Torga *et al.* 2002). These mice were used at 8 months at which time the pituitaries from Drd2$^{-/-}$ (KO) females were hyperplastic (Díaz-Torga *et al.* 2002).

All studies were performed in hCGβ and Drd2 mice of both genders and genotypes. Animals were housed in groups of four or five with mixed genotypes in a temperature-controlled room with lights on at 07:00 h and off at 19:00 h and were given free access to laboratory chow and tap water. All experimental procedures were performed according to the NIH Guidelines for Care and Use of Experimental Animals (Division of Animal Welfare, Office for Protection of Research Risks, National Institutes of Health, A#5072-01) and were approved by the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental (IBYME).

Mice were killed by decapitation and trunk blood was collected. All pituitaries were weighted after removal. Serum samples were separated by centrifugation and stored at −20°C for biochemical analyses. Anterior pituitaries of different experimental groups were stored at −70°C for posterior analyses.

RNA isolation and gene expression assays

Anterior pituitaries were collected and processed in TRIzol Reagent (Invitrogen), and total RNA was isolated according to the manufacturer's protocol. One microgram of RNA was reverse-transcribed in a 20 µL reaction volume using MMLV-RT (Promega) and random primers (Biodynamics). For quantitative real-time RT-PCR (QRT-PCR) primers sets were designed for the specific amplification, and primer sequences used are shown in Table 1.

To normalize the differences in the amount of starting template between samples, we previously assayed the appropriate internal control (housekeeping gene). Two reference genes were evaluated: the 60S ribosomal protein L38 (*Rpl38*) and Cyclophilin B (*Cypb*). The qPCR efficiency of each pair of primers was tested using serially diluted samples and was established through calibration curves. *Cypb* was selected as the most proper housekeeping gene due to the parallelism presented between its slope of the regression line (and consequently on the value of the correlation coefficient) with the slope of other genes evaluated. Moreover, as we show in Table 2, the expression levels of *Cypb* did not change regardless of the experimental group, ensuring a proper normalization within the samples and a robust QRT-PCR analysis.

Each sample was assayed in duplicate using the Fast Start Universal SYBR Green Master Rox (Roche) on a CFX96 Touch Real-Time PCR Detection System (BioRad). Differences in the cDNA target gene expression were quantified by comparing the threshold cycle (CT) with that of *Cypb* using the comparative CT method (ΔΔCT).

FST288 isoform was amplified using a specific QuantiTect Primer Assay (QIAGEN, catalogue number QT00105483). Since *FST288* exhibited CT values near the detection limit of the QRT-PCR assay, a semi-quantitative

Table 1 qPCR primer sequences.

Gene	Accession no.	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Inhba	NM_008380.2	GATCATCACCTTTGCCGAGT	TGGTCCTGGTTCTGTTAGCC
Inhbb	NM_008381.4	TCAGCTTTGCAGAGACAGATGG	ACACCTTGACCCGTACCTTC
Inha	NM_010564.5	GGGGATCCTGGAATAAGGCG	GTGGCACCTGTAGCTGGGAA
Betaglycan	NM_011578.4	AGCTGCCAAAGTGTGTGACTC	CGGACTGGACTCCTTCATGTT
Acvr1b	NM_007395.4	CAAATCAGAGGGTGGGGACC	TGGACTCCTCCAGAATTGCAT
Acvr2b	NM_001313757.1	TTTGGCTGCGTTTGGAAAGCTC	ACGACTGCTTGTCCTGAAGTGG
Pit-1	NM_008849.5	GTGATGTCCACAGCGACAGG	ACTCAGGGTGTGGTCTGGAA
CyclophilinB	NM_011149.2	GACCCTCCGTGGCCAACGAT	ACGACTCGTCCTACAGATTCATCTC
Gapdh	NM_008084.3	GTGCCAGCCTCGTCCCGTAG	GTGCCGTTGAATTTGCCGTGAGTG

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Table 2 Average of Ct values showing stable expression of *Cypb* within samples of different groups.

Ct values ($\bar{x}$)	***Cypb***	***InhBB***	**ΔCt values**
F wt	26.89	30.12	3.23
F β+	27.01	31.60	4.59
M wt	26.95	29.93	2.98
M β+	26.76	29.42	2.66

(sqPCR) following a protocol of 40 cycles was used. Agarose gels were digitalized, and the density of bands was measured with the software ImageJ (NIH, National Institutes of Health). The sqPCR results are expressed relative to *Gapdh*.

Radioimmunoassay (RIA)

Serum PRL, FSH and LH levels were measured by RIA using mouse-specific reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases and National Hormone and Pituitary Program (Dr. A. F. Parlow, NHPP, Torrance, CA). Assays were performed using 10 µL (PRL), 70 µL (FSH and LH) serum samples in duplicate. Results are expressed in nanograms per millilitre. The inter- and intra-assay coefficients of variation were 6.9 and 11.6% (PRL), 8.0 and 12.2% (FSH) and 7.5 and 11.2% (LH), respectively.

Western blot

Anterior pituitaries were homogenized and processed as previously described (Recouvreux *et al.* 2011). Membranes were incubated overnight at 4°C with mouse anti-FST antibody (1:150, sc 365003; Santa Cruz Biotechnology, Inc.), and then 1-h incubation with mouse anti-IgGK secondary antibody (1:200, sc 516102; Santa Cruz Biotechnology, Inc.) was performed. After washing in PBS 0.05% Tween 20 (PBS-T), the immunoreactive bands were detected by the chemiluminescence released by the oxidation of the luminol substrate (250 mM), in the presence of H_2O_2. The chemiluminescence was detected in a G:box chemi-HR16 (Syngene, Frederick, MD, USA) and bands intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as loading control: anti-GAPDH primary antibody (1:150, sc 365062; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Between protocols, membranes were stripped of bound antibodies with stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.8) and incubated at 50°C for 30 min with agitation, and then washed three times for 10 min in PBS-T at room temperature.

Confocal laser scanning microscopy

Pituitary glands of WT and hCGβ+ female mice were placed in Crioplast (Biopack, Buenos Aires, Argentina) and immersed in liquid nitrogen for a few minutes until the solidification of the embedding medium. Pituitaries were sectioned using a cryostat and slices were fixed in absolute methanol as previously described by Perez *et al.* (2018). Tissue sections were blocked for 1 h in 5% PBS–BSA, and incubated overnight with primary antibodies (anti-ActRIB, Abcam, USA, dilution 1:100; or anti phospho-p38, Cell Signalling, 1:200). After that, tissues were incubated with anti-PRL or anti-FSH, Dr A. 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 antibody (1:1000; Invitrogen) for 1 h. Finally, sections were mounted with fluoromount (Sigma) containing 4′,6-diamidino-2-phenylindole (DAPI). Negative controls were performed replacing the primary antibody with 1% PBS–BSA to validate the specificity of the immunostaining. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1000 (Olympus). The analysis of confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer.

