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Elevated hypothalamic aromatization at the onset of precocious puberty in transgenic female mice hypersecreting human chorionic gonadotropin: Effect of androgens

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ABSTRACT

Transgenic female mice overexpressing the α- and β- subunits of human chorionic gonadotropin (hCGαβ+) exhibited precocious puberty, as evidenced by early vaginal opening. Chronically elevated hCG in 21-day-old hCGαβ+ females stimulated gonadal androgen production, which exerted negative feedback over the endogenous gonadotropin synthesis, and activated the hypothalamic GnRH pulsatility and gene expression. Transgenic females also exhibited elevated hypothalamic aromatization in the preoptic area (POA), which is the sexually-differentiated area that controls the LH surge in adulthood. Ovariectomy at 14 days of age was unable to rescue this phenotype. However, the blockade of androgen action by flutamide from postnatal day 6 onwards reduced the aromatase levels in the POA of hCGαβ+ females. Our results suggest that early exposure of females to androgen action during a critical period between postnatal days 6–14 induces sex-specific organizational changes of the brain, which affect the aromatase expression in the POA at the onset of precocious puberty.

1. Introduction

In mammals, including humans, puberty is defined as the beginning of reproductive competence, and it is driven by the (re)awakening of the hypothalamic–pituitary–gonadal (HPG) axis, after its first active phase in the fetal-neonatal period, that culminates in the activation of gonadal function. At puberty onset the hypothalamus becomes activated and accelerates the pulsatile release of gonadotropin releasing hormone (GnRH), which impacts on the pituitary by stimulating the production of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Pituitary gonadotropins promote gonadal steroid production and gametogenesis (Ojeda and Urbanski, 1994), and gonadal steroids and inhibins participate in the endocrine regulation of the female HPG axis, by exerting feedback mechanisms at the pituitary and/or the hypothalamus (Bilezikjian et al., 2006).

The hypothalamus contains two anatomically distinct areas responsive to the feedback action of gonadal steroids: the arcuate nucleus (ARC), localized in the mediobasal hypothalamus (MBH), and the anteroventral periventricular nucleus (AVPV), localized in the preoptic area (POA) of the anterior hypothalamus (AH). Many lines of evidence indicate that the ARC contains the neuronal substrate mediating the negative feedback of gonadal steroids over the reproductive axis in both sexes, whereas in the AVPV resides a sexually-differentiated neuronal substrate responsive to the positive estradiol feedback that triggers the preovulatory GnRH-LH surge only in females (Kauffman, 2010; Roa et al., 2008).

Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; hCGαβ+, transgenic mice over-expressing hCGα and hCGβ subunits; HPG, hypothalamic–pituitary–gonadal; MBH, mediobasal hypothalamus; Ovx, ovariectomy; POA-AH, preoptic area-anterior hypothalamus; (C)PP, (central) precocious puberty; PPP, peripheral precocious puberty; TG, transgenic; WT, wild-type.

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The population of GnRH neurons serves as the final common pathway through which the brain regulates the secretion of gonadotropins from the pituitary (Herbison, 2005). During the prepubertal period, the GnRH neurons are highly sensitive to negative feedback, which permits only a slow frequency of GnRH release, but at puberty onset the sensitivity to the negative feedback decreases and the GnRH pulse frequency accelerates. Although much attention has been paid to the factors responsible for the onset of puberty, still the primary mechanism involved in GnRH neuron activation remains unclear, with key roles being proposed for specific neuronal inputs releasing amino acids and neuropeptides (Terasawa and Fernandez, 2001), as well as neuronal–glial interactions (Ojeda et al., 2008). The discovery of kisspeptin, a peptide encoded by the *Kiss1* gene that acts through the GPR54 receptor (KISS1R), revolutionized the knowledge of the neuroendocrine mechanisms that control GnRH release. Kisspeptin is a potent activator of GnRH neurons and essential for puberty onset in several species (Han et al., 2005; Tena-Sempere, 2010). Kisspeptin neurons localize in both the ARC and the AVPV, and they have been implicated in the control of both tonic and preovulatory GnRH discharge by gonadal steroids (Herbison, 2008; Kauffman, 2010). A normal regulation of the negative feedback by sex steroids is essential for the correct functioning of the reproductive neuroendocrine axis. The mechanisms by which androgens alter the female neuroendocrine function is not fully understood, but it has been shown that exposure of females to supraphysiological levels of androgens during critical periods of development may increase the risk of reproductive disorders, such as PP and polycystic ovarian syndrome (Abbott et al., 2005; Witham et al., 2012).

Precocious puberty (PP), defined in humans by the appearance of the secondary sexual characteristics before the age of 8 years in girls and 9 years in boys, is one of the most common endocrine disorders in children, being 10–20 times more common in girls than in boys (Teilmann et al., 2005). There are two general categories of this condition: *Central* PP (CPP, or gonadotropin-dependent PP), characterized by an early activation of the pulsatile release of hypothalamic GnRH that increases gonadotropin secretion, and *Peripheral* PP (PPP), caused by autonomous secretion of sex steroids from the gonads or adrenal glands (Parent et al., 2003; Stephen et al., 2011; Teilmann et al., 2005). CPP represents four fifths of the total number of patients with PP and it is responsive to the treatment with long-acting GnRH agonist analogs, which suppress the pulsatile secretion of LH and FSH. Although CPP is characterized by increased gonadotropin secretion, particularly of LH, this condition needs to be differentiated from other forms of PP in which the LH/hCG receptor (LHCGR) is activated independently from hypothalamic–pituitary signals, and it stimulates gonadal androgen production, such as in activating LHCGR mutations or human chorionic gonadotropin (hCG)-secreting tumors (Carel et al., 2004; Kitanaka et al., 1994; Latronico et al., 2000; Meehan et al., 2005; Starzyk et al., 2001). On this respect, the study of hCG-dependent PP in animal models may therefore lead to a better understanding of the HPG axis regulation in conditions of hormonal disruption.

In the present study, the etiology of hCG-dependent PP was further investigated, by using a transgenic (TG) mouse model with chronic hypersecretion of hCG. These mice present with elevated sex steroids and advanced pubertal onset, evidenced by the age of vaginal opening about 6 days before wild-type (WT) animals. Mice were analyzed at 21 days of age, when hCGαβ+ females developed PP, and WT littermates still remained at the prepubertal stage. In addition, we used immature hCGαβ+ females subjected to ovariectomy or treated with the antiandrogen flutamide to investigate the influence of gonadal signals prior to the puberty onset over the transgenic HPG axis phenotype.

