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Endogenously elevated androgens alter the developmental programming of the hypothalamic–pituitary axis in male mice

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ABSTRACT

Transgenic male mice that express human chorionic gonadotropin (hCG) α and β subunits constitutively hypersecrete hCG and produce elevated levels of androgens. The aim of this study was to characterize the hypothalamic–pituitary function of these transgenic (hCGαβ+) males by focusing on FSH regulation. Serum FSH levels and pituitary mRNA expression of *Fshb*, *Lhb*, *Cga*, *Gnrhr* and *Esr1* were reduced, whereas *Fst* expression was increased in prepubertal hCGαβ+ males as compared with wild-type. In the hypothalamus, *Cyp19a1* expression, GnRH concentration and *ex-vivo* GnRH pulsatility were elevated in prepubertal hCGαβ+ mice, whereas *Kiss1* expression was decreased prepubertally and *Gad67* expression was elevated neonatally. The effect of androgens on the developmental programming of the hypothalamic–pituitary axis of hCGαβ+ males was evaluated by perinatal and prepubertal antiandrogen (flutamide) administration. Our studies identified a critical window between gestational day 18 and postnatal day 14, during which chronically elevated androgens and/or their locally produced metabolites activate the hypothalamus and concomitantly shut-down the gonadotropin axis.

1. Introduction

The integrated function of the hypothalamic–pituitary–gonadal (HPG) axis during male development is essential for acquiring normal reproductive performance at adulthood. Perturbations in this process can result in precocious or delayed puberty, infertility, and other alterations associated with elevated or reduced levels of steroid hormones (Achermann and Jameson, 1999; Themmen and Huhtaniemi, 2000). The hypothalamus acts as a pulse generator, synthesizing and releasing gonadotropin-releasing hormone (GnRH), which in turn stimulates the production and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland. Gonadotropins bind to their respective cognate G protein-coupled receptors to induce gonadal steroidogenesis and gametogenesis (Ascoli et al., 2002). In males, testosterone is the predominant gonadal steroid in circulation, but it is often converted in target tissues via aromatization to estradiol or via 5α-reduction to 5α-dihydrotestosterone (DHT) (Celotti et al., 1997). Gonadal steroids and inhibins are important in the endocrine regulation of gonadotropin secretion in the male, exerting negative feedback at the level of the pituitary and/or the hypothalamus, and reducing their synthesis and release (Purvis et al., 1977; Bilezikjian et al., 2006). Accordingly, castration results in increased circulating levels of gonadotropins that are reversed by administration of testosterone, DHT or estradiol (Lindzey et al., 1998).

In most mammals, transient activation of the HPG axis during perinatal life results in an increase in circulating gonadal steroids, which participate in the sexual differentiation and programming of the nervous system. GnRH secretion declines soon thereafter, and throughout the prepubertal period its pulse frequency is low (Sisk and Foster, 2004). After this period of quiescence, the puberty begins when the sensitivity of the HPG axis to steroid negative feedback declines and GnRH becomes reactivated to stimulate gonadotropin and steroid hormone secretion, this time resulting in complete gonadal maturation with the advent of fertility and mature reproductive behavior (Foster et al., 2006; Sisk and Foster, 2004). In male rodents, testosterone levels increase progressively exhibiting two peaks, one in late gestation (gestational days 17–19) (Weisz and Ward, 1980), and the other in early postnatal life (within

Abbreviations: HPG, hypothalamic–pituitary–gonadal; hCGαβ+, transgenic mice over-expressing hCGα and hCGβ subunits; WT, wild-type; PND, post natal day; dpc, days post-coitum; AVPV, anteroventral periventricular nucleus; ARC, arcuate nucleus.

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few hours after birth) (Corbier et al., 1978). The perinatal testosterone surge is responsible for masculinization and defeminization of the brain, and a broad spectrum of experimental data have demonstrated that estrogens and DHT derived from testosterone through aromatization and 5α-reduction, respectively, are critical for these processes (Negri-Cesi et al., 2008; Sakuma, 2009). It has been proposed that the default neuroendocrine phenotype is female, and the acute perinatal testosterone secretion programs the feedback actions of sex steroids on GnRH secretion (Foster et al., 2006). These modeling processes induced by testosterone and/or its metabolites permanently alter the circuitry in the developing forebrain, preventing the male from exerting the female estradiol positive feedback response that generates the GnRH/LH surge and triggers ovulation (Kauffman, 2009; Tena-Sempere, 2010). In contrast, rodent males castrated during the critical window of perinatal development can produce a GnRH/LH surge at adulthood (Kauffman, 2009).

The mechanism through which GnRH neuron activation is achieved at the onset of puberty is presently under intense investigation. Several factors have been proposed as central regulators of GnRH neurons (Ojeda and Skinner, 2006; Ojeda et al., 2003). One of those is the kisspeptin, a peptide encoded by the *Kiss1* gene that acts through the GPR54 receptor to induce the release of GnRH. Several studies have demonstrated that kisspeptin-GPR54 are the most potent activators of GnRH neurons (Han et al., 2005) and essential for the onset of puberty in several species (Herbison, 2008; Tena-Sempere, 2010). In rodents, kisspeptin is expressed in the arcuate nucleus (ARC), localized in the ventromedial hypothalamus, and in the hypothalamic anteroventral periventricular nucleus (AVPV), localized in the preoptic area and extending probably as a continuum along the rostral periventricular area of the third ventricle (RV3P) (Herbison, 2008; Kauffman, 2009). In rodents, sheep and primates, it has been proposed that the ARC contains the neuronal substrate mediating the negative feedback regulation of the reproductive axis by estrogens and androgens in both sexes, whereas the AVPV provides the anatomical substrate/circuitry for generating the sexually differentiated preovulatory LH surge in females (Kauffman, 2009; Roa et al., 2008). In addition to the dimorphic pattern of the kisspeptin-GPR54 circuitry, the GABAergic and glutamatergic neurons also provide major synaptic inputs to GnRH neurons and are also implicated in the establishment of the sexual differences in the developing brain by perinatal sex steroids (McCarthy et al., 2002). In addition, these two systems are closely related, since glutamate is the natural precursor for GABA synthesis.