Three slides (corresponding to three pituitaries) were analyzed for each group. For the morphometric analysis, a total of 5000 pituitary cells were examined in randomly chosen fields of each glass slide and quantified in order to establish the percentage of lactotroph (PRL+) immunoreactive for ActRIB ($n=3$, $P=0.05$).

Ex vivo assay

Female hCGβ+ and WT littermates (6-month-old) were killed and anterior pituitaries were collected in 300 µL of Dulbecco Eagle's Modified Medium (DMEM) supplemented with 2.5 v/v foetal bovine serum (Natocor, Argentina), 15 v/v horse serum (Internegocios, Argentina), and 20 µg/mL gentamicin (Sigma-Aldrich). Anterior pituitaries were cut into small fragments and washed to remove traces of blood. After 2 h of incubation with supplemented media at 37°C in a CO_2 incubator, to stabilize the tissue, tissues were washed with fresh medium and stimulated for 45 min at 37°C with 20 ng/mL of Recombinant Human Activin A (NIH, Lot # NU1-4345) in 250 µL of serum-free DMEM or with 250 µL of serum-free DMEM alone (control). Medium samples (20 µL) were taken at 15, 30, and 45 min, and stored at −20°C until measurement of PRL by RIA.

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Statistical analysis

Results are expressed as mean ± S.E.M. Data were analyzed by Student's *t*-test when two groups were compared. Two-way ANOVA was performed for female vs male analyses, as the effects of two factors (genotype and sex) were evaluated, followed by Tukey's *post hoc* test when interaction was significant. The morphometric study of ActRIB protein expression in the lactotroph population was performed using the chi-square test. The degree of activin-inhibitory action in the *ex vivo* assay was evaluated by two-way ANOVA followed by a Šídák *post hoc* analysis. $P<0.05$ was considered significant. Data were transformed when required.

Results

Sexual differences in the development of lactotroph hyperplasia in hCGβ and Drd2 mice

As previously described by Rulli *et al.* (2002) and Kelly *et al.* (1997), only hCGβ+ and Drd2ko (KO) female mice develop lactotroph hyperplasia at 6 and 8 months of age, respectively, evidenced by an enlargement of pituitary size (Fig. 1A and B) and hyperprolactinemia (Fig. 1C and D) when compared to their WT littermates.

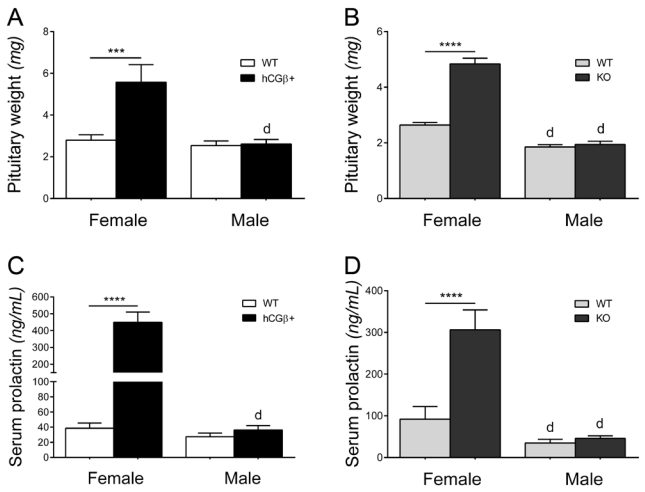

Figure 1
Pituitary weight (A and B) and serum PRL levels (C and D) of 6-month-old hCGβ and 8-month-old Drd2 mice. Data analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when the interaction effect was significant. Data are expressed as mean ± S.E.M. n = 8–10/group. (A) hCGβ mice. Interaction $P = 0.0338$. ***$P < 0.001$ hCGβ+ vs WT female. $^{d}P < 0.0001$ hCGβ+ male vs hCGβ+ female. (B) Drd2 mice. Interaction $P < 0.0001$. ****$P < 0.0001$ KO vs WT female. $^{d}P < 0.0001$ male vs female. (C) hCGβ mice. Interaction $P < 0.0001$. ****$P < 0.0001$ hCGβ+ vs WT female. $^{d}P < 0.0001$ hCGβ+ male vs hCGβ+ female. (D) Drd2 mice. Interaction $P = 0.0257$. ****$P < 0.0001$ KO vs WT female. $^{d}P < 0.0001$ male vs female.

Pituitary βA-subunit and βB-subunit mRNA expression is reduced in hCGβ+ and Drd2ko (KO) females

To better understand the role of activins in prolactinoma development, we first analyzed, by quantitative real-time PCR (qRT-PCR), the pituitary mRNA expression of β (beta) subunits in both animal models of lactotroph hyperplasia. We found a markedly reduced pituitary mRNA expression of both βA (*Inhba*) and βB (*Inhbb*) subunits in hCGβ+ (Fig. 2A and C, respectively) and KO (Fig. 2B and D, respectively) females when compared to their wild type (WT) littermates. Moreover, a significant gender difference was observed in both experimental models. Male pituitaries presented significantly higher expression of both, *Inhba* and *Inhbb*, compared to females, regardless of the genotype, and concomitant with the absence of lactotroph hyperplasia development in this gender (Fig. 2).