2. Materials and methods

2.1. Animals

All studies were performed in double TG female mice carrying both α and β subunits of hCG (hCGαβ+ mice), under the control of ubiquitin C promoter (Gonzalez et al., 2011; Rulli et al., 2003). Production, breeding and genotyping of the hCGαβ+ mice have been described previously (Rulli et al., 2002, 2003). hCGαβ+ mice were created on a FVB/n genetic background and WT females were used as controls. Mice were maintained under controlled conditions (12 h light/dark cycle, 22–23 °C), and were given free access to laboratory chow and tap water. All experimental procedures were in compliance with the NIH guidelines for care and use of experimental animals, and 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). Cardiac blood was obtained immediately after the mice were sacrificed with CO_2 asphyxiation, and serum samples were separated by centrifugation and stored at −20 °C until hormone measurements. Pituitaries and ovaries were collected, snap frozen and stored at −70 °C for hormone measurement or RNA isolation. The hypothalami were dissected out into the POA-AH and the MBH, snap frozen and stored at −70 °C for further analyses.

2.2. Animal treatments

Ovx: Fourteen-day-old WT and hCGαβ+ female mice were anesthetized with a ketamine:xilacin solution (60:10 mg/kg body weight) and ovaries were removed through a lateral incision. Mice were maintained for 7 days following castration and euthanized at 21 days of age. Sham-operated WT and hCGαβ+ females were used as controls.

Antiandrogen treatment: Six-day-old hCGαβ+ females were implanted s.c. with 20-mg flutamide pellets (Schering Canada Ltd., Quebec, Canada). Mice were sacrificed at 21 days of age. Sham-operated hCGαβ+ females were used as controls.

2.3. Hormone measurements

FSH concentration in serum was measured by double antibody radioimmunoassay (RIA), according to the method described previously (Ratner et al., 2012). The results were presented in terms of the rat-FSH-RP-2 standard supplied by the NIDDK (Bethesda, MD, USA). The sensitivity of the assay was 0.24 ng/tube, and intra- and inter-assay coefficients of variation were 7% and 11%, respectively. GnRH RIA was performed as described by Mongiat et al. (2006); the sensitivity of the assay was 1.5 pg/tube, and intra- and inter-assay coefficients of variation were 7% and 12%, respectively. The intraovarian testosterone was determined by homogenizing the ovaries in 200 μl PBS. The homogenates and serum samples were extracted twice with 2 mL diethyl ether and evaporated to dryness. After reconstitution into PBS, testosterone was measured by conventional RIA (Ratner et al., 2012). Intra- and inter-assay coefficients of variation were less than 12%. Serum estradiol was determined with the Ultra-sensitive Estradiol RIA Kit DSL-4800 (Diagnostic Systems Laboratories).

2.4. Neuronal cultures and treatments

Female fetal donors were obtained from pregnant mice at embryonic day 16 and identified by the absence of the spermatic artery on the developing gonad. Under a dissecting microscope, the hypothalamic region (delimited by the optic chiasm, the lateral

hypothalamic sulcus and the mammillary bodies) was dissected out and stripped off the meninges. Culture conditions of hypothalamic neurons were prepared as described (Cambiasso et al., 1995, 2000; Gorosito and Cambiasso, 2008). The medium was Neurobasal without phenol red supplemented with B-27 and GlutaMAX I (Invitrogen). The cultures were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO2. After seeding the neurons were treated with 6.5 IU/ml recombinant hCG (Ovidrel, Merck-Serono Co.) or vehicle for 48 h. The cells were collected and RNA isolated for gene expression assays, as indicated below.

2.5. Western blot

POA-AH homogenates were prepared into Ripa's buffer (150 mM NaCl, 0.1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) with protease inhibitor cocktai1 (1:500; P8340, Sigma–Aldrich Chemical Co.). The homogenates were combined with an equal volume of Laemmli's buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 125 mM Tris, pH 6.8) and boiled at 100 °C for 5 min. Protein samples (50 μg/lane) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Argentina). Immunoblottings were performed using a rabbit polyclonal anti-aromatase antiserum (1:100; Abcam, Cambridge, UK; no. ab18995) and a mouse monoclonal anti-α-tubulin antibody (1:3000; T9026, Sigma Aldrich). Secondary antibodies conjugated to near-infrared fluorophores were used to visualize signals in a two-channel infrared scanner (Odyssey®). The resulting images were quantified with the software Scion Image (NIH).

2.6. RNA isolation and gene expression assays

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of RNA were treated with DNAseI (Invitrogen) and reverse-transcribed in a 20 μL reaction using M-MLV reverse transcriptase (Promega) and random hexameres (Biodynamics). For quantitative real-time RT-PCR (qRT-PCR) primers sets were designed for the specific amplification of murine *Fshb*, *Lhb*, *Cga*, *Lhcgr*, *Gnrhr*, *Gnrh*, *Esr1*, *Pr*, *Cyp19a1* and *Gapdh* was used as housekeeping control gene (Supplementary material, Table 1). Each sample was assayed in duplicate using 4 pmol of each primer, 1X SYBR Green Master Mix (Applied Biosystems) and 2–20 ng of cDNA in a total volume of 13 μL. Amplification was carried out in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). For the assessment of quantitative differences in the cDNA target between samples the mathematical model of Pfaffl (2001) was applied. An expression ratio was determined for each sample by calculating $(E_{\text{target}})^{\Delta Ct(\text{target})}/(E_{\text{GAPDH}})^{\Delta Ct(\text{GAPDH})}$, where E is the efficiency of the primer set and $\Delta Ct = Ct_{(\text{referencecDNA})} - Ct_{(\text{experimental cDNA})}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction vs. Ct value ($E = 10^{-(1/\text{slope})}$). Efficiencies of 2 ± 0.1 were considered optimal. Results were expressed relative to a reference sample (a WT female chosen *ad random*). Since *Fst* and *Kiss1* exhibited Ct values reaching the detection limit of the qRT-PCR assay, a semi-quantitative (sq) RT-PCR following a protocol of 40 cycles was used. Agarose gels were digitalized and the density of bands was measured with the software Scion Image (NIH). The sqPCR results were expressed relative to *Gapdh*.

