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Research report

Neonatal inhibition of androgen activity alters the programming of body weight and orexinergic peptides differentially in male and female rats

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ABSTRACT

The involvement of androgens in the regulation of energy metabolism has been demonstrated. The main objective of the present research was to study the involvement of androgens in both the programming of energy metabolism and the regulatory peptides associated with feeding. For this purpose, androgen receptors and the main metabolic pathways of testosterone were inhibited during the first five days of postnatal life in male and female Wistar rats. Pups received a daily s.c. injection from the day of birth, postnatal day (P) 1, to P5 of Flutamide (a competitive inhibitor of androgen receptors), Letrozole (an aromatase inhibitor), Finasteride (a 5-alpha-reductase inhibitor) or vehicle. Body weight, food intake and fat pads were measured. Moreover, hypothalamic Agouti-related peptide (AgRP), neuropeptide Y (NPY), orexin, and proopiomelanocortin (POMC) were analyzed by quantitative real-time polymerase chain reaction assay. The inhibition of androgenic activity during the first five days of life produced a significant decrease in body weight in females at P90 but did not affect this parameter in males. Moreover, the inhibition of aromatase decreased hypothalamic AgRP mRNA levels in males while the inhibition of 5α-reductase decreased hypothalamic AgRP and orexin mRNA levels in female rats. Finally, food intake and visceral fat, but not subcutaneous fat, were affected in both males and females depending on which testosterone metabolic pathway was inhibited. Our results highlight the differential involvement of androgens in the programming of energy metabolism as well as the AgRP and orexin systems during development in male and female rats.

1. Introduction

Gonadal steroids are involved in the regulation of energy metabolism with differential effects in males and females (Mauvais-Jarvis, 2011; Clegg, 2012; Mauvais-Jarvis et al., 2013; Santollo and Eckel, 2013; López and Tena-Sempere, 2015; Wang and Xu, 2019). The influence of estrogens on feeding has been studied for much longer than that of other steroids (Asarian and Geary, 2006; 2013). It has been reported that estradiol, through estrogen receptors (ER) α, ERβ and G-protein coupled ER (GPER) inhibits food intake and regulates adipose tissue distribution in males and females (Roepke, 2009; Shi et al., 2009; Asarian and Geary, 2013; Mauvais-Jarvis et al., 2013; Meyer et al., 2011; Frank et al., 2014; Santollo and Daniels, 2015; Ponnusamy et al., 2017; Butler et al., 2018) and some of the mechanisms underlying this regulation are known (Gao and Horvath, 2008).

Androgens promote abdominal fat deposition, regulate lipolytic activity and their receptors are expressed in adipose tissue, (Fan et al., 2005; Shi et al., 2009; Asarian and Geary, 2013). Moreover, androgens also participate in energy metabolism by regulating meal frequency, central leptin OBRB-STAT3 signalling, and energy expenditure, (Chai

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et al., 1999; Fan et al., 2005; 2008; Asarian and Geary, 2013). It is important to take into account that some of these studies were performed with testosterone treatments following orchidectomy. Considering that testosterone can have estrogenic effects, as it is susceptible to metabolization to estradiol by the action of aromatase, some of those effects may be due to estradiol aromatized from testosterone. However, some direct or indirect action of androgens via androgen receptors (AR) have also been reported. Male AR deficient mice develop obesity (Fan et al., 2005), as well as leptin and insulin resistance (Lin et al., 2005), and AR favours central leptin signalling in the hypothalamus throughout AR activity (Mauvais-Jarvis, 2011). Nevertheless, although there is some evidence that androgens are involved in the regulation of energy metabolism, their mechanisms are poorly understood.

Hypothalamic circuits regulate food intake through the balance of anorexigenic and orexigenic peptides and several nuclei of the hypothalamus participate in this regulation. Signalling from the periphery about the energy status of the body reaches the proopiomelanocortin (POMC) and neuropeptide Y/Agouti-related protein (NPY/AgRP) neurons in the arcuate nucleus (Arc). Second order neurons located in the perifornical area (PFA) and lateral (LH), dorsomedial (DMH) and paraventricular (PVH) nuclei of the hypothalamus receive anorexigenic signals via POMC derived α-MSH, and/or orexigenic input via NPY and AgRP neuropeptides. The integration of all these signals determines the initiation or cessation of food intake (Schwartz et al., 2000; Krashes et al., 2014; Morton et al., 2014; Stuber and Wise, 2016).

