Programming of the reproductive axis by hormonal and genetic manipulation in mice

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Abstract

In mammals, the reproductive function is controlled by the hypothalamic–pituitary–gonadal axis. During development, mechanisms mediated by gonadal steroids exert an imprinting at the hypothalamic–pituitary level, by establishing sexual differences in the circuits that control male and female reproduction. In rodents, the testicular production of androgens increases drastically during the fetal/neonatal stage. This process is essential for the masculinization of the reproductive tract, genitals and brain. The conversion of androgens to estrogens in the brain is crucial for the male sexual differentiation and behavior. Conversely, feminization of the brain occurs in the absence of high levels of gonadal steroids during the perinatal period in females. Potential genetic contribution to the differentiation of brain cells through direct effects of genes located on sex chromosomes is also relevant. In this review, we will focus on the phenotypic alterations that occur on the hypothalamic–pituitary–gonadal axis of transgenic mice with persistently elevated expression of the human chorionic gonadotropin hormone (hCG). Excess of endogenously synthesized gonadal steroids due to a constant hCG stimulation is able to disrupt the developmental programming of the hypothalamic–pituitary axis in both transgenic males and females. Locally produced estrogens by the hypothalamic aromatase might play a key role in the phenotype of these mice. The ‘four core genotypes’ mouse model demonstrated a potential influence of sex chromosome genes in brain masculinization before critical periods of sex differentiation. Thus, hormonal and genetic factors interact to regulate the local production of the neurosteroids necessary for the programming of the male and female reproductive function.

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Introduction

The hypothalamic–pituitary–gonadal (HPG) axis is essential for the normal function of the reproductive system in both males and females. Any alteration in the regulation of the different hormones or receptors involved in this process is enough to cause infertility. If these alterations occur during a critical period of fetal or postnatal development, the effects can be permanent and trigger, in addition to infertility, pathologies such as cancer, metabolic disorders and cardiovascular dysfunctions that are manifested in adulthood (Gluckman *et al.* 2008, Homburg 2009, Gore *et al.* 2015). It is remarkable that while the critical period of sexual differentiation includes fetal and early postnatal stages, the manifestation of certain pathologies and dysfunctions occurs later in life, beginning at puberty. This hypothesis, based on the developmental origin of adult diseases has become increasingly relevant in the field of reproductive endocrinology. The most significant cases in humans are infertility, polycystic ovary syndrome (PCOS), metabolic syndrome, cardiovascular diseases and diabetes (Gluckman *et al.* 2008, Homburg 2009).

In recent years, there has been growing interest in identifying potential adverse human health effects arising from environmental endocrine disruptors (Gore *et al.* 2015). The major disrupting agents described are those that mimic or antagonize the action of steroid hormones. However, endogenous hormones can also be considered endocrine disruptors when these are produced beyond the normal range of concentration or outside the critical time-window and may affect the normal development of the fetus/neonate. Pathologies such as congenital adrenal hyperplasia or PCOS that cause hyperandrogenism in the maternal environment are clear examples of inappropriate exposure to endogenous hormones during human development (Homburg 2009). Women exposed to excess androgens during early gestation exhibit hyperandrogenism, oligomenorrhea, polycystic ovary, in addition to LH hypersecretion, deficient embryonic development, insulin resistance with abdominal obesity, and hyperlipidemia (Homburg 2009). Epidemiological and experimental studies have revealed that *in utero* exposure to steroid hormones or endocrine disruptors may also influence the risk of tumor development

in adult life (Halakivi-Clarke *et al.* 2000, Palmer *et al.* 2006). However, the relationship between the intrauterine exposure and tumorigenesis is still poorly understood, and the identification of these mechanisms is of particular interest. Experimental animal models with excess of natural or synthetic steroid hormones have been instrumental in deciphering their impact on the reproductive physiology, both in males and females (Hakim *et al.* 2017, Paixao *et al.* 2017). The induction of prenatal hyperandrogenism in female Rhesus monkeys by testosterone administration has been pivotal for understanding the basis of PCOS (Abbott *et al.* 2009). Studies on sheep and rodents have also provided important information on this subject (Padmanabhan & Veiga-Lopez 2013, Paixao *et al.* 2017).