2.7. Ex vivo GnRH pulsatility assays

We performed pulsatility studies *ex vivo* as described previously (Catalano et al., 2010; Gonzalez et al., 2011). Briefly, total hypothalami, including the POA, were collected and incubated in 1.5 mL microfuge tubes containing 250 μL Krebs-Ringer bicarbonate buffer with 4.5 mg/mL glucose and 16 mM HEPES at 37 °C for 6 h. After 30 min pre incubation, the medium from each flask was renewed at 9 min intervals, and medium was stored at −20 °C until GnRH measurement by RIA. GnRH pulses were identified and their parameters defined using Cluster8 computer algorithm analysis developed by Veldhuis and Johnson (1986) (obtained from M.L. Johnson, University of Virginia; http://mljohnson.pharm.virginia.edu/home.html). Cluster8 compares clusters of points by pooled *t*-testing to look for nadirs and peaks in time series data. Using peak and nadir clusters of one and two points, respectively, peaks were identified and considered as GnRH pulses, peak width as pulse width, and the area under the peak as the mass of the pulse (in arbitrary units). The pulse areas obtained during the experiment were defined as total mass released. The experiment was repeated four times, including one animal from each group per experiment.

2.8. Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis for comparing two sets of data was performed with Student's t test for two independent groups. For the comparison of three or more sets of data, the one-way ANOVA was applied. In those experiments where the effects of two factors (genotype and age, or genotype and treatment) were studied, the two-way ANOVA was performed. In both cases Fisher's least significance difference (LSD) post-hoc test was used to establish the level of significance between group pairs. Data were transformed when required. Statistics were done with the software InfoStat 2008 (available in www.infostat.com.ar). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Organ weight and hormonal profiles of WT and hCGαβ+ females

The 21-day-old hCGαβ+ females exhibited increased body, ovary and uterus weights, as compared with the age-matched WT controls (Table 1). As previously seen in the hCGβ+ TG females (Rulli et al., 2002), hCGαβ+ exhibited vaginal opening already at 20 ± 1 days of age, accompanied by cornified cells in the vaginal smears, which is indicative of estrogen action; WT littermates attained the vaginal opening and puberty onset at 26 ± 1 days of age. The serum levels of testosterone were significantly elevated in hCGαβ+ females (Table 1). The serum concentration of FSH and estradiol, as well as the intraovarian testosterone levels, were evaluated in 14-, 21- and 28-day-old WT and hCGαβ+ females (Fig. 1). The ages were chosen in order to evaluate the hormonal status before, during and after the onset of puberty of TG females. The serum FSH profile of TG females (Fig. 1A) showed a significant

Table 1
Body and reproductive organs weight, vaginal opening and serum testosterone of 21-day-old WT and hCGαβ+ females.

	WT	hCGαβ+	hCGαβ+Ovx
Body weight (g)	11.7 ± 0.2a	13.1 ± 0.4b	12.3 ± 0.3a
Uterus weight (mg)	13.8 ± 1.3a	71.4 ± 2.8b	15.6 ± 2.4a
Ovary weight (mg)	1.72 ± 0.2	3.76 ± 0.28***	ND
Pituitary weight (mg)	0.97 ± 0.13	1.24 ± 0.05	ND
Testosterone (pmol/ml)	0.15 ± 0.01	0.63 ± 0.08**	ND
Vaginal opening	No	Yes	No

Data are presented as the mean ± SEM. Asterisks: Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$, $N = 4$–6. Letters: one way ANOVA-Fisher's LSD, $N = 4$, different letters indicate statistical differences between groups ($p < 0.05$). ND: not determined.

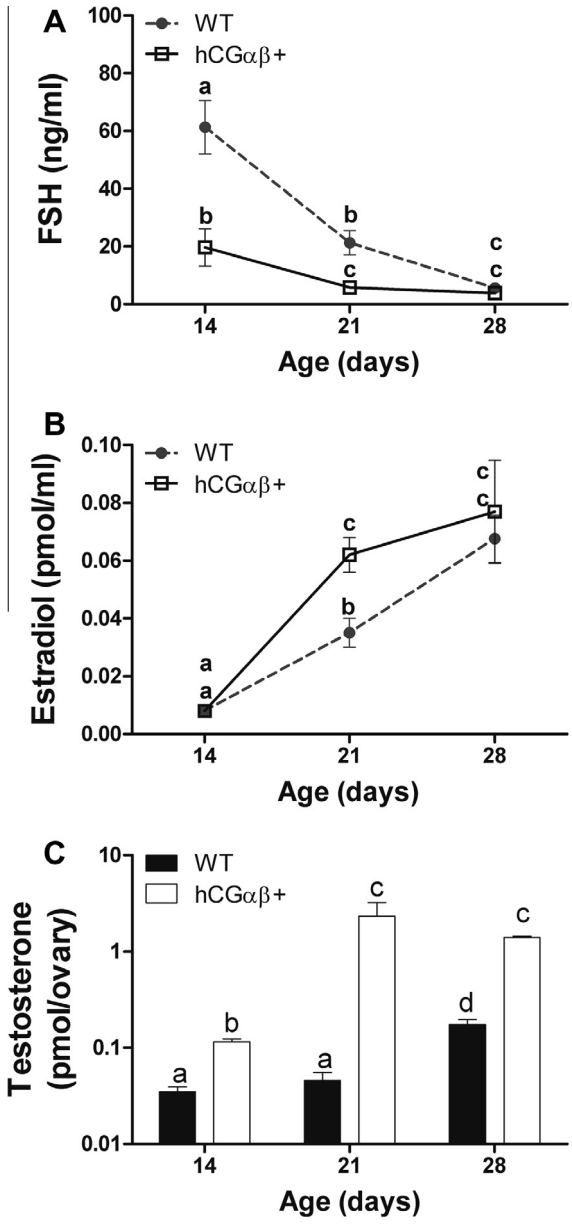

Fig. 1. *Hormonal profile of WT and hCGαβ+ females.* (A) Serum FSH levels, (B) serum estradiol levels, and (C) intraovarian testosterone levels. Two way ANOVA-Fisher's LSD for FSH and testosterone demonstrated significant interactive, genotype and age effects ($p < 0.05$), and for estradiol significant interactive and age effects ($p < 0.001$). $N = 5–7$. Different letters indicate statistical differences between groups (at least $p < 0.05$).

decrease at both 14 and 21 days of age ($p < 0.01$), which were normalized at 28 days of age, as compared with age-matched WT controls. Similar to hCGβ+ females (Rulli et al., 2002), estradiol levels were transiently elevated at 21 days in the hCGαβ+ females (Fig. 1B, $p < 0.05$), but similar to WT controls on days 14 and 28 of age. The intraovarian testosterone levels were highly (up to 100-fold) elevated in the hCGαβ+ females at all ages analyzed, as compared with age-matched WT controls (Fig. 1C, $p < 0.0001$).