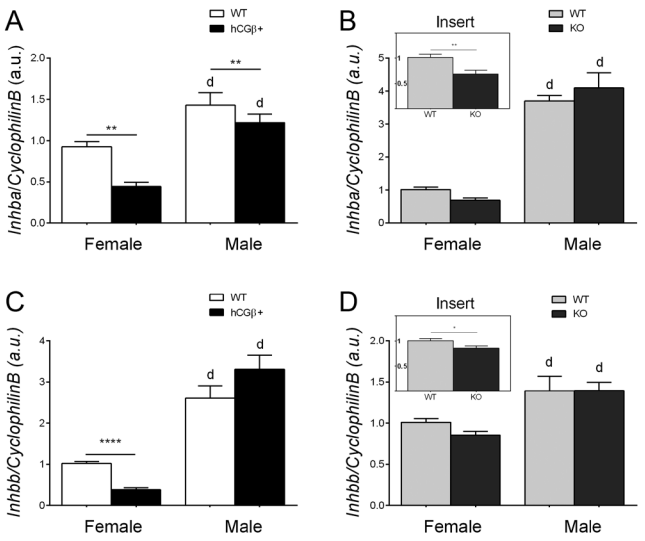

Figure 2
Pituitary *Inhba* and *Inhbb* expression in hCGβ and Drd2 mice pituitaries. mRNA transcripts were amplified with specific primers by qRT-PCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females. Data analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when interaction was significant. Data are expressed as mean ± S.E.M. n = 8–10/group. (A) hCGβ mice. *Inhba* mRNA expression. Interaction ns. **$P < 0.01$ (genotype). $^{d}P < 0.0001$ (sex). (B) Drd2 mice. *Inhba* mRNA expression. Interaction ns. $^{d}P < 0.0001$ (sex). Insert: *p$P < 0.01$ KO vs WT female. Data analyzed by Student's *t* test. (C) hCGβ mice. *Inhbb* mRNA expression. Interaction $P < 0.0001$. ****$P < 0.0001$ hCGβ+ vs WT female. $^{d}P < 0.0001$ male vs female. (D) Drd2 mice. *Inhbb* mRNA expression. Interaction ns. $^{d}P < 0.0001$ (sex). Insert: *$P < 0.05$ KO vs WT. Data analyzed by Student's *t* test.

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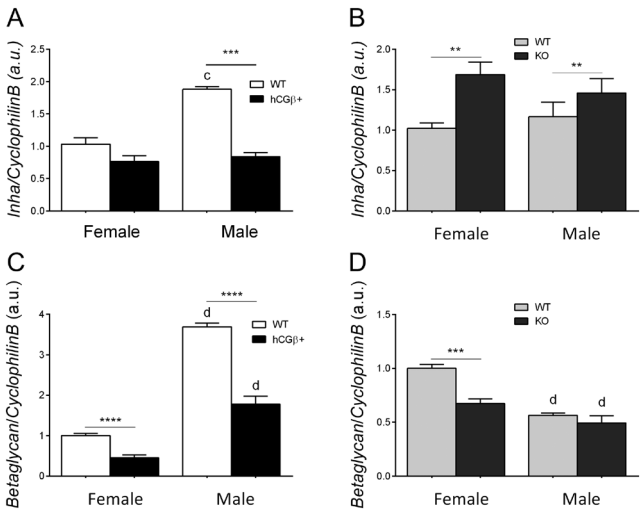

Figure 3
Pituitary mRNA expression of α-subunit (*Inha*) and *betagycan* in hCGβ and Drd2 mice. mRNA transcripts were amplified with specific primers by qRT-PCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females. Data analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when the interaction effect was significant. Data are expressed as mean ± S.E.M. $n = 6$–8/group. (A) *Inha* mRNA expression in 6-month-old hCGβ mice pituitaries. Interaction $P < 0.001$. $^{c}P < 0.001$ WT male vs WT female. $^{***}P < 0.001$ hCGβ+ male vs WT male. (B) *Inha* mRNA expression in 8-month-old Drd2 mice pituitaries. Interaction ns. $^{**}P < 0.01$ (genotype). (C) *Betaglycan* mRNA expression in pituitaries from hCGβ mice. Interaction ns. $^{****}P < 0.0001$ (genotype). $^{d}P < 0.0001$ (sex). (D) *Betaglycan* mRNA expression in pituitaries from Drd2 mice. Interaction $P < 0.05$. $^{***}P < 0.001$ KO vs WT female. $^{d}P < 0.0001$ (male vs female).

Pituitary inhibin α-subunit and betaglycan expression in hCGβ and Drd2 mice

To determine levels of locally synthesized inhibins, antagonists of activin inhibitory functions on lactotrophs, we evaluated, by qRT-PCR, the mRNA expression of the specific inhibin α-subunit (*Inha*) in pituitaries from both mouse models. *Inha* expression was found to be model specific. In hCGβ mice, higher levels of *Inha* were found in WT males compared to WT females (Fig. 3A). On the other hand, decreased pituitary *Inha* mRNA expression was observed in hCGβ+ male mice when compared to their WT littermates. However, no genotype differences were found in females. In contrast, when we analyzed the pituitary *Inha* mRNA expression in Drd2 mice, we found increased expression levels in Drd2ko (KO) mice in both sexes, when compared to the respective WT littermates (Fig. 3B), without gender differences.

We next determined the pituitary mRNA expression of betaglycan, the co-receptor responsible for increasing the affinity of inhibins for ActRII. As observed in Fig. 3, decreased *betaglycan* mRNA expression levels was observed in the hyperplastic pituitaries from both, hCGβ+ and Drd2ko (KO) females, when compared to their WT littermates (Fig. 3C and D, respectively). Moreover, decreased levels of pituitary *betaglycan* were also found in hCGβ+ males when compared to their WT littermates (Fig. 3C). Additionally, and as observed for *Inha* expression, mouse model-specific gender differences were also found, with higher *betaglycan* expression in hCGβ males, but, in contrast, lower *betaglycan* expression in Drd2 males when compared to WT females (Fig. 3C and D, respectively). These results suggest that activin antagonism is differentially exerted in hCGβ and Drd2 mice.

Even though pituitary inhibins (A and B) can act as an autocrine and/or paracrine factor on lactotroph cells, it is known that inhibins are primarily synthesized by the gonads and mostly antagonize pituitary activin functions in an endocrine manner. In this context, we next evaluated the gonadal mRNA expression of the three subunits (*Inhba*, *Inhbb*, and *Inha*) in both hCGβ and Drd2 female mice by qRT-PCR. As observed in Fig. 4, ovaries from hCGβ+ females showed a decreased mRNA expression of the three subunits when compared to their WT littermates (Fig. 4A, B and C).

In contrast, the gonadal mRNA expression of *Inhba*, *Inhbb*, and *Inha* was found increased in Drd2KO female mice ovaries (Fig. 4D, E and F) when compared to their WT littermates, suggesting that higher levels of gonadal inhibins may reach the pituitary by circulation in this model. This result, in addition to the elevated pituitary *Inha* expression found in KO female mice pituitaries (Fig. 3B), suggests an increased antagonism of pituitary activins by local and gonadal inhibins in KO female mice, concomitant with the development of lactotroph hyperplasia.