It is well established that in females, exposure to androgens brings about profound alterations in the neuroendocrine control of ovulatory cycles that lead to loss of the ability to generate GnRH/LH surges as adults (Robinson, 2006). In males, the putative influence of excess of androgens has been largely neglected, most likely because their neuroendocrine regulation is simpler than that of the females, and due to the fact that males are normally exposed to testosterone from early development (Foster et al., 2006). It is however possible that multiple sources of steroids, such as endogenous androgens derived from the mother or the fetus, or exogenous endocrine active compounds from the environment, may potentially cause developmental defects in the male reproductive axis that can be manifested in later life (Gore, 2008). Transgenic male mice with chronically elevated circulating concentrations of the human chorionic gonadotropin (hCGαβ+ mice) have been previously characterized and shown to be infertile (Rulli et al., 2003). The hCGαβ+ males exhibit prepubertal Leydig cell hypertrophy/hyperplasia and enhanced testicular steroidogenesis, evidenced by elevated levels of testosterone from early postnatal age to adulthood (Ahtiainen et al., 2005; Rulli et al., 2003). In contrast to the protocols with exogenously administered steroids, which can by far exceed the physiological levels, this transgenic model guarantees that the maximum steroid levels that may be reached remain within those produced *in vivo*. Consequently, the hCGαβ+ mice can provide novel information about the role of abnormal levels of gonadal steroids on the developmental programming of the hypothalamic–pituitary axis in males. For this purpose, the hCGαβ+ mice were subjected to perinatal and prepubertal antiandrogen treatment and castration, and the hypothalamic and pituitary functions were analyzed.

Table 1
Seminal vesicle (SV) and epididymis (Epi) weight, relative to the body weight (BW) of 28-day-old WT, hCGαβ+, hCGαβ+ treated with flutamide from PND14 (F) and from 18dpc (F18dpc).

	WT	hCGαβ+	hCGαβ+F	hCGαβ+F18dpc
SV (mg/BW)	2.25 ± 0.06 a	10.14 ± 0.36 b	2.71 ± 0.15 a	0.82 ± 0.11 c
Epi (mg/BW)	0.61 ± 0.01 ac	0.95 ± 0.02 b	0.74 ± 0.02 c	0.56 ± 0.03 a

Data are presented as the mean ± SEM. One way ANOVA followed by Bonferroni's post hoc test was conducted. $N = 4–6$, Different letters: $p < 0.05$.

2. Materials and methods

2.1. Animals

All studies were performed in double transgenic male mice that overexpress the glycoprotein hormone common α and hCGβ subunits, under the control of the human ubiquitin C promoter. 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 wild-type (WT) males were used as controls. Mice were maintained under controlled conditions (12-h light/dark cycle, 21 °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. Hypothalami, pituitaries and testes were collected, snap frozen and stored at −70 °C for hormone measurement or RNA isolation. Pituitaries were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry.

2.2. Animal treatments

2.2.1. Castration

Fourteen- or 75-day-old WT and hCGαβ+ males were anesthetized with a ketamine:xilacin solution (60:10 mg/kg body weight) and testes were removed through a transabdominal incision. Mice were maintained for 14 days following castration and euthanized at 28 or 90 days of age. Sham-operated mice were used as controls.

2.2.2. Antiandrogen treatments

Fourteen-day-old WT and hCGαβ+ males were implanted s.c. with 20-mg-flutamide pellets (Schering Canada Ltd., Quebec, Canada). Pellets were replaced every 15 days until sacrifice at 28 or 90 days of age and the weight of seminal vesicles and epididymides was recorded (Table 1). To evaluate the effect of perinatal flutamide treatment, hCGα+ females were mated with hCGβ+ males and the day of appearance of vaginal plug was considered as 1 day *post-coitum* (dpc). At 18 dpc pregnant females were implanted s.c. with a 20-mg-flutamide pellet. The day of birth was taken as post natal day (PND) 1. Pups were injected s.c. with flutamide (50 mg/kg body weight, dissolved in castor oil) at PND 1, 3 and 5. Control mice were injected s.c. with vehicle following the same schedule. At PND 7 a flutamide pellet was implanted s.c. and replaced with a new pellet after 15 days. Mice were sacrificed at 28 days of age, and the weight of seminal vesicles and epididymides was recorded (Table 1). As was previously characterized, the hCGαβ+ males exhibit elevated androgen concentration, which stimulates the growth of the androgen-dependent organs seminal vesicles and epididymides (Ahtiainen et al., 2005; Rulli et al., 2003). The weight of these organs at sacrifice was taken as the principal criterion to evaluate the efficacy of the perinatal and prepubertal flutamide treatments (Dhar and Setty, 1987; Rulli et al., 1995). Table 1 shows that flutamide blocked the androgen effect on the seminal vesicle and epididymis at 28 days of age in both perinatal and prepubertal treatments.

2.3. Hormone measurements

The FSH concentration in serum and pituitary extracts was measured by a double antibody radioimmunoassay (RIA), according to a method described previously (Rulli et al., 1996). Individual pituitaries were homogenized in 100 μL of PBS and a

Table 2
Primer sequences used for gene expression assays.

Gene	GenBank access number	Primer forward 5′–3′	Primer reverse 5′–3′	Product size (bp)	Annealing temp. (°C)
Fshb	NM_008045	AAACGACGATGGACATTGCC	GTCAGGACAGCGGCAAACAGT	139	60
Lhb	NM_008497	TGTCAACGCAACTCTGGCC	GGCAGTACTCGGACCATGCT	105	55
Cga	NM_009889	GACTTTATTATTCAGGGTTGCCCA	AGAAGCAACAGCCCATACACTG	100	55
Esr1	NM_007956	ATGAAAGGCGGCATACGGAAAG	CACCCATTTCATTTCGGCCTTC	94	60
Gnrhr	NM_010323	GCCCACAGTCTTCTCGCAATG	ATGAGGAGGGGGATGATGA	111	55
Fst	NM_008046	GCTGCTACTCTGCCAGTTCAT	CACATCCTCCTCGGTCCA	166	55
Gnrh	NM_008145	GAACCCCAGCACTTCGAATGT	TGGCTTCCTCTTCAATCAGACTTT	94	55
Cyp19a1	NM_007810	CGGGCTACGTGGATGTGTT	GAGCTTGCCAGGCGTTAAAG	135	55
Srd5a2	NM_053188	GTTTAGCGTCGGTGTCTTCT	GCCCATCCATTCAATAATCT	175	55
Kiss1	NM_178260	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATACC	140	60
Gad67	NM_008077	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC	79	58
Gapdh	NM_008084	CCAGAACATCATCCCTGCAT	GTTCAGCTCTGGGATGACCTT 67	67	60

dilution of the homogenate was assayed for pituitary FSH content. 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. To measure hypothalamic GnRH concentration, individual hypothalami were homogenized in 100 μL of ice-cold 0.1 N HCl as described by Heger et al. (2003), and centrifuged at 13,000 × *g* at 4 °C for 30 min. Supernatants were recovered and the procedure was repeated once with the precipitates. A dilution of the supernatant obtained was assayed for GnRH concentration by RIA, and an aliquot was separated for protein determination by the method described by Lowry et al. (1951). The 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. Intratesticular testosterone and estradiol were determined by homogenizing the testis in 200 μL PBS. The homogenates were extracted twice with 2 mL diethyl ether and evaporated to dryness. After reconstitution into PBS, testosterone and estradiol were measured by conventional RIAs (Rulli et al., 1995). Intra- and inter-assay coefficients of variation were less than 12%.