3.2. Effect of Ovx on FSH levels and pituitary gene expression in 21-day-old WT and hCGαβ+ females

To analyze the influence of gonadal signals on the TG phenotype, WT and hCGαβ+ females were subjected to Ovx at postnatal day 14, being sacrificed at 21 days of age, and serum FSH and pituitary gene expression were analyzed (Fig. 2). As was already demonstrated in Fig. 1A, the hCGαβ+ females showed a decrease in serum FSH ($p < 0.0001$) as compared to WT controls. The removal of ovarian inputs by Ovx increased serum FSH in WT and hCGαβ+ females to the same level ($p < 0.05$). Ovx in TG females also prevented the premature vaginal opening at 21 days of age, and their body and uterine weights were normalized (Table 1). The gene expression of the gonadotropin subunits *Fshb*, *Lhb*, and the common *Cga* were also studied in all experimental groups at 21 days of age. The same pattern observed in serum FSH was found in *Fshb* expression, where the hCGαβ+ females showed a decrease in *Fshb* mRNA levels as compared with WT ($p < 0.001$), and Ovx increased *Fshb* expression in both the WT and hCGαβ+ females to similar levels ($p < 0.0001$). *Lhb* expression was also decreased in the hCGαβ+ females as compared with the WT controls ($p < 0.0001$), indicating that the endogenous LH production was also compromised in TG mice. Interestingly, although Ovx was able to elevate *Lhb* expression in both genotypes ($p < 0.01$), the hCGαβ+ females showed lesser increase after Ovx, only reaching levels comparable to WT females. *Cga* expression showed no differences between genotypes, and Ovx was able to increase its expression to the same degree in both the WT and hCGαβ+ females ($p < 0.05$). The expression of key genes involved in gonadotropin regulation, i.e. GnRH receptor (*Gnrhr*) and follistatin (*Fst*) was also analyzed. *Gnrhr* was found diminished in WTOvx, hCGαβ+ and hCGαβ+ Ovx pituitaries as compared with WT ($p < 0.01$). *Fst* showed no differences between genotypes, but Ovx was able to significantly increase its expression in WT and hCGαβ+ females ($p < 0.01$).

3.3. Effect of Ovx on the hypothalamic ex vivo GnRH pulsatility in 21-day-old WT and hCGαβ+ females

To evaluate the hypothalamic status in TG females, *ex vivo* studies were conducted on WT and hCGαβ+ hypothalami in order to analyze the pulsatility of GnRH release in this TG model (Fig. 3). Representative GnRH pulsatility patterns in 21-day-old WT, WTOvx, hCGαβ+ and hCGαβ+ Ovx females are shown in Fig. 3A. An increase in pulse frequency was observed in WTOvx, hCGαβ+ and hCGαβ+ Ovx females, as compared with the WT controls ($p < 0.05$; Fig. 3B). The median pulse mass and total mass released during the experiment were found elevated in the hCGαβ+ Ovx as compared with the WT, WTOvx and hCGαβ+ females ($p < 0.05$; Fig. 3B). Interestingly, the median GnRH pulse width showed an inverse tendency to that found in the frequency, being elevated in WT as compared to WTOvx, hCGαβ+ and hCGαβ+ Ovx mice ($p < 0.05$).

3.4. Effect of Ovx on the hypothalamic gene expression and aromatase protein levels in 21-day-old WT and hCGαβ+ females

The gene expression of *Gnrh*, *Kiss1*, *Cyp19a1*, *Esr1* and *Pr* was evaluated in the hypothalamus of 21-day-old WT, WTOvx, hCGαβ+ and hCGαβ+ Ovx females (Fig. 4). The hypothalamic tissue was separated into the two areas responsible for the ovulatory GnRH surge and the tonic GnRH control: the POA-AH and the MBH, respectively (Catalano et al., 2010). *Gnrh* in the POA-AH showed elevated expression levels in hCGαβ+ females as compared with WT ($p < 0.05$). Interestingly, Ovx in WT mice increased *Gnrh* expression in this area ($p < 0.001$), whereas in the hCGαβ+ no change was observed, even though the level remained elevated as compared to WT mice ($p < 0.05$). We also observed elevated *Kiss1*, *Cyp19a1* and *Pr*, and decreased *Esr1* expression in the POA-AH of hCGαβ+ mice, as compared with WT ($p < 0.05$). Ovx in the hCGαβ+ females was able to decrease *Kiss1* expression to levels comparable to those found in WT controls. In both POA-AH of

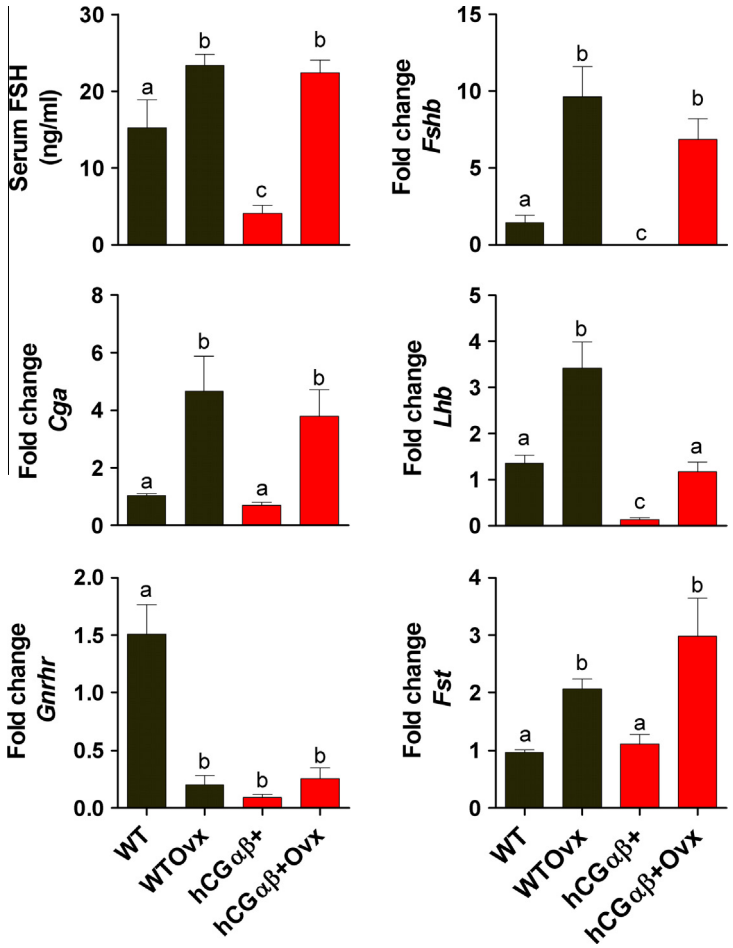

Fig. 2. *Serum FSH levels and pituitary gene expression profile in 21-day-old WT and hCGαβ+ females subjected to Ovx.* The mRNA expression of FSHβ subunit (*Fshb*), LHβ subunit (*Lhb*), common gonadotropin α subunit (*Cga*) and GnRH receptor (*Gnrhr*) was evaluated by qRT-PCR, whereas follistatin (*Fst*) was evaluated by sqRT-PCR. Results were expressed as fold changes relative to WT. Two way ANOVA-Fisher's LSD demonstrated significant interactive and genotype effects for FSH, *Fshb*, *Lhb* and *Gnrhr* ($p < 0.05$), and significant treatment effects for FSH, *Fshb*, *Lhb*, *Cga* and *Fst* ($p < 0.001$). $N = 3–5$. Different letters indicate statistical differences between groups (at least $p < 0.05$).