Pituitary FST288

Since FST288 is a functional and physiological antagonist of activin inhibitory function, we analyzed its local expression by semi-quantitative PCR (sq-PCR) and Western blot in pituitaries from female and male mice of both experimental models of prolactinoma. We found increased levels of FST288 (mRNA and protein) in hCGβ+ female mice pituitaries when compared to their WT littermates (Fig. 5A and B), suggesting an antagonism of activin inhibitory function by FST288 in this group concomitant with the development of lactotroph hyperplasia.

On the other hand, neither gender nor genotype alterations in pituitary FST288 mRNA expression were observed in the Drd2 mouse model (Fig. 5C).

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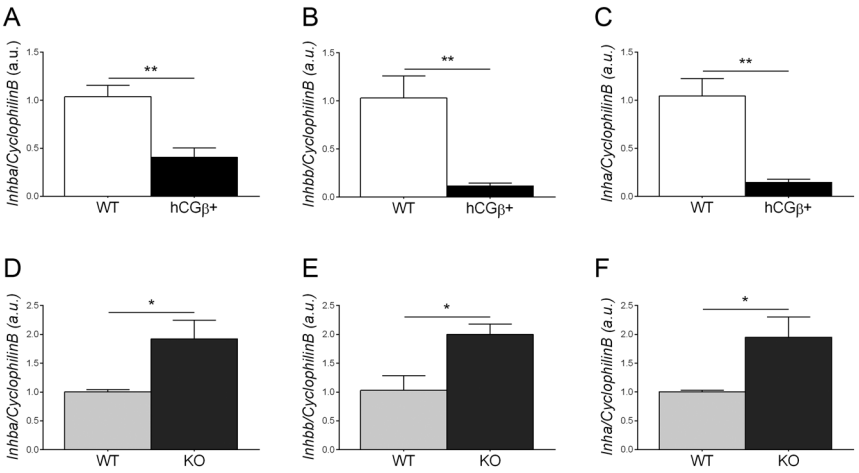

Figure 4
mRNA expression of *Inhba*, *Inhbb* and *Inha* in hCGβ+ and Drd2ko (KO) female mice ovaries. mRNA transcripts were amplified with specific primers by qRT-PCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females. Data were analyzed by Student's *t* test and expressed as mean ± S.E.M. (n = 6/group). (A, B and C) mRNA expression in 6-month-old hCGβ female mice ovaries; (A) *Inhba* (**P = 0.01); (B) *Inhbb* (**P = 0.01); (C) *Inha* (**P = 0.01). (D and E) mRNA expression in 8-month-old Drd2 female mice ovaries; (D) *Inhba* (*P = 0.05); (E) *Inhbb* (*P = 0.05); (F) *Inha* (*P = 0.05).

Serum gonadotrophins levels in hCGβ and Drd2 mice

Due to the differences found among the two animal models of prolactinoma in the pituitary and ovary expression (Figs 3 and 4) and knowing that inhibin synthesis is closely related to gonadotrophins production (especially FSH), we measured serum FSH and LH levels in both mice. As expected, higher levels of serum FSH were found in males, compared with females, in both animal models (Fig. 6A and B, respectively). Mice overexpressing hCGβ subunit presented lower FSH levels when compared to WT littermates (Fig. 6A), as previously described (Rulli *et al.* 2002). In contrast, Drd2ko (KO) females presented increased FSH levels (Fig. 6B) compared to WT, with no genotype differences in males.

Serum LH levels were found decreased in male and female Drd2KO mice without gender differences (Fig. 6D). In the hCGβ mouse model, males presented higher serum

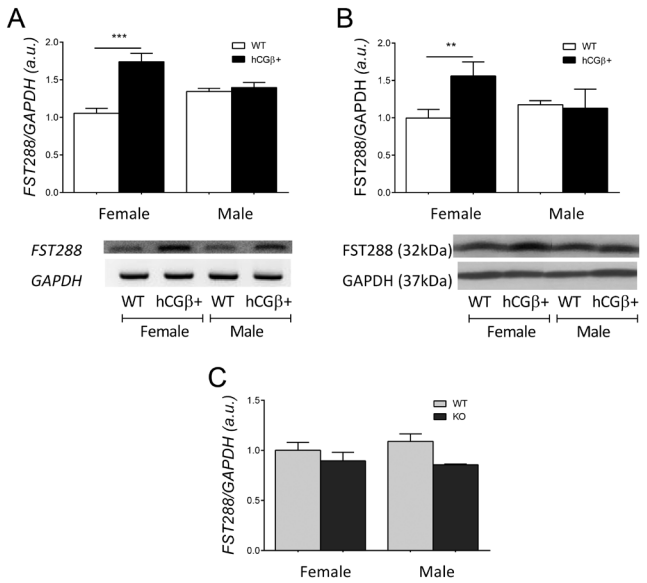

Figure 5
Pituitary FST288 expression in hCGβ (A and B) and Drd2 (C) mice. Data analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when the interaction effect was significant. Data are expressed as mean ± S.E.M. n = 6–8/group. (A and C) *FST* (62 pb) mRNA transcripts were amplified by sqPCR, normalized to *Gapdh* (171 pb) and quantified by densitometry. Results are expressed relative to those for WT females (n = 6/group). (A) hCGβ mice. Interaction P < 0.01. ***P < 0.001 hCGβ+ vs WT female. Representative immunoreactive bands are shown. (C) Drd2 mice. Interaction ns. No differences were found between groups. (B) Protein FST288 expression in hCGβ mice pituitaries. Interaction P < 0.05. **P < 0.01 hCGβ+ vs WT female. Representative immunoreactive bands are shown.