The precise mechanisms by which steroid hormones program the reproductive neuroendocrine axis and cause reproductive dysfunctions are not completely understood and are still under investigation. In contrast to the experimental models with exogenously administered androgens, which can exceed the physiological levels, the challenge is to identify the pattern of response to endogenously produced steroids under different experimental conditions. In addition to the gonadal hormones, recent evidence indicates that numerous sex-specific, genetic and epigenetic factors modulate sex differences in the brain and other tissues during development and may impact on the reproductive and metabolic processes in adulthood (Arnold *et al.* 2013, Arnold 2017).

In this review, we will summarize the neuroendocrine changes affecting the reproductive axis in genetically manipulated male and female mice. A transgenic mouse model with endogenously elevated sex steroids induced by human chorionic gonadotropin (hCG) became a useful tool to study the influence of gonadal steroids on the developmental programming of the hypothalamic–pituitary–gonadal function in males and females (Rulli *et al.* 2002, 2003, Gonzalez *et al.* 2011, 2014). On the other hand, studies with the ‘four core genotypes’ (FCG) mouse model demonstrated a potential influence of sex chromosome genes in brain masculinization before critical periods of sex differentiation and contribute to discriminate between the effects of sex chromosome complement and the gonadal phenotype (Arnold & Chen 2009, Cisternas *et al.* 2015, Itoh *et al.* 2015, Cisternas *et al.* 2017).

The influence of steroids on the neuroendocrine regulation during development

The onset of the reproductive function in mammals comprises a series of events that include the activation of the gonadotropic axis, the sexual differentiation of the brain and the development of the reproductive tract. Proper functioning in adulthood depends on adequate development of the axis during a critical

time-window, which, in rodents ranges from fetal to early postnatal stage (Weisz & Ward 1980, Rhoda *et al.* 1984, Huhtaniemi 1994, O’Shaughnessy *et al.* 1997, O’Shaughnessy *et al.* 1998, Arnold 2017). This phenomenon is sexually dimorphic and depends on gonadal steroids (Negri-Cesi *et al.* 2008, Gore *et al.* 2015): prenatal androgens masculinize the reproductive tract and perinatal estradiol (derived from testosterone) masculinizes the brain. Previous to this critical time window in which hormones have a key role, a genetic sex-chromosome component appears to be involved in the brain sexual differentiation (Cisternas *et al.* 2015, Arnold 2017).

The normal fetal testis is steroidogenically active and produces substantial levels of androgens that, in rodents, peak at the end of gestation (days 17–18 in mice; 18.5–19.5 in rats) (Weisz & Ward 1980, Huhtaniemi 1994, O’Shaughnessy *et al.* 1998). This process is necessary for the masculinization of the reproductive tract, and can be exerted directly through the androgen receptor, either by testosterone or by its metabolite 5 α -dihydrotestosterone (DHT), synthesized by the enzyme 5 α -reductase (Wilson & Davies 2007). A second surge of testosterone occurs soon after birth (Rhoda *et al.* 1984). Neonatally produced testosterone is aromatized to estradiol by the enzyme P450 aromatase in the brain, thus allowing estradiol to exert its effect through estrogen receptors (McCarthy 2008, Ruiz-Palmero *et al.* 2013). The conversion of gonadal testosterone to locally produced estradiol is crucial for the male sexual differentiation of the brain and affects sexual behavior (Negri-Cesi *et al.* 2008, Konkle & McCarthy 2011). Castration of males during the critical period of neonatal differentiation causes brain feminization (acquisition of typically female responses) or brain demasculinization (loss of typically male responses) (McCarthy 2008, Negri-Cesi *et al.* 2008).

In female development, the absence of testicular hormones is considered sufficient to achieve the differentiation of a brain with female characteristics. Although it has been proposed that the fetal ovary is relatively quiescent compared to the testis, androgens and estrogens have been shown to be detectable in the late fetal stage (Wilson & Davies 2007). Interestingly, the brain feminization is maintained by the active suppression of masculinization via DNA methylation (Nugent *et al.* 2015). In females, abnormal exposure to testosterone during critical periods of differentiation may cause masculinization (acquisition of typically male responses) or defeminization (loss of typically female responses), ovulatory and virilizing genital alterations. Depending on the time window of exposure, behavioral and ovulatory dysfunctions can coexist without virilized genitalia, such as during late prenatal stage (Gorski 1985, Rhees *et al.* 1997, Robinson 2006).