Androgens can modulate the balance of neuropeptides that control feeding in adults. Administration of nandrolone decanoate, an anabolic androgenic steroid, leads to a decrease in POMC mRNA expression (Lindblom et al., 2003). In addition, other authors have shown the relationship between androgens and neuropeptide Y (NPY), observing that gonadectomy decreased the levels of this peptide and testosterone treatment increased them in the Arc and the ventromedial nucleus of the hypothalamus (VMH) (Sahu et al., 1990, 1992). Finally, it has also been shown by *in situ* hybridization that the decrease in testosterone levels produced by gonadectomy reduces the levels of cocaine- and amphetamine-related transcript (CART) mRNA in the PVH in young and old rats, and that NPY levels increase in the rostral area of the Arc, especially in young animals (Sohn et al., 2002).

It has also been demonstrated that leptin (Bouret et al., 2004) and ghrelin (Steculorum et al., 2015) are involved in the establishment of hypothalamic circuits that regulate feeding in mice and that estradiol is involved in the programming of energy metabolism and the proopiomelanocortin system differentially in male and female rats, early after birth (Carrillo et al., 2020). Androgens, like estrogens, have been implicated in the organization of estrogen and androgen receptor expression in pituitary and adrenal glands (Lagunas et al., 2022) as well as that of hypothalamic circuits and the vomeronasal system during the first days of life in rats. Specifically, estradiol aromatized from testosterone on the day of birth promotes the differentiation of the volume and number of neurons of different brain structures including the sexodimorphic nucleus of the medial preoptic area, the nucleus of the stria terminalis, the cortical posteromedial amygdala or the locus coeruleus, among others (Gorski, 1985; Collado et al., 1992; Valencia et al., 1992; Guillamón and Segovia, 1997), but to our knowledge there are no studies on the participation of androgens in the programming of the feeding system in rats. Only Nohara et al.'s (2011) study showed the participation of testosterone in the programming of some parameters of energy metabolism and the control of feeding in mice in the first day of life.

Therefore, considering the involvement of androgens in the regulation of feeding as well as their actions on the Arc in mice early after birth, the main aim of the present research is to study the possible involvement of androgens in the programming of the hypothalamic circuits that regulate feeding. For this purpose, androgen receptors and their main metabolic pathways were inhibited during the first five days of postnatal life in rats and several physiological parameters, along with the gene expression of various neuropeptides related to food intake were analysed. Finally, this study will include males and females, firstly because sex differences have been shown in several processes underlying energy metabolism and the programming of the systems that regulate food intake and secondly, because the presence of androgens and its receptors, as well as aromatase and 5α-reductase activity has been reported during the first days of life in rats of both sexes in plasma and in different brain areas, including the hypothalamus (Weisz and Ward, 1980; Karolczak et al., 1998; Konkle and McCarthy, 2011; Brock et al., 2015; Cisternas et al., 2017).

2. Material and methods

Throughout the study, special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required. All experiments were designed and conducted according to the guidelines presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, the European Union legislation (Council Directives 86/609/EEC and 2010/63/UE), and the Spanish Government Directive (R.D. 1201/2005). Experimental procedures were approved by the Local Institutional Bioethical Committee (UNED, Madrid).

2.1. Animals and experimental treatments

Wistar male and female rats were kept in an automatically controlled room, under stable temperature, humidity and light conditions (22º ± 2º C; 55 ± 10% humidity; 12 h light/12 h dark cycle, lights on from 08:00–20:00), and received food and water *ad libitum*.

For mating, a male was placed in a cage with two females for 1 week. Pregnant females were housed individually in plastic maternity cages with wood shavings as nesting material. The twelve pregnant females were monitored and on postnatal day (P) 1 (P1), pups from litters born within the same 24 h were weighed, sexed, and randomly distributed to the different experimental conditions, assigning five females and five males to each mother.

Pups received a daily s.c. injection (volume of 0.01 ml/kg) from P1 to P5. Flutamide groups: 10 males (FluM) and 10 females (FluF) were injected with the competitive inhibitor of AR, flutamide, that creates an inactive form that cannot translocate into the cell nucleus, (25 mg/kg, F9397, Merk); letrozole groups: 10 males (LetM) and 10 females (LetF) were injected with the aromatase inhibitor, letrozole (1 mg/kg, L6545, Merck); finasteride groups: 10 males (FinM) and 10 females (FinF) were injected with the competitive inhibitor steroid type II 5-α-reductase, that interferes with the enzymatic conversion of testosterone to 5-DHT, (5 mg/kg, NB-48–0403–100MG, Quimigen); and control groups: 10 males (CM) and females (CF) were injected with vehicle (corn oil). All doses were selected based on previous studies (Fanaei et al., 2013; Yamada et al., 2015; Cataldi et al., 2018; 2022).