2.4. Immunohistochemistry

Immunohistochemistry for the FSHβ subunit was carried out in pituitary glands as previously described (Rulli et al., 2002). Paraffin sections were dehydrated and peroxidase-inactivated by using 0.5% (v/v) H_2O_2 in methanol for 20 min at room temperature. Sections were pre-treated with saponin 0.5% for 5 min, blocked with 10% normal goat serum in 1% BSA for 1 h at room temperature, and incubated with a rabbit antiserum against FSH (NIDDK-anti-rFSH-IC-1) overnight at 4 °C, at a 1:500 dilution in 2% normal goat serum. Negative control was incubated with 2% normal goat serum in the absence of primary antiserum. After 3 washes with TBS-0.005% Tween, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc., Burlingame, CA) at a 1:1000 dilution for 2 h at room temperature, washed with TBS and incubated in avidin–biotin–horseradish peroxidase-conjugate (Vectastain Elite ABC kit, Vector Laboratories Inc.). Staining was carried out with 0.055% (w/v) 3,3′-diaminobezidine in 0.1% (v/v) H_2O_2 in Tris–HCl. FSHβ-positive cells were counted in one field per pituitary. Data are presented as number of FSHβ-positive cells per field ($N = 4$).

2.5. 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 PCR (qPCR) primers sets were designed for the specific amplification of murine *Fshb*, *Lhb*, *Cga*, *Gnrhr*, *Gnrh*, *Esr1*, *Cyp19a1*, *Srd5a2*, *Kiss1r*, *Gad67* and *Gapdh* as housekeeping control gene (Table 2). 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 7700 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 $\Delta Ct = Ct_{(\text{reference cDNA})} - Ct_{(\text{experimental cDNA})}$. 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 male chosen *ad random*). Since *Fst* and *Kiss1* exhibited Ct values reaching the detection limit of the qPCR assay for prepubertal male samples, a semi-quantitative (sq) 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*.

2.6. Ex vivo GnRH pulsatility assays

We performed pulsatility studies *ex vivo* as described by Heger et al. (2003). Briefly, individual hypothalami (including the preoptic area-anterior hypothalamus–medial basal hypothalamus) 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 interpeak interval calculated. Peaks were considered as GnRH pulses, peak amplitude as pulse amplitude, interpeak interval as interpulse interval, and the area under the peak as the mass of the pulse. The pulse areas obtained during the experiment were defined as total mass released (in arbitrary units). The experiment was repeated four times, including one animal from each group per experiment.

2.7. 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 Bonferroni's 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 significant.

3. Results

3.1. Serum and pituitary FSH levels are decreased in hCGαβ+ males

Serum FSH levels were evaluated in WT and hCGαβ+ males at the age of 7, 10, 21, 28 and 90 days (Fig. 1A). Serum FSH concentration showed a significant increase in WT mice between PND 10 and 28, but in hCGαβ+ mice the levels remained low and unchanged at all ages, being significantly lower than those measured in WT mice from PND10 onward. The pituitary FSH content (Fig. 1B) and the immunolocalization of FSHβ-producing cells (Fig. 1C) were also determined in WT and hCGαβ+ pituitaries at PND28. In agreement with the serum profile, the pituitary FSH content of the hCGαβ+ males was significantly decreased as compared with the WT mice ($p < 0.001$), and the number and intensity of FSHβ-producing cells was reduced in hCGαβ+ pituitaries as compared with WT males (WT: 109.25 ± 15.35, hCGαβ+: 28.50 ± 3.12 FSHβ-positive cells/field, $p < 0.001$). The pituitary weights were lower in hCGαβ+ males compared with WT controls (WT: 0.104 ± 0.006, hCGαβ+: 0.057 ± 0.008 mg/body weight, $p < 0.01$). The intratesticular testosterone and estradiol concentration were evaluated at PND10 and PND28 (Fig. 1D). In agreement with previous reports, testosterone was elevated at both ages ($p < 0.05$) (Ahtiainen et al.,