During gestation, the fetus is exposed to its own hormones, placental steroids and hormones of maternal

origin that are capable of crossing the placental barrier. However, the α -fetoprotein produced by the fetus and the yolk sac has affinity for estradiol and sequesters it, thus protecting the female brain from the effects of maternal or placental estrogens (Bakker *et al.* 2006). During a period between birth and puberty, estrogens play a role in feminization of neural and behavioral traits of female rodents (Bakker & Baum 2008).

The 'FCG' model: a transgenic mouse model for studying the sex chromosome influence

A genetic contribution of sex chromosomes to the differentiation of brain cells has been demonstrated with the FCG mouse model (Carruth *et al.* 2002, Scerbo *et al.* 2014, Arnold 2017). In this model, the testis-determining *Sry* gene is deleted from the Y chromosome and inserted onto an autosomal chromosome. The Y and autosomal chromosomes segregate independently and give rise to four different genotypes (Fig. 1). As a result, mice bearing the *Sry* gene develop testes and are masculinized, whereas mice lacking the *Sry* gene develop a female phenotype, irrespective of their sex chromosome (DeVries *et al.* 2002, Arnold & Chen 2009). This model allows comparing the effects of gonadal sex and chromosome sex complement.

The first neural phenotype reported to be influenced by sex chromosome was the expression of the rate-limiting enzyme in catecholamine biosynthesis, the tyrosine hydroxylase, in mesencephalic neurons harvested from embryonic day 14 (before the critical period of brain masculinization). Using the FCG mouse model, Carruth *et al.* (2002) demonstrated that XY cultures have more tyrosine hydroxylase-immunoreactive neurons than XX cultures irrespective of the gonadal type of the embryos. At the same time, the FCG model was used to study sexually dimorphic brain and behavioral phenotypes in adulthood. Most of the sexually dimorphic phenotypes

(male copulatory behavior, social exploration behavior and sexually dimorphic neuroanatomical structures in the hypothalamus and lumbar spinal cord) correlated with the presence of ovaries or testes, and therefore, reflect the hormonal output of the gonads. However, the density of vasopressin-immunoreactive fibers in the lateral septum of both male and female mice with XY sex chromosomes were more masculine than XX mice (De Vries *et al.* 2002), indicating that sex chromosome genes contribute directly to the development of a sex difference in the brain. Moreover, another sex chromosome effects have also been detected on aggression, body weight, habit formation and response to brain injury (Arnold & Chen 2009, Forger *et al.* 2015). Importantly, many reports indicate that sex chromosomes impact the incidence and progression of diverse models of brain diseases such as multiple sclerosis (Palaszynski *et al.* 2005, Smith-Bouvier *et al.* 2008), systemic lupus erythematosus (Smith-Bouvier *et al.* 2008), major depressive disorders (Seney *et al.* 2013a,b) and neural tube closure defects (Chen *et al.* 2008).

Recently, we have demonstrated sex chromosome-induced differences in P450 aromatase expression in the developing mouse brain as early as gestational day 16 (GD16) (Cisternas *et al.* 2015). Using the FCG mouse model, we found that XY mouse embryos show higher P450 aromatase expression than the brain of XX embryos, independently of gonadal sex. Furthermore, estradiol or DHT increases P450 aromatase expression in cultures of anterior amygdala neurons derived from XX embryos, but not in those derived from XY embryos. The mechanism of P450 aromatase regulation by hormones involves ER β since the antiandrogen flutamide is not able to prevent P450 aromatase increase by DHT, while the ER β antagonist PHTPP blocked the effect of both estradiol and DHT (Cisternas *et al.* 2017). In addition, 3 β -diol, which has been reported to preferentially bind ER β (Kuiper *et al.* 1998), mimics the effects of estradiol and DHT on P450 aromatase expression (Cisternas *et al.* 2017). Thus, hormonal and genetic factors interact to regulate the expression of the key enzyme necessary for brain masculinization during development. Differences in the local production of estradiol by aromatization of testosterone due to genetic factors could impact on the arrangement of neural circuits underlying male and female behavior.