WT and hCGαβ+ mice, Ovx had no effect on *Cyp19a1*, *Esr1* and *Pr* expression.

In the MBH, the hCGαβ+ females also showed elevated expression levels of *Gnrh* as compared with WT mice ($p < 0.05$). In contrast to what was observed in the WT POA-AH for *Gnrh* expression, in the MBH Ovx had no effect. In hCGαβ+ MBH, Ovx was able to diminish *Gnrh* expression to values similar to those found in WT ($p < 0.01$). We also found a decreased expression of *Kiss1* in the TG MBH ($p < 0.05$), whereas *Cyp19a1*, *Esr1* and *Pr* expression showed no differences as compared with WT. Ovx in the TG MBH elevated *Kiss1* expression to values similar to those found in WT controls ($p < 0.01$). Interestingly, Ovx in the WT MBH increased *Esr1* expression ($p < 0.001$), but had no effect in hCGαβ+ mice. Neither *Cyp19a1* nor *Pr* expression showed differences between the groups in this area.

The *Cyp19a1* gene expression in the POA-AH of hCGαβ+ females after Ovx (from 14 to 21 days of age) was corroborated by changes in the aromatase protein levels by immunoblot (Fig. 5A). The same tendency of increased expression in the hCGαβ+ and hCGαβ+ Ovx females was detected as compared with WT ($p < 0.05$).

3.5. Effect of flutamide treatment on the vaginal opening and the hypothalamic aromatase protein levels in 21-day-old hCGαβ+ females

In order to analyze if the early puberty onset and elevated levels of aromatase in the POA-AH of TG females were due to the high androgen levels prior to the age of 14 days, hCGαβ+ females were treated with the antiandrogen flutamide from postnatal day 6 until sacrifice at 21 days of age. In these conditions, vaginal opening occurred at 20 ± 1 days of age, whereas FSH serum levels and uterus weight exhibited similar values in flutamide- treated hCGαβ+ females as compared with control hCGαβ+ (Table 2). In contrast, the protein levels of aromatase in POA-AH of 21-day-old hCGαβ+ females subjected to flutamide treatment were significantly reduced as compared with control hCGαβ+ mice (Fig. 5B).

3.6. Direct effect of hCG on female hypothalamic neurons in vitro

In order to analyze whether changes in *Cyp19a1* expression observed in hCGαβ+ and hCGαβ+ Ovx depend on the direct action of hCG, we cultured female hypothalamic neurons in basal or hCG-stimulated (6.5 IU/ml) conditions. After 48 h *in vitro*, *Cyp19a1* expression was not statistically different between basal and hCG-treated cultures (Fig. 5C), indicating that hCG was unable to directly stimulate *Cyp19a1* expression in female hypothalamic neurons after 48 h *in vitro*. *Lhcgr* expression was detected in the hypothalamic cultures, without changes after hCG stimulation.

4. Discussion

The reproductive function in mammals requires the coordinated action of sex steroids on the hypothalamic sites, which