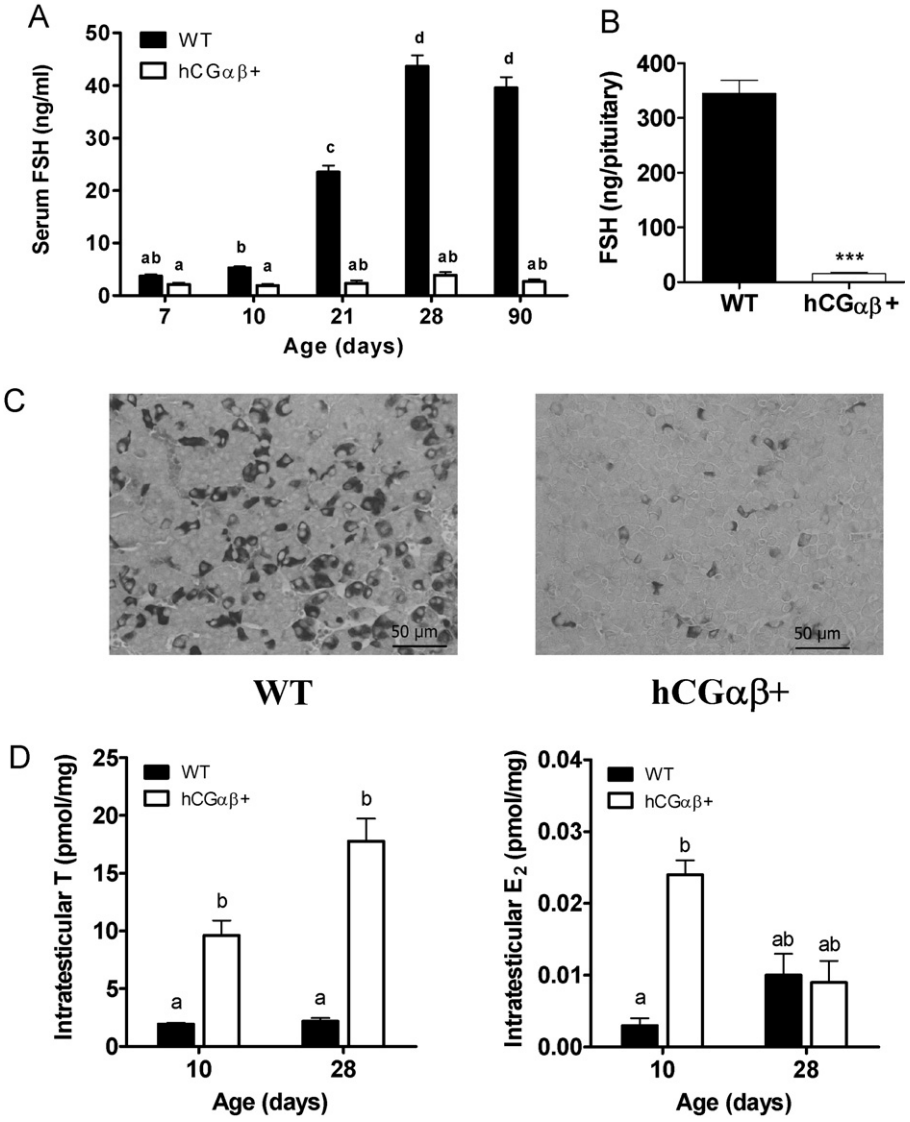

Fig. 1. *FSH expression, intratesticular testosterone and estradiol studies.* (A) Serum FSH profile of WT and hCGαβ+ males was evaluated between days 7 and 90 of age. Two-way ANOVA, followed by Bonferroni's post hoc test, demonstrated significant genotype, age, and interactive effects ($p < 0.001$); $N = 5$–7. Different letters: $p < 0.05$. (B) The FSH content in the pituitary glands was evaluated in 28-day-old WT and hCGαβ+ males. Data were analyzed by Student's *t*-test, $N = 5$, $^{***}p < 0.001$. (C) Immunohistochemistry for FSHβ subunit was performed in the pituitary of 28-day-old WT and hCGαβ+ males. (D) Intratesticular testosterone (T) and estradiol (E_2) were evaluated at 10 and 28 days of age in WT and hCGαβ+ males. Two-way ANOVA, followed by Bonferroni's post hoc test, demonstrated significant genotype effect for T ($p < 0.001$) and interactive effects for E_2 ($p < 0.001$). $N = 4$. Different letters: $p < 0.05$.

2005; Rulli et al., 2003). In contrast, although the hCGαβ+ testes showed elevated levels of estradiol at PND10, no difference was observed at PND28 between the genotypes.

3.2. The pituitary gene expression is altered in hCGαβ+ males

Expression of the gonadotropin subunits FSHβ (*Fshb*), LHβ (*Lhb*), and the common α (*Cga*) were studied in the pituitaries of WT and hCGαβ+ males at PND28 (Fig. 2). In agreement with the reduced serum FSH levels of the hCGαβ+ males, the expression levels of *Fshb* and *Cga* were 50-fold ($p < 0.001$), and the *Lhb* was 100-fold supressed in the hCGαβ+ males as compared with WT controls. The key genes involved in gonadotropin regulation, i.e. estrogen receptor α (*Esr1*), GnRH receptor (*Gnrhr*) and follistatin (*Fst*) were also measured (Fig. 2). *Gnrhr* and *Esr1* expression was decreased 4- and 2-fold, respectively (*Gnrhr*: $p < 0.001$, *Esr1*: $p < 0.05$), and *Fst* expression was 2-fold increased in hCGαβ+ males compared with WT mice ($p < 0.01$).

3.3. Hypothalamic GnRH concentration and Cyp19a1 expression are elevated in hCGαβ+ males

To evaluate if the suppression of the gonadotropin synthesis and secretion in the hCGαβ+ males was due to functional alterations at the hypothalamic level, different parameters involved in GnRH expression and release were evaluated in WT and hCGαβ+ mice at PND28 (Fig. 3). *Gnrh* mRNA expression did not show significant differences, whereas the hypothalamic GnRH concentration was significantly elevated in the hCGαβ+ group as compared with WT males ($p < 0.01$). The hypothalamic mRNA expression of the key androgen-regulated enzymes involved in testosterone metabolite conversion 5α-reductase type II (*Srd5a2*) and p450aromatase (*Cyp19a1*) was also evaluated (Negri-Cesi et al., 2008). In agreement with previous reports that have shown that androgens induce the expression of aromatase in the hypothalamus (Abdelgadir et al., 1994; Hutchison, 1997), a 20-fold increase in *Cyp19a1* mRNA expression was observed in hCGαβ+ compared with WT males

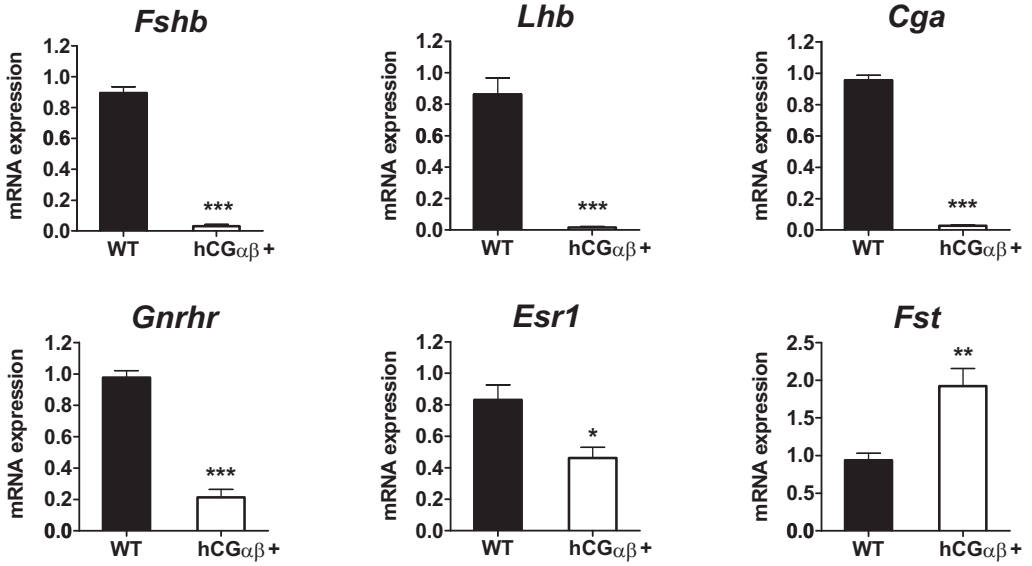

Fig. 2. *Pituitary gene expression profile of 28-day-old WT and hCGαβ+ males*. The mRNA expression of FSHβ subunit (*Fshb*), LHβ subunit (*Lhb*), common gonadotropin α subunit (*Cga*), estrogen receptor α (*Esr1*) and GnRH receptor (*Gnrhr*) was evaluated by qPCR, whereas follistatin (*Fst*) was evaluated by sqPCR. Data were analyzed by Student's *t*-test, $N = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

($p < 0.05$). In contrast, *Srd5a2* expression showed no differences between the two genotypes at this age.