Body weight and food intake were registered every 7 days from postweaning (P28) to P90. Every 7 days, a fixed amount of food was supplied to the animals. To calculate the intake, the difference between the food supplied and the leftover food measured in grams was measured.

On postnatal P90 the animals were weighed and decapitated between 9:00 and 11:00 a.m. All female rats were decapitated in the diestrus phase. The hypothalamus and subcutaneous (abdominal) and visceral (perigonadal) were rapidly removed, weighed, and frozen at −80°C.

2.2. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the isolated tissues with an illustra RNAspin mini-RNA isolation kit (GE Healthcare) following the manufacturer's instructions to measure orexin, agouti, NPY and POMC mRNA, and Pgk1 as control housekeeping gene.

Two μg of total RNA was used to synthesize cDNA using M-MLV

reverse transcriptase (Promega, United States) and random primers (Invitrogen, United States), following the manufacturer's instructions.

For quantitative real-time PCR, assay-on-demand gene expression products (Applied Biosystems) were used: Agouti-related peptide (AgRP: RN014311703_g1), NPY (Rn01410145_m1), orexin (Hcrt Rn00565995), POMC (Rn00595020_m1), and phosphoglycerate kinase (pgk1; Rn00569117_m1). For amplification, TaqMan Universal PCR Master Mix (Applied Biosystems) was used in accordance with the manufacturer's instructions in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). All samples were amplified in duplicate. Values were normalized to the housekeeping gene Pgk1 (Dheda et al., 2004; Pohjanvirta et al., 2006) that did not show differences between groups. Following the manufacturer's guidelines, the ΔΔCT method was used to determine relative expression levels. Statistics were performed using ΔΔCT values.

2.3. Statistical analysis

The evolution of body weight during development was analysed using a repeated measure ANOVA with sex and treatment as the within-subject factors and body weight as the between-subject factor. After that, in order to know when differences between each treatment with respect to the control group began, one-way ANOVA in each timepoint was performed. To determine sexual dimorphism in this parameter, ANOVAs between males and females with treatment as a factor were carried out. The significance level was set at $p<0.05$. To determine intra-sexes differences, male and female groups were analysed independently using a one-way ANOVA, with the significance level set at $p<0.05$.

Energy intake, body weight at sacrifice and Hypothalamic peptides mRNA levels were analysed following the same procedure as that used in body weight analyses: to determine sexual dimorphism, ANOVAs between males and females with treatment as a factor were carried out. The significance level was set at $p<0.05$. To determine intra-sexes differences, male and female groups were analysed independently using a one-way ANOVA, with the significance level set at $p<0.05$.

3. Results

3.1. Body weight

3.1.1. Body weight during development and P90

The evolution of body weight showed the expected differences that always occur between male and female rats. Our data detected a main effect of sex in body weight during development in control ($F1,15=80.075$; $p<0.0001$), flutamide ($F1,15=16.255$; $p<0.0001$), letrozole ($F1,15=88.955$; $p<0.0001$) and finasteride ($F1,15=0160$; $p<0.0001$) groups. However, some of the treatments altered the emergence of the differences between the sexes. Post hoc analyses showed an increase in body weight in all groups studied, with significant difference from P35 onwards between CM and CF, FluM and FluF; FinM and FinF ($p<0.0001$ in all cases), although males had higher body weight than females in all cases (Table 1). In the case of Letrozole groups, sex differences were observed from P42 onwards ($p<0.0001$) with males weighing more than females (Table 1). It is important to note that, since the weight of the animals was recorded once a week, these differences do not necessarily appear exactly one week later, however, the results allow us to verify that there are significant differences in the appearance of sexual dimorphism.

When analysed separately, males and females presented different developmental patterns depending on the treatment administered. In males, a transient difference was shown in P21, P35, P42 and P49 between CM and FluM, with CM weighing more than FluM ($p<0.05$ in all cases) However, no significant differences were found from P56 to P84 (Fig. 1A). Regarding the male letrozole group, no significant differences were found in body weight during development with respect to CM. Finally, treatment with finasteride showed significant differences with respect to the control group at P77 and P84, with FinM having greater body weight than CM (Fig. 1B).