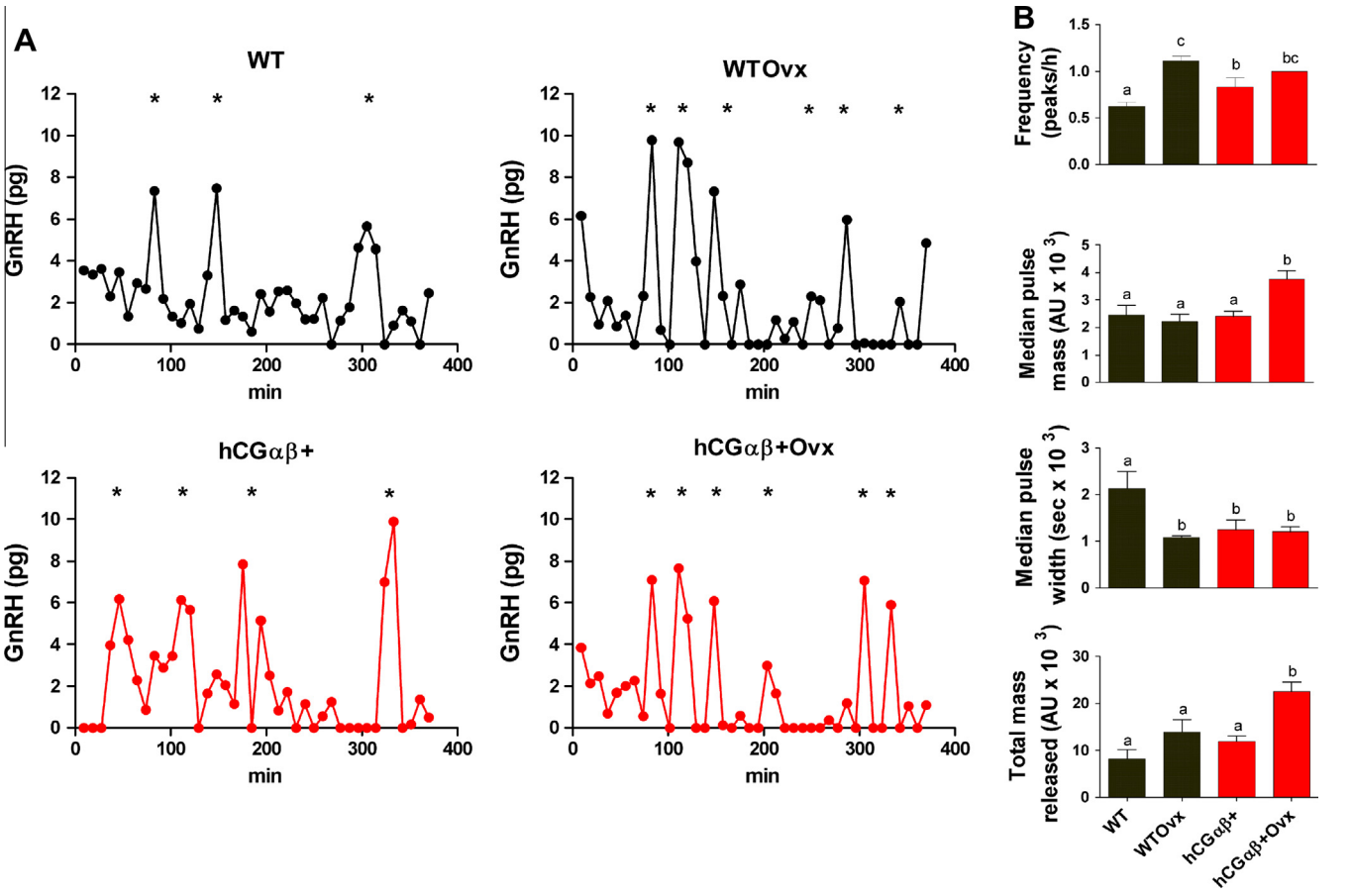

Fig. 3. *GnRH pulsatility studies ex-vivo.* (A) Representative GnRH pulsatility patterns from WT, WTOvx, hCGαβ+ and hCGαβ+ Ovx hypothalamic explants studied *ex vivo*. The results were expressed as pg of GnRH per incubate. The GnRH pulses detected by the Cluster8 algorithm are indicated by asterisks. (B) GnRH frequency, as number of pulses per hour; GnRH median pulse mass; GnRH median pulse width (sec), and GnRH total mass released during the experiment (6 h). Two way ANOVA-Fisher's LSD showed significant interactive and treatment effects for frequency, interactive and genotype effects for median pulse mass, interactive and treatment effects for the median pulse width and genotype and treatment effects for total mass released ($p < 0.05$). $N = 4$. Different letters indicate statistical differences between groups ($p < 0.05$).

modulate the pituitary gonadotropin secretion and sexual behavior. These brain areas are programmed perinatally and are potentially susceptible to endocrine disruption (Gore, 2008). In females, elevated sex steroids due to PPP would accelerate the hypothalamic maturation and induce CPP secondarily (Kukuvitis et al., 1995; Pasquino et al., 1987; Pescovitz et al., 1984; Schmidt and Kiess, 1998). Accordingly, different experimental models of female PP have been developed in rats and mice by early exposure to sex steroids, such as neonatal injection of testosterone, dihydrotestosterone or estradiol (Clark et al., 2003; Foecking et al., 2005; Matagne et al., 2004; Mathews et al., 1987; Witham et al., 2012). Also TG mouse models for LH and hCG overproduction, as well as for LHCGR activating mutations, exhibited PP along with elevated sex steroid production, particularly androgens (Meehan et al., 2005; Risma et al., 1997; Rulli et al., 2002). In the present study we characterized the HPG axis of hCGαβ+ females at the age of 21 days, when they develop premature vaginal opening, which in experimental rodent models is a sign of PP.

The hCGαβ+ mice showed signs of premature negative feedback of gonadal steroids upon the pituitary, as evidenced by diminished serum FSH levels at 14 and 21 days of age, as well as by diminished gene expression of *Fshb* and *Lhb*, as compared with WT controls. These findings indicated enhanced negative feedback regulation on both gonadotropins, a characteristic feature of PPP. Interestingly, despite the inhibitory action of sex steroids over the pituitary, at the hypothalamus we found elevated GnRH expression and pulsatility, indicative of CPP. Testosterone was elevated in the TG ovary at all ages studied, as a consequence of the direct action of hCG on theca cells, which respond to LHCGR stimulation by producing androgens (Richards and Pangas, 2010). Estradiol was not found elevated in the 14-day-old hCGαβ+ females, indicating that the diminished serum FSH observed at this age would be a consequence of the inhibitory feedback action of androgens. A similar hormonal profile was previously described in the hCGβ+ and bLHβ-CTP mouse models with high hCG or LH, respectively, where elevated testosterone was present at 14 days of age and led to precocious vaginal opening, transient estradiol elevation and uterine enlargement at 21 days of age (Risma et al., 1997; Rulli et al., 2002).

Estrogens are key regulators of kisspeptin expression in pre- and postpubertal females, and the majority of kisspeptin neurons in both hypothalamic regions co-express estrogen receptor α (Smith et al., 2006), which directly modulates the progression of puberty in females (Mayer et al., 2010). Previous studies demonstrated that estrogens potently exert inhibitory effects on ARC and stimulatory effects on AVPV kisspeptinergic neurons (Clarkson et al., 2009; Kauffman, 2010; Smith et al., 2005). Accordingly, *Kiss1* expression was found elevated in the POA-AH and diminished in the MBH of our TG females. These changes in *Kiss1* expression are expected to be caused by the premature elevation of serum and/or locally-produced estradiol, since Ovx was able to reverse these changes. The increase in *Kiss1* expression in the TG POA-AH appears to be related to PP in this model, since kisspeptin administered exogenously, as well as activating mutations of