3.4. Hypothalamic GnRH pulsatility is elevated in hCGαβ+ males

Since GnRH exerts its physiological actions on gonadotropin release in a pulsatile manner, *ex vivo* studies were conducted in WT and hCGαβ+ hypothalami in order to clarify if the GnRH pulsatility was also affected. Representative GnRH pulsatility patterns in WT and hCGαβ+ males at PND28 are shown in Fig. 4A, where a larger number of GnRH peaks in the hCGαβ+ group was observed, as compared with WT males. The hCGαβ+ mice also showed a higher pulse

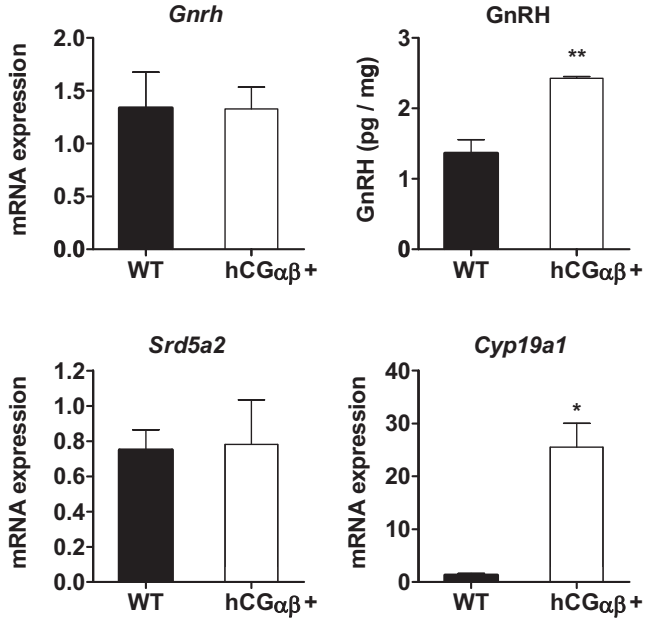

Fig. 3. *Hypothalamic GnRH concentration and gene expression profile*. The hypothalamic GnRH concentration (pg/mg protein) and hypothalamic gene expression were evaluated in 28-day-old WT and hCGαβ+ males. The mRNA expression of hypothalamic GnRH (*Gnrh*), p450aromatase (*Cyp19a1*) and 5α-reductase type II (*Srd5a2*) was evaluated by qPCR. Data were analyzed by Student's *t*-test, N = 4, $^{**}p < 0.01$.

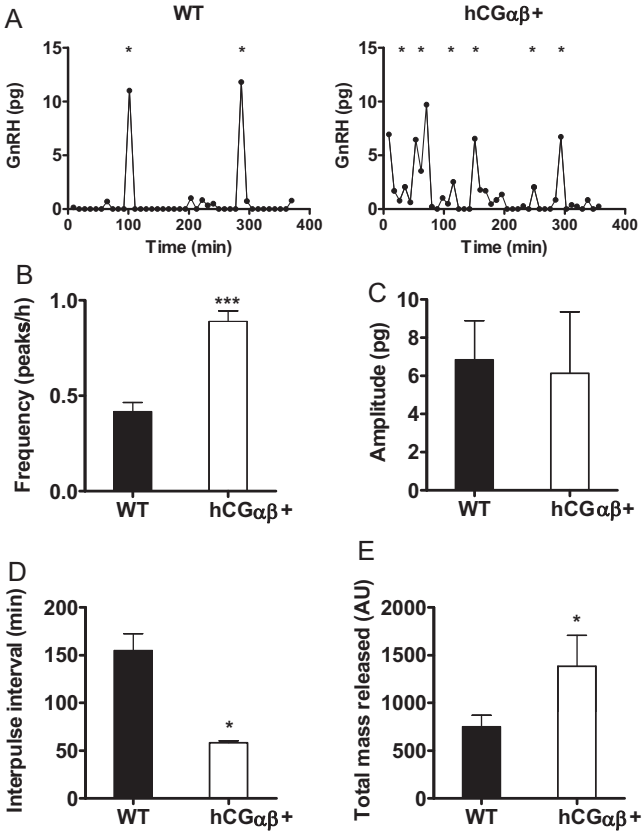

Fig. 4. *GnRH pulsatility studies ex vivo*. (A) Representative GnRH pulsatility patterns from WT and hCGαβ+ hypothalamic explants were studied *ex vivo*. Individual hypothalami were incubated in 250 μL buffer for 6 h, the medium was changed every 9-min intervals and the GnRH released to the medium was measured by RIA (for details see Section 2.6). 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; (C) GnRH pulse amplitude; (D) GnRH interpulse interval; E) GnRH total mass released during the experiment (6 h). Data were analyzed by Student's *t*-test, $N = 3$–4, $^{*}p < 0.05$, $^{***}p < 0.001$.

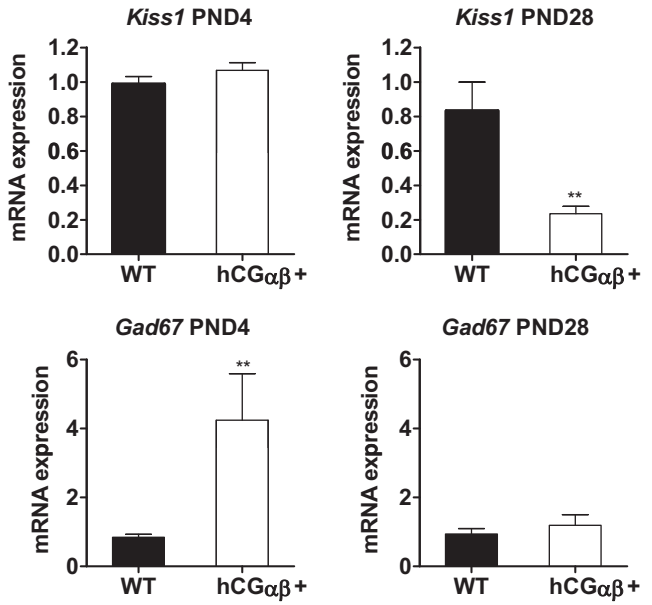

Fig. 5. *Expression of Kiss1 and Gad67 in early postnatal (PND4) and prepubertal (PND28) WT and hCGαβ+ hypothalamus. Kiss1* mRNA expression was evaluated by sqPCR, whereas *Gad67* mRNA expression was evaluated by qPCR. Data were analyzed by Student's *t*-test, $N = 4$, $^{**}p < 0.01$.

frequency ($p < 0.001$; Fig. 4B), lower interpulse interval ($p < 0.05$; Fig. 4D) and higher total mass released ($p < 0.05$; Fig. 4E) than those obtained for WT mice. The pulse amplitude did not show significant differences between genotypes (Fig. 4C).

3.5. *Gad67* expression is elevated early postnatally and *Kiss1* expression is diminished prepubertally in the hCGαβ+ hypothalamus

The hypothalamic expression of kisspeptin (*Kiss1*) was evaluated in early postnatal (PND4) and prepubertal (PND28) WT and hCGαβ+ hypothalami (Fig. 5). At PND4, hypothalamic *Kiss1* expression showed no differences between genotypes. However, at PND28 the hCGαβ+ males exhibited diminished levels of hypothalamic *Kiss1* expression as compared with WT controls ($p < 0.01$). The expression of the kisspeptin receptor GPR54 gene (*kiss1r*) was evaluated at PND28, and showed no differences between the genotypes (data not shown). The gene expression of *Gad67*, the rate-limiting enzyme in GABA synthesis (Davis et al., 1996), was also evaluated (Fig. 5). The data revealed that at PND4, hypothalamic *Gad67* expression was found elevated in the hCGαβ+ males as compared with WT males ($p < 0.01$), whereas at PND28 no differences were observed between the genotypes.