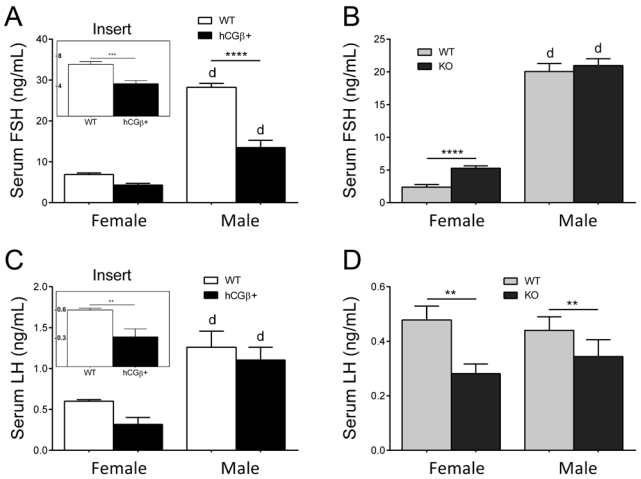

Figure 6
Serum gonadotrophin levels in hCGβ and Drd2 mice. (A and B) Serum FSH levels measured by RIA (ng/mL). (A) hCGβ mice. Interaction P < 0.0001. ^{d}P < 0.001 male vs female. ****P < 0.0001 hCGβ+ male vs WT male. Insert: ***P < 0.001 hCGβ+ female vs WT female (with data analyzed by Student's *t* test). (B) Drd2 mice. Interaction P = 0.0002. ****P < 0.0001 KO female vs WT female. ^{d}P < 0.0001 male vs female. (C and D) Serum LH levels measured by RIA (ng/mL). (C) hCGβ mice. Interaction ns. ^{d}P < 0.01 (sex). Insert: **P < 0.01 hCGβ+ female vs WT female (with data analyzed by Student's *t* test). (D) Drd2 mice Interaction ns. **P < 0.01 (genotype). (A, B, C and D) Data analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when interaction was significant. Data are expressed as mean ± S.E.M. n = 8–10/group.

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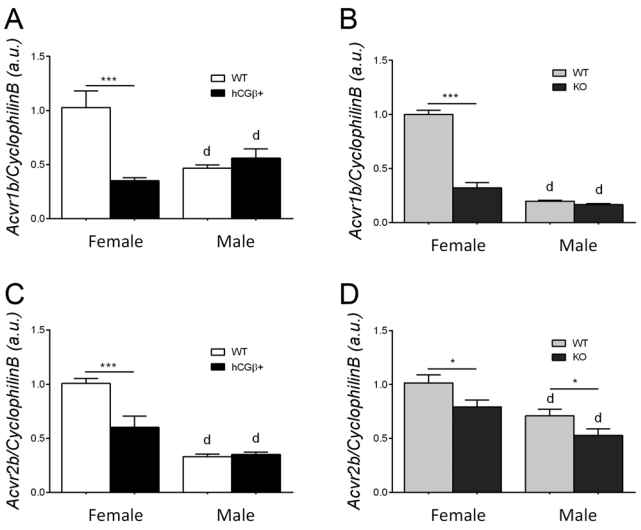

Figure 7
Pituitary mRNA expression of activin receptors in 6-month-old hCGβ and 8-month-old Drd2 mice. mRNA transcripts were amplified with specific primers by qRT-PCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females. Data were analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when the interaction effect was significant. Data are expressed as mean ± S.E.M. $n = 6–8$/group. (A and B) Activin type IB receptor mRNA expression (*Acvr1b*). (A) hCGβ mice. Interaction $P < 0.001$. $^{***}P < 0.001$ hCGβ+ vs WT female. $^{d}P < 0.05$ male vs female. (B) Drd2 mice. Interaction $P < 0.001$. $^{***}P < 0.001$ KO vs WT female. $^{d}P < 0.0001$ male vs female. (C and D) Activin type IIB receptor mRNA expression (*Acvr2b*). (C) hCGβ mice. Interaction $P < 0.05$. $^{***}P < 0.001$ hCGβ+ vs WT female. $^{d}P < 0.001$ male vs female. (D) Drd2 mice. Interaction ns. $^{*}P < 0.05$ (genotype). $^{d}P < 0.001$ (male vs female).

LH than females, without genotype differences. However, hCGβ+ females presented lower LH levels compared to their WT littermates (Fig. 6C).

These results suggest that, in females, pituitary and ovary inhibins expression follows FSH but not LH serum levels in each mouse model.

Pituitary mRNA expression of activin receptors in hCGβ and Drd2 mice

The activin inhibitory action on lactotroph function also depends on the expression of activin receptors. Then, we next analyzed the mRNA expression of activin receptors in pituitaries from hCGβ and Drd2 mice by qRT-PCR. As observed in Fig. 7, pituitary mRNA expression of activin type IB (*Acvr1b*) and type IIB (*Acvr2b*) receptors was found reduced in hyperplastic pituitaries from both mouse models (hCGβ+ and KO females) when compared to their WT littermates. Moreover, we also found a significant gender difference, as the expression of *Acvr1b* and *Acvr2b* was significantly lower in male pituitaries compared to females in both mouse models (Fig. 7A, B, C and D).

Activin type IB receptor (ActRIB) expression in lactotroph and gonadotroph cells of WT and hCGβ+ female mice

The expression of ActRIB in gonadotroph population is well known. However, this receptor was also described in lactotroph cell lines and human prolactinomas (Lacerte *et al.* 2004, de Guise *et al.* 2006). Then, we next assayed the ActRIB protein expression, specifically in the lactotroph population from normal and hyperplastic βhCG female pituitaries, through double immunostaining of PRL/ActRIB (Fig. 8A and B), using confocal laser scanning microscopy. Additionally, ActRIB expression in gonadotrophs was evaluated by double immunostaining of FSH/ActRIB (Fig. 8C and D).

As shown in Fig. 8, the ActRIB remains expressed normal as well as in hyperplastic lactotrophs (PRL+), and in gonadotroph population (FSH+) from WT as well as hCGβ+ pituitaries.

The morphometric analysis (Table 3) shows that, as expected, the lactotroph population significantly increased in hCGβ+ hyperplastic pituitaries (column A). The pituitary ActRIB expression was not significantly altered (column B). However, the proportion of lactotrophs ActRIB+/total lactotrophs (column C) was found decreased in hyperplastic pituitaries from hCGβ+ females (Fig. 9).

Inhibition of prolactin secretion by activin in an *ex vivo* assay

To evaluate whether the activin inhibitory action on prolactin secretion could be impaired in hyperplastic pituitaries due to the decreased ActRIB expression observed in this group, female WT and hCGβ+ pituitaries were incubated in serum-free medium containing human recombinant Activin A (Act A) or medium alone (control). Samples were taken at different times, and prolactin release was assayed by RIA. Prolactin release increased with time, in both groups (Table 4). As expected, hyperplastic tissues secreted higher levels of prolactin than WTs, and the increase in prolactin secretion was lower in tissues treated with activin A, as a result of its inhibitory action on lactotroph function. Then, activin A inhibited PRL secretion in tissues from both genotypes. To compare the degree of activin inhibitory action in each group, data was evaluated as fold change 45 min/15 min (Fig. 10). The two-way ANOVA showed a significant interaction ($P=0.0039$), indicating that the inhibitory effect was different in each group. In fact, de degree of inhibition