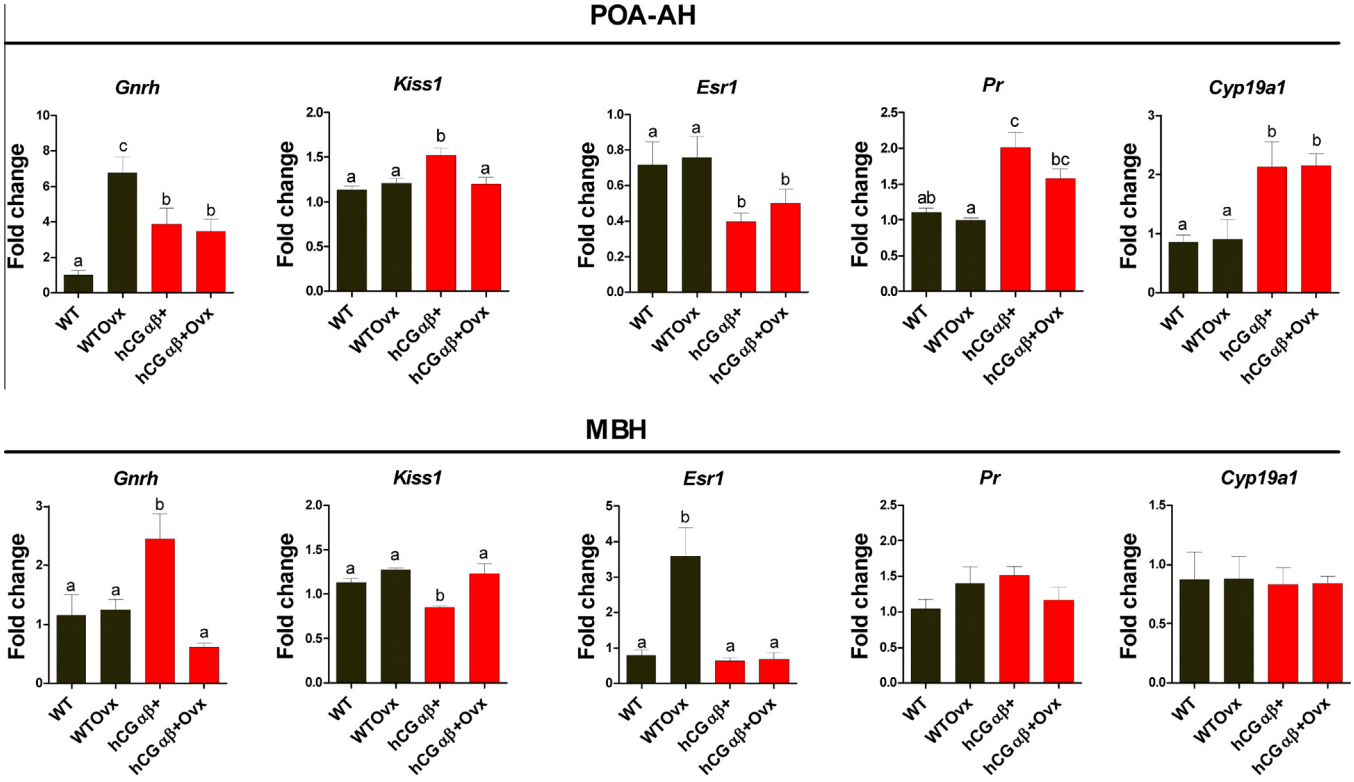

Fig. 4. *Hypothalamic gene expression profile*. The preoptic area-anterior hypothalamus (POA-AH) and the mediobasal hypothalamus (MBH) gene expressions were evaluated in 21-day-old WT, WTOvx, hCGαβ+ and hCGαβ+ Ovx females. The mRNA expression of GnRH (*Gnrh*), Estrogen receptor α (*Esr1*) and Progesterone receptor (*Pr*) was evaluated by qRT-PCR, whereas kisspeptin (*Kiss1*) mRNA expression was evaluated by sqRT-PCR. Results were expressed as fold changes relative to WT. Two way ANOVA-Fisher's LSD in the POA-AH demonstrated significant interactive effects for *Gnrh* and *Kiss1* ($p < 0.05$), genotype effect for *Kiss1*, *Esr1*, *Cyp19a1* and *Pr* ($p < 0.05$), and treatment effect for *Gnrh* ($p < 0.01$). Two way ANOVA-Fisher's LSD in the MBH demonstrated significant interactive effects for *Gnrh* and *Esr1* ($p < 0.05$), genotype effect for *Kiss1* and *Esr1* ($p < 0.05$) and treatment effect for *Gnrh*, *Kiss1* and *Esr1* ($p < 0.05$). $N = 3$–4. Different letters indicate statistical differences between groups (at least $p < 0.05$).

GPR54, trigger PP in females (Navarro et al., 2004; Teles et al., 2008).

The fact that elevated testosterone production and diminished serum FSH levels were already present at 14 days of age in hCGαβ+ females indicates that the rise in androgen production may have started earlier in postnatal development. Since LHCGR appears in the mouse ovary on postnatal day 5 (O'Shaughnessy et al., 1997), hyperandrogenemia is expected to appear around the first week of life in hCGαβ+ females. According to our results, the hCGαβ+ females would be exposed to elevated androgens early postnatally from day 5, but not perinatally. This was demonstrated by the presence of estradiol-induced features in hCGαβ+ females that are known to be abolished by perinatal exposure to androgens, like elevated *Pr* and *Kiss1* expression in the POA-AH, diminished *Kiss1* expression in the MBH and GnRH frequency acceleration (Foecking et al., 2005; Kauffman et al., 2007; Matagne et al., 2004).

In males, the perinatal androgen surge stimulates aromatase expression and activity in the hypothalamus, and locally-produced estradiol is required for both masculinization and defeminization of the male brain (Abdelgadir et al., 1994; Domínguez-Salazar et al., 2002; Gonzalez et al., 2011; Lephart et al., 1992). In the present study, together with elevated androgen production, we found elevated aromatase expression in the POA-AH of hCGαβ+ females at peripuberty, suggesting possible postnatal masculinizing and/or defeminizing effects in this model. Some results have suggested that the activation of androgen receptors may play a role in the defeminization process (Lund et al., 2000). Some evidence demonstrated that female rats treated postnatally with testosterone displayed increased aromatase activity even if they were ovariectomized before puberty or in adulthood (Roselli and Klosterman, 1998). Since the induction of aromatase by testosterone is an androgen receptor- mediated mechanism (Roselli and Resko, 1997), it is possible that androgen receptor activation in TG females enhances the expression of aromatase in the POA-AH, and thereby potentiates the local accumulation of estrogen and the activation of estrogen receptors. Previous studies indicated that the implantation of an estradiol pellet in the POA, but not in the MBH, is able to cause premature vaginal opening and PP in rats (Döcke et al., 1984). Along the same line, it was found that estradiol administration to female rats on postnatal day 10 causes premature vaginal opening and stimulates GnRH pulsatility (Matagne et al., 2004). The putative role of estradiol as trigger of PP in hCGαβ+ females is also in agreement with our results, in which the androgen blockade by flutamide administration was unable to prevent the premature vaginal opening or the increased uterus weight. Moreover, Ovx in hCGαβ+ mice was ineffective in changing these parameters, despite elevated POA-AH aromatase expression, since the removal of gonadal steroids eliminate the androgen substrate for local aromatization.