3.6. Antiandrogen treatment or castration is not able to restore FSH secretion when applied in prepuberty or adulthood

The effect of androgens on the regulation of FSH expression and secretion was evaluated by administration of the antiandrogen flutamide to WT and hCGαβ+ males at PND14, following sacrifice at the age of 28 or 90 days. In addition, we conducted castration in order to compare its effect with that of antiandrogen treatment. Since the endocrine effects of gonadal steroids, activins and inhibins are eliminated 14 days after gonadectomy (Kumar et al., 1992), WT and hCGαβ+ males were castrated two weeks before sacrifice at the ages of 28 or 90 days. As expected, serum FSH levels increased in response to castration in WT males at both ages, and flutamide also induced a significant increase at 28 days of age (Dhar and Setty, 1987) (Fig. 6A). In contrast, in flutamide-treated

Table 3
Pituitary and hypothalamic gene expression profile, GnRH hypothalamic concentration and GnRH pulse frequency of control (hCGαβ+) and castrated (hCGαβ+Cx) 28-day-old males.

	hCGαβ+	hCGαβ+Cx
Pituitary (mRNA expression)		
Fshb	0.031 ± 0.012	0.024 ± 0.003
Lhb	0.016 ± 0.005	0.012 ± 0.002
Cga	0.027 ± 0.006	0.070 ± 0.004***
Gnrhr	0.362 ± 0.062	0.313 ± 0.021
Esr1	0.462 ± 0.067	0.446 ± 0.087
Fst	2.057 ± 0.249	2.340 ± 0.138
Hypothalamus		
Kiss1 mRNA expression	0.29 ± 0.02	0.23 ± 0.02
Gnrh mRNA expression	1.33 ± 0.21	0.75 ± 0.32
GnRH concentration (pg/mg protein)	2.43 ± 0.04	3.41 ± 0.89
GnRH frequency (peaks/h)	0.89 ± 0.06	0.50 ± 0.10*

Data are presented as the mean ± SEM. The pituitary and hypothalamus mRNA expression are presented relative to intact WT males. Student's *t*-test, $N = 3$.
* $p < 0.05$.
*** $p < 0.001$.

and castrated hCGαβ+ males serum FSH values remained lower than those measured in the WT controls ($p < 0.001$) at both ages, indicating that castration or flutamide treatment were not able to rise serum FSH levels. In order to evaluate in more detail the effect of castration on 28-day-old hCGαβ+ males, several parameters of the pituitary–hypothalamic axis were analyzed (Table 3). The mRNA expression of *Fshb*, *Lhb*, *Esr1*, *Gnrhr*, *Fst*, *Gnrh* and *Kiss1*, as well as the hypothalamic GnRH concentration remained unaltered, whereas the GnRH pulse frequency significantly decreased after castration ($p < 0.05$). An increase in Cga expression was detected after castration ($p < 0.001$), which is in agreement with the slight increase in serum FSH after castration at both the prepubertal and adult age (Fig. 6A). These results indicate that the withdrawal of gonadal steroids by castration influenced mainly the GnRH pulsatility in this model.

3.7. Perinatal antiandrogen treatment on hCGαβ+ males increases FSH and Gnrhr expression and normalizes Cyp19a1 and Kiss1 expression

The effect of the antiandrogen treatment on serum FSH levels was evaluated perinatally in hCGαβ+ males by the administration of flutamide from 18 dpc (F18dpc) until sacrifice at PND28 (Fig. 6B). The serum FSH levels in hCGαβ+F18dpc were elevated, as compared with hCGαβ+ mice ($p < 0.05$). In agreement with the serum concentration, pituitary *Fshb* mRNA expression was also elevated in hCGαβ+F18dpc as compared with the untreated hCGαβ+ males ($p < 0.05$). This increase of *Fshb* expression in hCGαβ+F18dpc mice was accompanied by a concomitant increase in *Gnrhr* expression ($p < 0.05$). In addition, *Cyp19a1* and *Kiss1* expression were normalized, while *Gad67* expression showed no difference after perinatal flutamide treatment in hCGαβ+F18dpc males ($p < 0.05$). The expression of *kiss1r* showed no change after the treatment (data not shown).

4. Discussion

Many lines of evidence indicate that steroid hormones permanently program the neuroendocrine function and behavior. These pathways can be disrupted during a critical period of brain sexual differentiation and manifested as reproductive dysfunctions in adulthood (Gore, 2008). Transgenic hCGαβ+ male mice, which are infertile and produce elevated androgen levels as a consequence of hCG hypersecretion from early development (Ahtiainen et al., 2005; Rulli et al., 2003), provide a useful model to study the influence of