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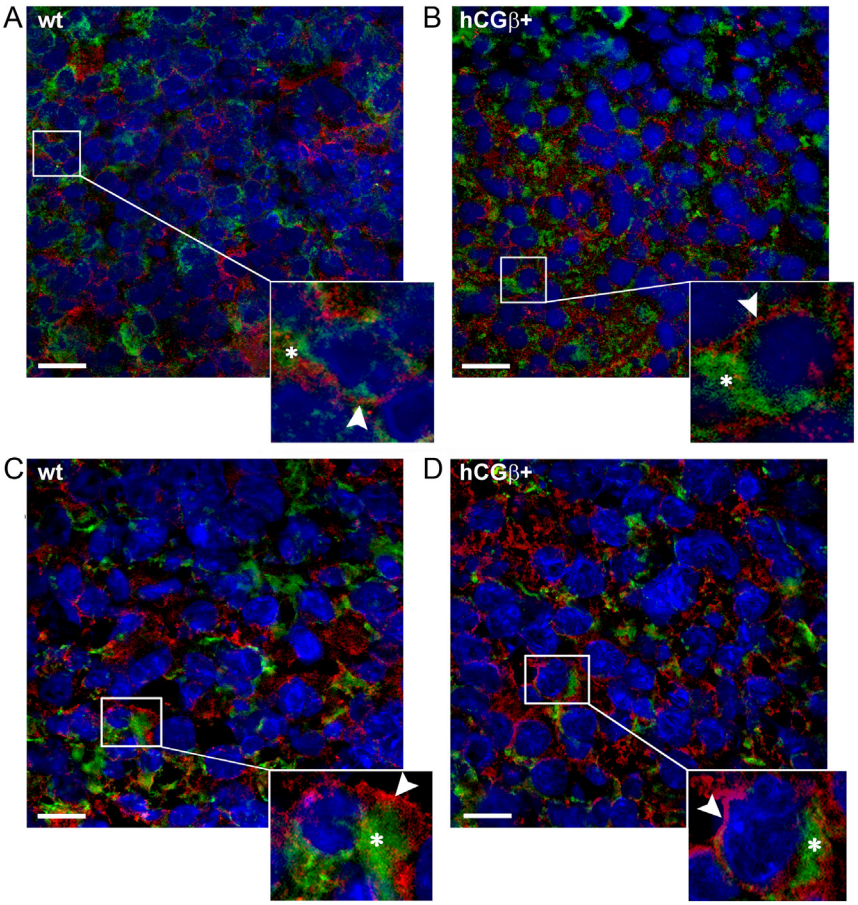

Figure 8
Activin type IB receptor expression in lactotrophs and gonadotrophs from hCGβ female mice pituitaries. (A and B) Identification of ActRIB (red, arrowhead) and prolactin (green, *) by double indirect immunofluorescence in WT (A) and hCGβ+ (B) female mice pituitaries. (C and D) Identification of ActRIB (red, arrowhead) and FSH (green, *) by double indirect immunofluorescence in WT (C) and hCGβ+ (D) female mice pituitaries. Nuclei were immunostained with DAPI (blue). Scale bar = 20 μm.

was about 53% in WT tissues while it was about 41% in hyperplastic pituitaries.

Signalling pathway involved

Although the pSmad2/3 pathway represents the canonical signalling used by activins as well as TGFβ1, activation of other signalling cascades have also been described (Engel *et al.* 1999, Derynck & Zhang 2003). In fact, activin-inhibition of lactotroph function involves the inhibition of *Pit-1* through the p38MAPK pathway in a Smad-independent manner (de Guise *et al.* 2006). Pit-1 (or POU class 1 homeobox 1, POU1F1) is a critical pituitary-specific transcription factor involved in the development of the pituitary gland and, specifically, in the differentiation and proliferation of lactotrophs, somatotrophs and thyrotrophs (Szeto *et al.* 1996).

When pituitary *Pit-1* expression was evaluated by real-time qPCR in the two mouse models, we observed, as expected, an increase of Pit-1 mRNA levels in hyperplastic pituitaries (hCGβ+ and KO females), compared with their WT littermates (Fig. 11A and B), in accordance with increased proliferation in lactotroph population, while no genotype difference in *Pit-1* expression was observed in males, despite genotype.

Then, we next assayed phospho-p38MAPK (p-p38) expression, specifically in lactotroph population (PRL+ cells), by double immunostaining using confocal laser scanning microscopy, in hCGβ female pituitaries. As observed in Fig. 12, upper panel, almost all PRL+ cells express p-p38 in female WT-pituitaries suggesting a marked activin inhibition in this group. However, few lactotrophs express p-p38 in hyperplastic pituitaries from

Table 3 Morphometric analysis: double immunostaining of ActRIB protein expression in lactotroph population (PRL+) from hCGβ female mice.

	A	B	C
	% Lactotrophs (PRL+)	% cells ActRIB+	% Lactotrophs ActRIB+/Total lactotrophs
WT	29.03 ± 2.13	33.99 ± 3.65	52.91 ± 1.62
hCGβ+	45.93 ± 5.04 (P = 0.03)	40.27 ± 4.06 (P = 0.22)	39.09 ± 2.0 (P = 0.005)

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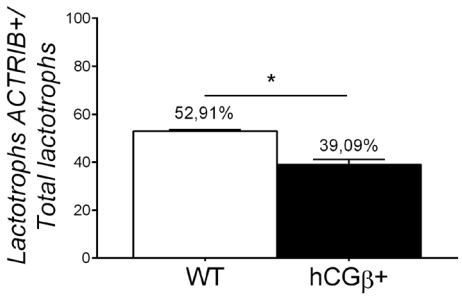

Figure 9
Percentage of lactotrophs expressing ActRIB/ total lactotrophs. A total of 5000 pituitary cells were examined in randomly chosen fields of each glass slide and quantified in order to establish the percentage of lactotroph (PRL+) immunoreactive for ActRIB in female WT and hCGβ+ pituitaries. Data analyzed by chi-square test, $n = 3$, $^*P = 0.05$, $P < 0.001$.

hCGβ+ female (lower panel), and this is concomitant with decreased activin expression and increased *Pit-1* expression, suggesting that a lower activin-inhibitory action could be involved in lactotroph hyperplasia development in this group.