One effect occurring after the removal of gonadal steroid by Ovx, with the consequent loss of negative feedback at the central level, is the concomitant increase in GnRH pulse frequency (Levine and Ramirez, 1980). In this sense, the elevated *Gnrh* expression found in the POA-AH of WTOvx, hCGαβ+ and hCGαβ+ Ovx mice was in agreement with the elevated GnRH pulse frequency found in these groups, as well as with the diminished *Gnrhr* expression found in the pituitary, which reflects receptor downregulation (Mo et al., 2010). The loss of gonadal signals by Ovx in the TG females was able to elevate serum FSH levels as well as pituitary *Fshb*, *Cga* and *Fst* expression. However, some of the parameters analyzed were not reversed in TG females after Ovx: (i) the GnRH pulse mass *ex vivo* was found significantly elevated in hCGαβ+ Ovx

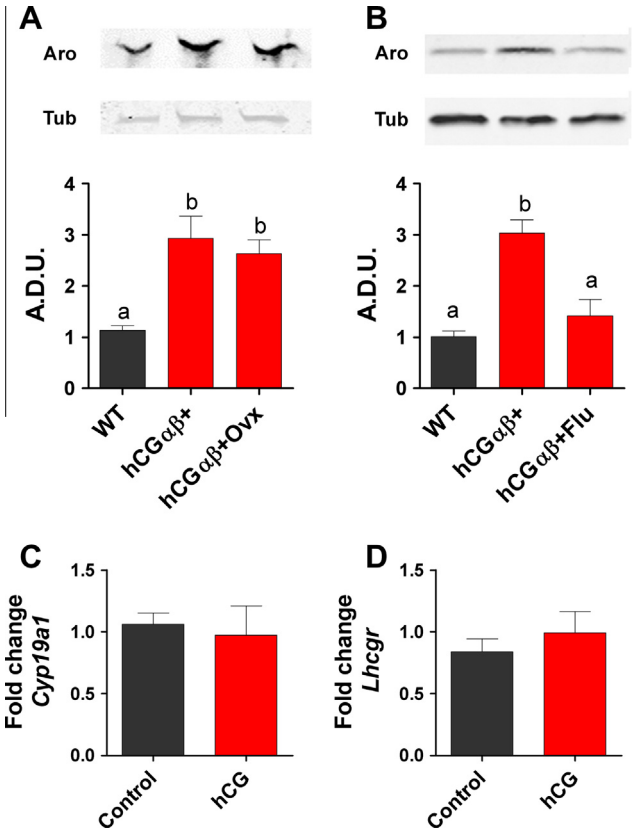

Fig. 5. (A) Protein levels of aromatase in the preoptic area-anterior hypothalamus (POA-AH) of 21-day-old WT, hCGαβ+ and hCGαβ+ Ovx females. Ovx was performed at postnatal day 14. Aromatase protein levels (Aro) were determined by western blot and expressed in arbitrary densitometric units (A.D.U.) after relativization with α-tubulin (Tub). Data were analyzed by one way ANOVA-Fisher's LSD. $N = 3–4$. Different letters indicate statistical differences between groups ($p < 0.05$). (B) Protein levels of aromatase in the POA-AH of 21-day-old WT, hCGαβ+ and hCGαβ+ Flu females. Flutamide (Flu) pellet was administered from postnatal day 6. (C) Effect of recombinant hCG (6.5 IU/mL) on the gene expression of *Cyp19a1* and *Lhcgr* in female hypothalamic neurons after 48 h *in vitro*. Results were expressed as fold changes relative to WT. $N = 4$ independent cultures.

Table 2
Uterus weight, serum FSH and vaginal opening of 21-day-old hCGαβ+ and flutamide-treated hCGαβ+ females.

	hCGαβ+	hCGαβ+Flu[a]
Uterus weight (mg)	71.4 ± 2.8	75.2 ± 2.1
Serum FSH (ng/mL)	3.53 ± 0.91	2.12 ± 0.35
Vaginal opening	Yes	Yes

[a] hCGαβ+ Flu: hCGαβ+ females treated with flutamide from postnatal day 6 to 21. Data are presented as the mean ± SEM. $N = 4–8$.

mice, indicating that the TG hypothalamus responded differently than the WT to the ovarian signal removal, (ii) the expression of *Esr1* in the MBH and *Lhb* in the pituitary were not increased in the hCGαβ+ Ovx females to the same extent as in WTOvx controls, and (iii) the elevated expression of aromatase and diminished of *Esr1* in the POA-AH were not affected by Ovx at postnatal day 14 in the hCGαβ+ females. In contrast to the hCGαβ+ female phenotype, gonadectomy from days 14 to 28 of age in hCGαβ+ males was unable to increase serum FSH levels, pituitary expression of *Fshb*, *Lhb* or *Gnrhr*, and hypothalamic *Gnrh* or *Kiss1* (Gonzalez et al., 2011). The sexual dimorphism in the response of the hypothalamic–pituitary unit to gonadectomy found in this model could be explained by the different time at which hCG starts to stimulate gonadal androgen production, considering that the mouse testis starts expressing LHCGR at gestational day 16, whereas in the ovary it appears later, at postnatal day 5, as mentioned above (O'Shaughnessy et al., 1997, 1998). This difference would lead to irreversible functional alterations at the hypothalamic–pituitary axis occurring in the perinatal period in hCGαβ+ males, and reversible ones occurring later in postnatal development in hCGαβ+ females.

In the hCGαβ+ females, the removal of gonadal inputs by Ovx at postnatal day 14 had no effect on aromatase expression in the POA-AH. This could be explained by a direct effect of hCG on the hypothalamic nuclei, or by alterations of the developmental programming of the female brain by androgens prior to Ovx. Previous studies demonstrated neurotrophic effects of hCG in fetal rat brain *in vitro*, which contains functional LH/hCG receptors (Al-Hader et al., 1997). Our present results showed that, at least *in vitro*, hCG was not able to directly stimulate aromatase expression or alter *Lhrcg* in female hypothalamic neuronal cultures. This suggests that the elevated aromatase in the hypothalamic nuclei of hCGαβ+ females could reflect androgen organizational actions taking place prior to postnatal day 14, when androgen production was elevated. In this respect, the blockade of androgen action by flutamide between postnatal days 6 and 21 was able to reduce the aromatase levels in the POA-AH of hCGαβ+ females, indicating that this was an androgen receptor- mediated event. The same phenotype of elevated androgens and hypothalamic aromatase expression was observed in prepubertal hCGαβ+ males, which was normalized after antiandrogen treatment between gestational day 18 and postnatal day 14 (Gonzalez et al., 2011).

In conclusion, the hCGαβ+ mice provided a valuable model for understanding the impact of early and persistent LH/hCG hypersecretion, and consequently postnatal elevated androgens, on the onset of puberty and the HPG axis in females. Altogether, our results indicated that early exposure of females to androgen action during a critical period between postnatal days 6–14 induced permanent alterations on the sex-specific organization of the brain, and determine the masculinization/defeminization of the aromatase expression in the POA-AH in females at the onset of puberty.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2014.04.005.

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