With respect to the females, our results showed that a lack of activity of AR or aromatase altered the evolution of body weight, since treatment with flutamide significantly decreased body weight from P21 to P84, (except on P28) ($p<0.05$ in all cases) (Fig. 1C), and a significant difference was found from P70 onwards ($p<0.0001$ in all comparisons) in females treated with letrozole, with CF weighing more than LetF (Fig. 1D). However, no significant differences in body weight during development were detected in females treated with finasteride.

3.1.2. Body weight at P90

When body weight was analysed at P90, a main effect of sex was detected in control ($F1,8=190.822$; $p<0.001$), flutamide ($F1,15=16.255$; $p<0.0001$), letrozole ($F1,15=88.955$; $p<0.0001$) and finasteride ($F1,15=0.160$; $p<0.0001$) groups, with males being heavier than females in all cases (Table 2). When each sex was analysed separately, no differences were shown between the control group and any of the treatments administered; flutamide, letrozole or finasteride in male rats. However, all treatments had an effect in the case of the females, since a decrease in body weight was observed at P90 in FluF ($F1,8=8.621$; $p=0.019$), LetF ($F1,8=18.251$; $p=0.003$) and FinF ($F1,8=7.608$; $p=0.025$) groups (Fig. 1E,F,G, respectively).

3.2. Food Intake

Significant differences were found between males and females in food intake in all treatments studied. Differences between CM and CF ($F1,7=23.021$; $p=0.002$), FluM and FluF ($F1,8=111.05$; $p<0.0001$), LetM and LetF ($F1,7=16.918$; $p=0.003$) and FinM and FinF ($F1,8=278.811$; $p<0.0001$) were detected. In all cases males ate significantly more than females (Table 2).

When male groups were analysed separately, significant differences were detected between CM vs FluM ($F1,8=9.675$; $p=0.017$) and CM vs LetM ($F1,8= 8.560$; $p=0.022$). In both cases CM ate significantly more than FluM or LetM (Fig. 2A,B). However, no differences were observed with respect to the control group when males were treated with finasteride.

When the female groups were analysed, a significant decrease in energy intake was observed with finasteride treatment ($F1,8=14.325$; $p=0.005$) (Fig. 2C). However, no differences were observed between control females and flutamide or letrozole females.

3.3. Visceral and subcutaneous fat

While significant differences between sexes were not detected in

Table 1
Sex differences during development in body weight.

	P21	P28	P35	P42	P49	P56	P63	P70	P77	P84
CM *vs* CF			*	*	*	*	*	*	*	*
FLUM *vs* FLUF			*	*	*	*	*	*	*	*
LETM *vs* LETF				*	*	*	*	*	*	*
FINM *vs* FINF			*	*	*	*	*	*	*	*