Discussion

Although activins are known to induce FSH production from gonadotroph population, they were described as negative regulators of lactotroph function: cell proliferation and PRL production (Lacerte *et al.* 2004, Bilezikjian *et al.* 2012). In this work, we studied the pituitary activin-inhibin system in two animal models of lactotroph hyperplasia: hCGβ and Drd2 mice. The expression of activin βA and βB subunits was found significantly decreased in pituitaries from hCGβ+ and KO females, concomitant with the development of lactotroph hyperplasia. In contrast, higher mRNA expression of activin subunits was observed in male pituitaries, which would suggest increased bioavailability of activins, and thus, stronger activin inhibitory function on lactotroph population, protecting this sex from hyperplasia development in both mouse models (Kelly *et al.* 1997, Rulli *et al.* 2002). Additionally, a reduced proportion of lactotrophs expressing ActBIR was observed in hCGβ+ female pituitaries compared to WTs. In accordance, a weaker inhibitory action of activin on PRL secretion was found in hyperplastic pituitaries compared to that observed in WTs. All these results suggest that reduced activin inhibitory action could be involved in the development of lactotroph hyperplasia in females from both animal models.

Disruptions on activins inhibitory function have been previously proposed to be related to the development of endocrine and neuroendocrine tumours (Risbridger *et al.* 2001, Bilezikjian *et al.* 2004, Namwanje & Brown 2016). The decreased expression of *Inhba* and *Inhbb* found in pituitaries from hCGβ+ and Drd2KO females suggests a reduced bioavailability of activins in these groups, and this fact could be related to lactotroph hyperplasia development.

On the other hand, an essential role in the regulation of local activin function is given by its antagonists. Inhibins and follistatins antagonize activin function in the pituitary by preventing activin binding to its receptors (Suszko & Woodruff 2006, Olsen *et al.* 2015). Our present results show that, in addition to the decreased activin expression found in both models of lactotroph hyperplasia, an increased activin antagonism is also observed. Interestingly, this antagonism was found to be model specific.

In hCGβ+ female pituitaries, the primary activin-antagonism seems to be given by FST, as FST288 expression, mRNA and protein, were found increased compared to WTs, while no genotype difference was observed in males. However, decreased inhibin expression was observed in both the pituitary and the ovary, in this model.

The increase of FST expression observed in hCGβ+ females could be due to the decreased levels of ovarian inhibins found in this group, as was described that inhibins inhibit pituitary FST mRNA levels (Bilezikjian *et al.* 1996, Dalkin *et al.* 1998).

Table 4 Activin inhibition of prolactin secretion in an *ex vivo* assay.

Time (minutes)	**WT**		**hCGβ+**	
	Control	Act-A	Control	Act-A
15	221.12 ± 21.13	277.06 ± 35.34	520.81 ± 18.09	612.77 ± 1.48
30	559.19 ± 76.34	383.80 ± 92.86	1014.91 ± 3.88	724.32 ± 42.41
45	1142.08 ± 113.36	681.16 ± 124.65	1950.12 ± 22.57	1350.41 ± 114.98
Fold increase	5.16 ± 0.01	2.45 ± 0.08	3.75 ± 0.05	2.20 ± 0.11

Pituitaries from 6-month=old WT and hCGβ+ female mice were incubated in serum free medium (Control group) or medium containing with 20 ng/mL human recombinant Activin A (Act-A group). Medium samples were taken at 15, 30, and 45 min. Prolactin levels released at the medium were assayed by RIA. Data show secreted PRL (ng/mL/mg protein). Fold increase: Released PRL at 45 min/released PRL at 15 min) in each group.

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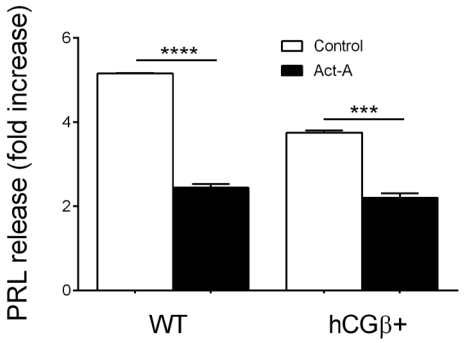

Figure 10
Inhibition of prolactin secretion by activin in an *ex vivo* assay. Fold increase in PRL release (45 min/15 min, data from Table 4) in each group. Data analyzed by two-way ANOVA (genotype × treatment), followed by a Šídák *post hoc* analysis. Interaction $P = 0.0039$. ****$P < 0.0001$ WT control vs WT Act-A; ***$P = 0.0007$ hCGβ+ control vs hCGβ+ Act-A. Data are expressed as mean ± S.E.M. $n = 3$/group.

In contrast, increased levels of pituitary and ovarian *Inha* were found in Drd2KO females compared to WTs, suggesting that both local and gonadal inhibins mediate activin antagonism in this mouse model.

The differences observed in *Inha* expression among sexes and genotypes in both models, and in particular, among hCGβ and Drd2 female mice, could be related to the differences found in serum FSH levels, as was previously demonstrated (Woodruff *et al.* 1996).

Due to the high hCG circulating levels (Rulli *et al.* 2002, Ratner *et al.* 2012), hCGβ+ females present high levels of estradiol, testosterone, and progesterone during their sexual development. From 2 months of age serum prolactin levels increase and females present lactotroph hyperplasia followed by the development of prolactinomas, probably due to the over-exposure to estrogens during peripuberty. At 6 months, hCGβ+-female

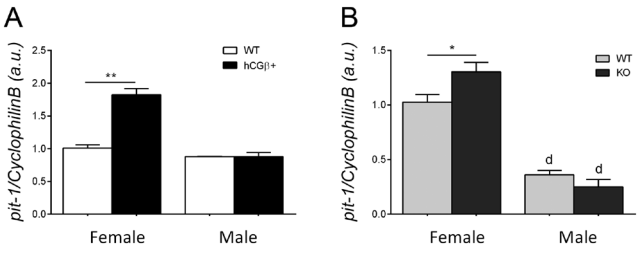

Figure 11
Pituitary mRNA expression of *Pit-1* in 6-month-old hCGβ and 8-month-old Drd2 mice. mRNA transcripts were amplified with specific primers by qRT-PCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females. Data were analyzed by two-way ANOVA (sex × genotype), followed by a Tukey's *post hoc* analysis when the interaction effect was significant. Data are expressed as mean ± S.E.M. $n = 6$–8/group. (A) hCGβ mice. Interaction $P = 0.0224$. **$P < 0.01$ hCGβ+ vs WT female. (B) Drd2 mice. Interaction $P = 0.0463$. *$P < 0.05$ KO vs WT female. $^{d}P < 0.01$ male vs female.

ovaries present massive luteinization with persistently elevated levels of androgens. This hormonal environment induces a significant decrease in GnRH as well as FSH secretion (Rulli & Huhtaniemi 2005), with concomitant decrease in ovarian and pituitary inhibin production (McLachlan *et al.* 1989, Smith *et al.* 1991, Shayya *et al.* 2014). In the same way, the reduced *Inha* expression found in hCGβ+ male mice pituitaries could be explained by the decreased FSH production and the abnormal development of reproductive organs characteristic of this genotype (Rulli *et al.* 2003, Gonzalez *et al.* 2011) as previously proposed by Dias *et al.* (2009).