Transgenic mice hypersecreting hCG: a mouse model for endogenously elevated gonadal steroids

An increased gonadal response to luteinizing hormone (LH) or hCG leads to enhanced steroidogenesis through the LH/hCG receptor. hCG has a higher receptor affinity and a longer circulating half-life than LH, due to the carbohydrates associated to its molecule (Banerjee & Fazleabas 2011, Choi & Smitz 2014). hCG is normally secreted by the human trophoblastic cells

Figure 1 Four core genotypes (FCG) mice obtained by crossbreeding XX females with XY-Sry transgenic males. Representative figure showing the genotypes of XX and XY mice with testes (XXSry, XY-Sry) and XX and XY mice with ovaries (XX, XY-).

Figure 2 Effect of castration on the hypothalamic-pituitary axis of transgenic male and female mice hypersecreting hCG (hCGαβ+). Serum FSH levels and gene expression of hypothalamic *Cyp19a1* and *Kiss1*, and pituitary *Gnhr* of prepubertal WT and hCGαβ+ mice after gonadectomy (Cx for males, Ovx for females) are shown. Intact prepubertal WT and hCGαβ+ males and females were used as controls. The relative mRNA expression was carried out by qRT-PCR ($n=4$). Data are presented as mean $\pm$ S.E.M. Two-way ANOVA, followed by Bonferroni's *post hoc* test. Different letters indicate a value of at least $P<0.05$ (adapted from Gonzalez *et al.* 2011, 2014).

(Fisheletal. 1984), being essential to maintain progesterone production by the corpus luteum of pregnancy. Various investigations have demonstrated the presence of LH/hCG receptors in numerous fetal and adult organs, as well as tumor tissues (Iles *et al.* 2010). Of note is the presence of these receptors in the brain, adipose tissue, pancreas, uterus and endothelial cells, in addition to the gonads (Lei *et al.* 1993, Cole & Butler 2012). However, the physiological relevance of these extra-gonadal LH/hCG receptors is not completely understood.

We have generated a transgenic mouse model carrying the common α - and hCG β subunit genes (hCGαβ+ mice) under a constitutive promoter that leads to the transgene expression from GD10.5 (Rulli *et al.* 2002, 2003). These mice hypersecrete hCG, are infertile and suffer significant alterations on the neuroendocrine regulation of the gonadotropin axis, particularly due to an increased gonadal steroid production (Rulli *et al.* 2002, 2003, Gonzalez *et al.* 2011, 2014). Even though males and females exhibit substantial differences in

their phenotypes, steroid hormones induce important alterations on the gonadotropin synthesis and secretion in both sexes. The main differences appear to be based on the time window in which each sex is particularly sensitive to hormonal changes during development. Whereas the functional LH/hCG receptor is normally expressed from GD17 in the mouse testis (O'Shaughnessy *et al.* 1998), it occurs at PND5 in the mouse ovary (O'Shaughnessy *et al.* 1997). Therefore, males and females exhibit differential responses to elevated hCG-induced steroids during development: prenatal/neonatal changes in the male and postnatal in the female.

Males

As a consequence of elevated hCG, transgenic hCGαβ+ males show high levels of testosterone and progesterone, accompanied by an increased weight of androgen-dependent organs, such as the prostate and seminal

Figure 3 Hypothalamic aromatase after flutamide administration in transgenic mice hypersecreting hCG (hCGαβ+). In males, flutamide normalized aromatase expression when administered from gestational day 18 (GD18) to postnatal day 28 (PN28); analyzed by qPCR. In females, flutamide normalized aromatase expression when administered from PND6 to PND21; analyzed by western blot. Data are presented as mean ± S.E.M. Two-way ANOVA, followed by Bonferroni's *post hoc* test. Different letters indicate a value of at least $P < 0.05$.

vesicles (Rulli *et al.* 2003, Gonzalez *et al.* 2011). These males exhibit reduced testicular size and develop Leydig cell adenomas derived from the fetal Leydig cell population (Ahtiainen *et al.* 2005). The hypothalamic-pituitary function of prepubertal hCGαβ+ males is also affected, as manifested by the high hypothalamic GnRH concentration and accelerated GnRH pulse frequency, which induces downregulation of the GnRH receptor and reduction of FSH synthesis and secretion at the pituitary level (Gonzalez *et al.* 2011). Other transgenic models of LH/hCG overexpression (Matzuk *et al.* 2003) or constitutive activation of LH/hCG receptor (Meehan *et al.* 2005) also showed low levels of FSH and reduced testicular weight.