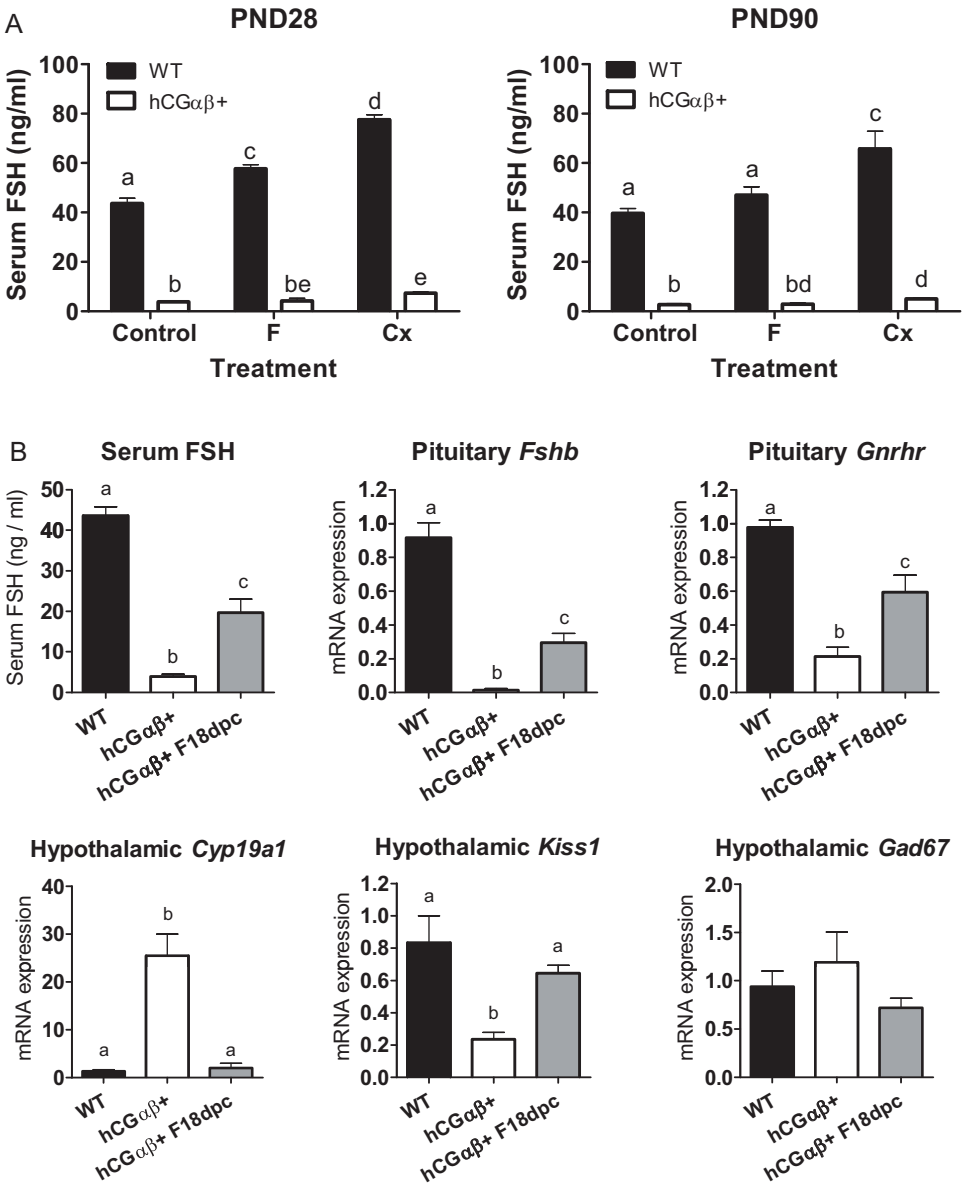

Fig. 6. (A) *Effect of prepubertal and adult flutamide treatment and castration.* Serum FSH levels were evaluated in control, flutamide-treated from PND14 (F) and castrated (Cx) WT and hCGαβ+ males at PND28 and PND90. Statistical analysis was conducted within each age group by two-way ANOVA, followed by Bonferroni's post hoc test. $N = 5–7$. Different letters: $p < 0.05$. At PND28, significant genotype, treatment and interactive effects were found ($p < 0.001$); at PND90, only genotype and treatment ($p < 0.001$), but not interactive effects were found. (B) *Effect of perinatal flutamide treatment*. Serum FSH levels (FSH), pituitary and hypothalamus gene expression were evaluated in WT, hCGαβ+ and hCGαβ+ treated with flutamide from 18 dpc (hCGαβ+F18dpc) at PND28. The pituitary FSHβ mRNA expression (*Fshb*), pituitary GnRH receptor mRNA expression (*Gnrhr*), hypothalamic p450aromatase mRNA expression (*Cyp19a1*) and hypothalamic GAD67 mRNA expression (*Gad67*) were evaluated by qPCR, whereas the hypothalamic kisspeptin mRNA expression (*Kiss1*) was evaluated by sqPCR. Data were analyzed by one way ANOVA followed by Bonferroni's post hoc test, $N = 4–6$, different letters: $p < 0.05$.

untimely high concentrations of androgens on the neuroendocrine control of gonadotropin secretion.

In the present study we found low FSH levels throughout the life in the hCGαβ+ males, as well as FSH unresponsiveness to castration or antiandrogen flutamide treatment in prepuberty or adulthood. These findings point to a profound and persistent malfunction of the neuroendocrine feedback control of the gonadotropin axis. Most of our observations were made at the age of 28 days, considering that this is a relevant point in the maturation of the HPG axis (Ojeda and Skinner, 2006). In addition, HPG function at this age is dependent on a proper perinatal neuroendocrine programming and is highly sensitive to the effects of sex steroids (Navarro et al., 2009). To analyze the gonadotropin function of hCGαβ+ males we focused on FSH rather than LH, since the similarities in structure and function between LH and hCG, together with the elevated hCG levels produced by these mice, made difficult the interpretation of the results based on LH secretion. However, the pituitary mRNA levels of *Fshb*, *Lhb* and *Cga* revealed that the expression of all gonadotropin subunits was suppressed in hCGαβ+ males, indicating that not only FSH but also LH production was affected.

There is evidence that the GnRH responsiveness of pituitary gonadotropes is correlated with pituitary GnRH receptor number and hypothalamic GnRH pulse frequency (Meidan et al., 1982). It has been demonstrated that pulsatile GnRH secretion in the short term leads to GnRH receptor up-regulation, whereas prolonged exposure to high concentrations and/or high pulse frequency of GnRH induces down-regulation of the cognate receptor; this is followed by suppression of gonadotropin synthesis and secretion (Cheng et al., 2000; Conn et al., 1984). It was also demonstrated that GnRH applied continuously or in pulses of high frequency

increases pituitary follistatin mRNA levels (Kirk et al., 1994). In this respect, we found that the hypothalamic function of prepubertal hCGαβ+ males was altered, with increased GnRH content and pulsatile secretion accompanied by increased expression of pituitary *Fst* and decreased expression of pituitary *Gnrhr*. It is, therefore, possible that the mechanisms of gonadotropin suppression in the hCGαβ+ males revolve, at least partly, around the increased GnRH discharge from the hypothalamus, which may lead to a premature down-regulation of *Gnrhr*, thus altering the development and/or functionality of gonadotropes. This is supported by the reduced number of FSHβ-producing gonadotropes observed in hCGαβ+ males. In this respect, previous studies have shown that GnRH stimulates the responsiveness of gonadotropes during early pituitary differentiation (Kudo et al., 1994) and gonadotrope proliferation (Childs and Unabia, 2001), and that hypothalamic input is required for the development of normal number of thyrotropes and gonadotropes during late gestation (Szarek et al., 2008).