In contrast, serum FSH levels remain higher in female mice lacking Drd2 receptors compared to WTs. This fact could be related to the hypoestrogenism presented in Drd2KO females compared to WTs (Saiardi *et al.* 1997). The higher FSH levels could explain the increased expression of pituitary and gonadal inhibins in this animal model.

On the other hand, the lower serum LH levels observed in both, hCGβ+ and Drd2KO females, could be related to the hyperprolactinemia observed in these groups that induces a significant decrease in GnRH secretion that impacts on LH levels (Rulli & Huhtaniemi 2005).

Inhibins antagonize activin signalling by competitive binding to ActRII receptors (Vale *et al.* 2004). However, inhibins binding to ActRII shows 10-fold lower affinity than activins. Then, it was demonstrated that this antagonism requires the presence of a co-receptor betaglycan (also known as TGFβ type III receptor) which markedly increases the affinity of inhibins for ActRII (Lewis *et al.* 2000, Bernard *et al.* 2002).

When the pituitary *betaglycan* expression was explored, in our mouse models, it was found reduced in hCGβ+ and KO female mice pituitaries, despite the levels of *Inha* observed in each model. This fact could limit the inhibin antagonism in the hyperplastic pituitaries. However, although betaglycan is expressed in almost all anterior pituitary cell types, the degree of expression is cell-type specific. While 97% of FSH-positive gonadotrophs are immune positive for betaglycan, only 23% of lactotrophs are positive cells (MacConell *et al.* 2002). This finding suggests that betaglycan is mainly involved in the control of gonadotroph population. Nevertheless, further studies are needed to assess the impact of this reduced betaglycan expression on activin antagonism, not only in gonadotrophs but also in lactotrophs.

Other components involved in the regulation of activin function are the activin membrane receptors and its intracellular effectors (Suszko & Woodruff 2006).

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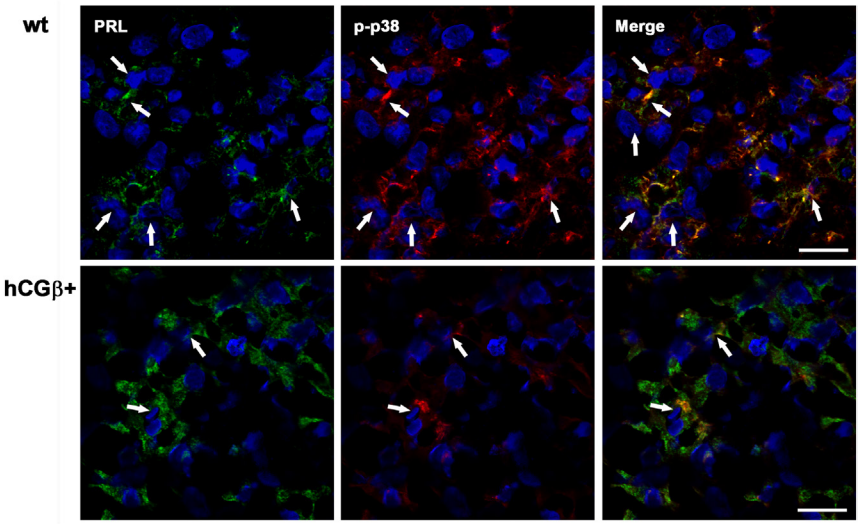

Figure 12
Phospho-p38MAPK (p-p38) expression in lactotrophs from hCGβ female mice pituitaries. Upper panel: wild type pituitary; Lower panel: hCGβ+ mice pituitary. White arrows show lactotrophs (PRL+) expressing p-p38. Nuclei were immunostained with DAPI. Scale bar = 20 μm.

Activin receptor type IB (ActRIB or ALK4) has been involved in the inhibition of PRL synthesis and secretion (Lebrun *et al.* 1999, Miyamoto *et al.* 1999, Olsen *et al.* 2015).

In the present work, we found reduced ActRIB and ActRII mRNA expression in hCGβ+ and Drd2KO female pituitaries (lactotroph hyperplasia) compared to WT counterparts. Additionally, we demonstrated, by double immunostaining, that ActRIB expression is present not only in gonadotrophs but also in lactotroph population in our experimental models, and that ActRIB remains expressed in lactotrophs from tumoural pituitaries. However, hyperplastic pituitaries (hCGβ+ female) present a lower proportion of lactotrophs expressing ActRIB/total lactotrophs compared to WTs. Moreover, in an *ex vivo* assay, we demonstrated that the inhibitory activin action on prolactin secretion is reduced in hyperplastic pituitaries compared to WT tissues.

Finally, and regarding the signalling involved, although the phosphorylation of SMAD2/3 proteins represent the canonical pathway, other intracellular effectors were also described to mediate activin actions, in a cell- and tissue-specific manner (Derynck & Zhang 2003). In fact, in lactotrophs, activins inhibit prolactin gene expression through repression of the transcription factor Pit-1 (Lacerte *et al.* 2004) involving the p38MAPK pathway, in a Smad-independent manner (de Guise *et al.* 2006). Our present results show that the strong expression of p-p38 observed in lactotroph population from female WT pituitaries is significantly reduced in tumoural pituitaries concomitant with the decrease in the inhibitory activin action, the increase in Pit-1 expression and the lactotroph hyperplasia development.

Summarizing, the present results suggest that decreased pituitary activin function is involved in the lactotroph hyperplasia development. Moreover, our work also suggests that the gender differences observed in the pituitary activin-inhibin system, in the two mice models studied, could be related to the gender differences in the appearance of prolactinomas.

Prolactinomas are the pituitary tumours with higher prevalence in humans. Even though, they generally respond well to the current therapy with dopamine agonists, a subset of patients exhibit resistance to dopaminergic drugs. Then, alternative therapies are desired.

The present work highlights the importance of the activin inhibitory action on lactotroph function and places the local activin system as new targets in the treatment of dopamine agonist resistant prolactinomas.

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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