Kisspeptin (*Kiss1*) and its G protein-coupled receptor 54 (*Kiss1r*) are essential components of the HPG axis and the onset of puberty, by controlling gonadotropin secretion through GnRH stimulation. Different studies show that kisspeptin control of GnRH occurs by modulating the negative feedback mechanism of gonadal steroids in both sexes (Clarkson 2013, Poling & Kauffman 2013). Neonatal administration of estrogenic compounds results in a dose-dependent decrease of *Kiss1* expression in prepubertal males and females (Navarro *et al.* 2009). Prepubertal hCGαβ+ males exhibit reduced hypothalamic levels of *Kiss1* expression, which is consistent with the suppressive action of high circulating levels of testosterone and locally converted estrogens through an increased hypothalamic P450 aromatase expression (*Cyp19a1*) (Fig. 2) (Gonzalez *et al.* 2011). It was demonstrated that estradiol is able to

alter the morphology and synapses of glial cells in the arcuate nucleus (Garcia-Segura *et al.* 2008). Since GnRH neurons express ERβ and LH/hCG receptor, elevated levels of agonists may alter the physiology of these neurons (Chu *et al.* 2009). Even though a direct effect of hCG *in vivo* cannot be discarded, studies performed on fetal hypothalamic neurons *in vitro* showed that *Cyp19a1* expression was not directly affected by hCG (Gonzalez *et al.* 2014).

Interestingly, FSH levels remain low after prepubertal castration in hCGαβ+ males, thus indicating that the FSH response to the androgen feedback regulation is severely affected in these mice. The mRNA expressions of pituitary *Gnrhr*, hypothalamic *Cyp19a1* and *Kiss1* also remain unaltered under these conditions (Fig. 2; Gonzalez *et al.* 2011). Since the testosterone surge by the time of birth is essential for the establishment of the male sexual behavior and reproductive physiology in mice, the next step was to analyze the effect of androgen deprivation perinatally. The administration of the antiandrogen flutamide to pregnant mothers from GD18 and then from birth to 28 days of age to hCGαβ+ males induced a recovery of FSH synthesis and secretion, accompanied by a normalization of hypothalamic *Cyp19a1* (Fig. 3; Gonzalez *et al.* 2011). These findings identified a critical time window in which androgens, by acting through their receptor, modulate the activation of the hypothalamic-pituitary axis in these mice. During this period, elevated levels of androgens would induce an irreversible impairment of the hypothalamic function, along with the synthesis and secretion of gonadotropins, processes in which kisspeptin and GnRH receptor regulation play a key role. In normal conditions, GnRH stimulates the gonadotropic response and cell proliferation during early pituitary differentiation. Moreover, a correct connection between the hypothalamus and the pituitary is necessary for the development of a normal number of thyrotropes and gonadotropes during late gestation, as was shown in sheep (Szarek *et al.* 2008). On the other hand, the absence of fetal GnRH signaling specifically inhibits the differentiation of FSH-producing gonadotropes (Wen *et al.* 2010).

This evidence shows that the low levels of FSH associated with the persistently elevated hCG would not be solely due to the negative feedback exerted by gonadal steroids, but to a failure in the perinatal programming exerted by androgens and/or their locally-produced neurosteroids on the developing hypothalamic-pituitary unit of hCGαβ+ males. All these changes ultimately impact on the fertility of these mice at adulthood.

Females

Numerous studies have been published about the effect of androgens on the female developmental

Figure 4 Summary of the impact of persistent hCG stimulation on the developmental programming of the hypothalamic-pituitary axis in both transgenic hCG hypersecreting male and female mice (hCGαβ+; perinatal in males, and postnatal in females). *Cyp19a1*, P450 aromatase (Gonzalez et al. 2011, 2014); E2, estradiol; FSH, follicle stimulating hormone; GD 16–18, gestational day 16–18; Gnhr, Gonadotropin releasing hormone; *Kiss1*, kisspeptin 1; LHR, luteinizing hormone receptor; PND6, postnatal day 6; SV, seminal vesicles; T, testosterone.

programming, which in turn would impact on the metabolic, behavioral and reproductive function at adulthood. Administration of testosterone propionate to female rats induces several reproductive alterations, depending on the time window of administration, that is, fetal, neonatal or late postnatal age (Tyndall et al. 2012, Paixao et al. 2017). Prenatal administration of testosterone or DHT to mice or rats induces acyclicity, advanced puberty and infertility at adulthood. Additionally, the early postnatal exposure to androgens is also capable of inducing ovulatory dysfunctions and alterations in mating behavior. Thus, early exposure to an excess of androgens during fetal or postnatal life would induce long-lasting alterations on the reproductive programming.