Unlike pituitary gonadotropin synthesis, the hypothalamic GnRH content and GnRH pulse frequency were increased in prepubertal hCGαβ+ males. This difference suggests the possibility that the negative feedback effects at the pituitary level may mask a central stimulatory effect that was revealed by the GnRH pulsatility studies. Such a masking effect has been observed previously with steroid treatments *in vivo*, where the combination of estradiol and DHT induced activation of the GnRH neurons in castrated males, despite their suppressive effect on gonadotropin secretion at the pituitary level (Pielecka and Moenter, 2006). Moreover, central synergism between estradiol and DHT has previously been reported for the control of aromatase activity in the male rat hypothalamus (Roselli, 1991). In our model, a similar scenario is expected for elevated estrogen and DHT, since the hypothalamic expression *Cyp19a1* and GnRH pulsatility were found elevated. This early activation of the GnRH pulse generator may be interpreted as a sign of precocious puberty in the hCGαβ+ males. However, our previous studies found no signs of precocious puberty, estimated by the time of balanopreputial separation, despite the elevated androgen levels (Ahtiainen et al., 2005), thus reinforcing the concept that the onset of puberty is a brain-dependent phenomenon including both gonadal hormone-dependent and independent neuronal mechanisms (Foster et al., 2006). Evidence to date suggests that kisspeptin signaling through its G protein-coupled receptor GPR54 is an essential component of the neuroendocrine reproductive axis, acting as gatekeeper in the awakening of reproductive function at puberty, by stimulating the secretion of the releasable pool of GnRH (Navarro et al., 2005). Several studies have pointed out the role of kisspeptin in mediating the negative feedback effects of gonadal steroids on GnRH secretion in both sexes (Smith et al., 2005a,b). Navarro and co-workers (2009) recently reported that neonatal administration of estrogenic compounds resulted in a dose-dependent decrease in *Kiss1* mRNA levels at the prepubertal stage in male and female rats. In the present study we found reduced expression of *Kiss1* in prepubertal hCGαβ+ compared with WT mice. These data are in agreement with the suppressive effects of both elevated circulating testosterone and the locally-produced estrogens on the hypothalamic *Kiss1* expression. However, the fall in *Kiss1* expression does not explain the elevated GnRH concentration and pulsatility observed in prepubertal hCGαβ+ males. These changes could be explained by the action of several other neurotransmitters, such as glutamate, GABA (McCarthy et al., 2002), and glial-derived factors (Garcia-Segura et al., 2008; Ojeda et al., 2003). Importantly, the circuitries of stimulating neurotransmitters and trans-synaptic glial-derived factors are dependent on the action of gonadal steroids during perinatal life, resulting in dimorphic cytoarchitectural differences in males and females (Davis et al., 1996; Garcia-Segura et al., 2008; McCarthy et al., 2002). It was previously demonstrated that there is a sensitive period in perinatal life when both GABA and glutamate are excitatory, after which GABA gradually switches to mediate inhibitory processes in the adult brain (McCarthy, 2008). Interestingly, the ability of estradiol to enhance GABA synthesis, and to extend the developmental duration of excitatory GABA in cultured hypothalamic neurons, has also been documented (McCarthy et al., 2002). In our model, we observed elevated gene expression of *Gad67*, the rate-limiting enzyme in GABA synthesis at PND4, suggesting that chronically elevated testosterone and/or its metabolites may alter the circuitries of stimulatory/inhibitory inputs perinatally, resulting in an accelerated GnRH pulsatility in prepubertal life. In addition to the ability of steroids to alter parameters of the GABAergic and glutamatergic neurotransmission perinatally, glia cells in the ARC have been shown to modulate synaptic function by changing their morphology and neuroplastic actions in response to estrogen treatment (Garcia-Segura et al., 2008). Finally, GnRH neurons express estrogen receptor β and LH receptor (Chu et al., 2009; Mores et al., 1996), which in the presence of elevated agonist levels may alter the GnRH neuron physiology. Wether any of the above-mentioned factors is compensating for the low *Kiss1* expression and the main cause for the premature activation of GnRH neurons in the hCGαβ+ mice is subject for future investigations.

One of the most striking findings in this study was the pronounced and permanent gonadal steroid-independent attenuation of the gonadotropin synthesis in the hCGαβ+ males, as evidenced by lack of response of the low FSH secretion to the release of gonadal negative feedback by castration or flutamide treatment at prepuberty and in adulthood. Moreover, also the levels of *Fshb*, *Lhb* and *Cga* expression remained low after castration from PND14 onward. In addition, the accelerated GnRH pulse frequency of the hCGαβ+ males diminished after castration indicating that, unlike gonadotropin regulation, the increased GnRH pulse frequency was dependent on gonadal signals. In contrast, when flutamide treatment began on gestational day 18 and was maintained until PND28, a partial recovery of both FSH synthesis and secretion occurred in the hCGαβ+ males, accompanied by increased *Gnrhr* expression. These results suggest the existence of a critical window in perinatal life when androgens determine the level of activation of the HPG axis. During this period, elevated androgen levels may induce an irreversible shut-down of the pituitary gonadotrope differentiation, gonadotropin synthesis and secretion, where the regulation of the GnRH receptor plays a key role. These findings strongly suggest that androgen excess is able to disrupt the developmental programming of the male HPG axis. A direct testosterone-dependent regulation of hypothalamic aromatase expression was demonstrated in hCGαβ+ males, indicating that locally produced estrogens might play a key role in the hypothalamic–pituitary phenotype of these mice. The fact that FSH recovery after perinatal flutamide treatment was partial suggests an incomplete blockade of androgen action by flutamide, or the involvement of other mechanisms besides androgens, probably mediated by an estrogen receptor (Chu et al., 2009; Lindzey et al., 1998). In this sense, our results suggest that, together with gonadal androgens, the hypothalamic formation of estrogens may be participating in the mechanism of gonadotropin suppression in the hCGαβ+ males during the critical early stage of development. In fact, active hypothalamic aromatization of testosterone may be a pre-requisite for the feedback regulation, because estradiol produced prenatally outside the central nervous system may not be bioavailable due to its avid binding to α-fetoprotein in serum (De Mees et al., 2006).

The perinatal exposure to steroids is sexually dimorphic and influences differently the selected hypothalamic nuclei involved in the control of reproductive hormone secretion and sexual behavior in adulthood (McCarthy, 2008). Since opposite effects of the gonadal steroids were described on AVPV and ARC in relation to

the GnRH/gonadotropin system, it is worth to define in future studies in which precise hypothalamic areas the aforementioned changes occur in our model. In this respect, we are also interested in comparing the impact of the hCG hypersecretion on the developmental programming of the female hypothalamic–pituitary axis. In the mouse testis, the LH/hCG receptor has been found around gestational day 17 (O'Shaughnessy et al., 1998), whereas in the mouse ovary it is expressed postanatally, around PND5 (O'Shaughnessy et al., 1997). We, thus, expect to observe a different response to the hCG-induced- elevated gonadal steroids on the hypothalamic–pituitary development between sexes. Consequently, the present model would shed light into the mechanisms whereby androgens and other sex steroids contribute to building up the sexual dimorphism in the gonadotropin axis function.

New research has identified the presence of chemicals in the environment that have the potential to activate the androgen signaling pathway (Gray et al., 2006). Since unexplained infertility is common, the possibility that exposure to abnormal concentrations of steroids during gestation would be responsible for disrupting the male reproductive neuroendocrine control system in adulthood should be explored in humans. In this sense, the hCGαβ+ model is a powerful tool to investigate novel mechanisms mediated by androgens and their locally-produced metabolites in the (dys)regulation of the male HPG axis.

5. Conclusions

In conclusion, we demonstrated in this study that chronically elevated androgens induce accelerated GnRH pulse release, and irreversible suppression of pituitary gonadotropin synthesis and secretion in the hCGαβ+ males that can be re-established by blockade of the androgen action perinatally. These results indicate that abnormal androgen levels are able to disrupt the developmental programming of the gonadotropin system in males.

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