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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