As demonstrated in males (Gonzalez et al. 2011), the regulation of the hypothalamic-pituitary unit is also affected in transgenic hCGαβ+ females (Gonzalez et al. 2014). These females show elevated testosterone levels at least from PND14 onward. These are expected to be high by the second week of life, considering that the LH receptor is active by PND5 in the mouse ovary (O'Shaughnessy et al. 1997). In normal conditions, a FSH surge occurs during the first two weeks, when the pituitary-gonadal feedback regulation is not fully

active (Dullaart et al. 1975). In hCGαβ+ females, this early FSH surge is suppressed, together with a reduced gene expression of *Fshb*, *Lhb* and *Gnrhr* at the pituitary level (Fig. 2; Gonzalez et al. 2014). In addition, precocious puberty is accompanied by a transient surge of estradiol. This evidence suggests that the negative feedback regulation of the gonadotropin axis occurs prematurely in these females. A similar phenotype was found in the bLHβ-CTP mouse model with chronically elevated LH, where high testosterone was present at 14 days of age and led to precocious puberty and transient estradiol elevation (Risma et al. 1997). The expressions of *Cyp19a1* and *Kiss1* in the preoptic area, which is the sexually-differentiated hypothalamic area that controls the ovulatory LH surge and displays estradiol-induced positive feedback, are elevated in 21-day-old transgenic females (Fig. 2). In contrast to the phenotype observed in males, hCGαβ+ females are able to respond to ovariectomy at PND14 by increasing serum FSH levels (Fig. 2) and gene expression of pituitary *Fshb* and *Lhb*, and also prevents the premature vaginal opening in these mice (Gonzalez et al. 2014). However, as was also demonstrated in hCGαβ+ males, castration was unable to affect the hypothalamic *Kiss1* and *Cyp19a1* expression in hCGαβ+ females (Fig. 2). Interestingly, when the antiandrogen flutamide is administered to hCGαβ+ females from PND6 until puberty, aromatase from the preoptic area is normalized (Fig. 3; Gonzalez et al. 2014). These results show that, in females, early exposure to androgens during a critical time-window between the second and third week of life induces sex-specific changes on the hypothalamus that alter the P450 aromatase expression at peripuberty. Consequently, changes in the locally-produced steroids may have implications in the occurrence of abnormal ovulatory LH surge of the reproductive cycles at adulthood, thus culminating in female infertility.

Conclusions

Differently from experimental models where hyperandrogenism is induced by exogenous administration, transgenic hCG-hypersecreting mice are a useful tool to study how the endogenously produced gonadal steroids *in vivo* may impact on the male or female reproductive axis (Fig. 4). Nevertheless, the precise mechanisms by which early exposure to steroid hormones affect the reproductive function in males and females are still under debate, even in the light of the new evidence showing the existence of genetic factors that precede gonadal influences during the genesis of differences between the sexes in brain structure (Cisternas et al. 2015, 2017, Arnold 2017). In males, information about the possible influence of elevated androgens on the developmental programming of the hypothalamic-pituitary axis is limited, probably because

males are normally exposed to androgens from early stages of fetal development and throughout their lives. Evidence derived from transgenic hCG males show that excess of endogenous androgens during a critical time- window between GD18 and PND14 induces long-lasting changes on the reproductive axis, resulting in a premature activation of the hypothalamus and a concomitant silencing of the pituitary gonadotropin production and seems to be the cause of infertility in adulthood. In females, early exposure of steroids during a critical period between PND6 to 14 induces sex-specific organizational changes of the hypothalamus. These changes would have an effect at the preoptic area and alter the P450 aromatase expression, which in turn, would have an impact on the ovulatory cycles and fertility at adulthood. In both sexes, androgens and their locally produced neurosteroids might play a key role in the dysregulation of the hypothalamic–pituitary function during development.

Declaration of interest

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

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