* = differences between males and females of the same groups

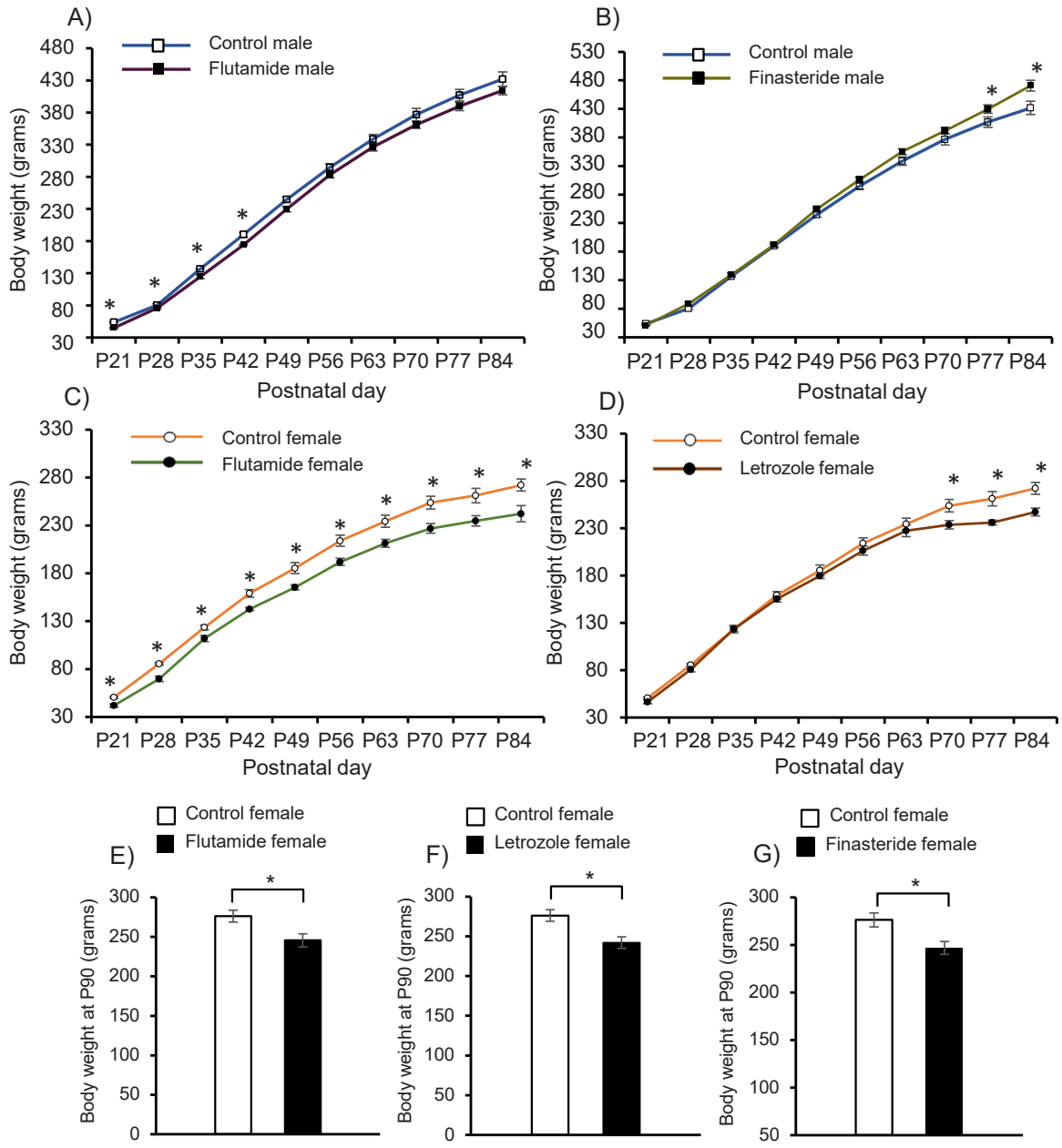

Fig. 1. Graphs show significant differences in the evolution of body weight from weaning to postnatal day 84 among groups due to treatment administered in each sex (A-D). Graphs (E-F) represent significant differences in final body weight in females since were not detected differences between treated males and their respective control groups. A) control male vs flutamide male; B) control male vs finasteride male; C) control female vs flutamide female; D) control female vs letrozole female. E) control female vs flutamide female; F) control female vs letrozole female; G) control female vs finasteride female. * shows statistically significant differences ($p<0.05$ in all cases). All values are expressed as mean ± S.D.

Table 2
Summary of sex differences.

	Control	FLU	FIN	LET
Food intake	♂>♀	♂♀	♂>♀	♂>♀
Body Weight (P90)	♂>♀	♂♀	♂>♀	♂>♀
Visceral fat	n.s.	n.s.	n.s.	n.s.
Subcutaneus fat	♂>♀	♂>♀	♂>♀	n.s.
Brown fat	n.s.	n.s.	n.s.	n.s.
Orexin	n.s.	n.s.	n.s.	♂>♀
Agouti	♂>♀	♂>♀	♂>♀	♂>♀
NPY	♂<♀	n.s.	n.s.	n.s.
POMC	n.s.	♀>♂	n.s.	♂>♀

visceral fat, our data showed significant sex differences between CM vs CF ($F1,7=868$; $p=0.02$), FluM vs FluF ($F1,8=30.060$; $p=0.001$) and FinM vs FinF ($F1,8=23.049$; $p=0.001$) in subcutaneous fat, with males having more subcutaneous fat than females. No sex differences were detected in subcutaneous fat between letrozole groups (Table 2).

When visceral fat depots were analysed separately in male and female rats, it was observed that there was a differential response between the sexes to this parameter. While no significant differences were detected in visceral fat in male groups, significant differences between CF vs FluF ($F1,8=6.222$; $p=0.037$) and CF vs LetF ($F1,8=5.453$; $p=0.48$) were found, with CF showing higher values than FluF and LetF (Fig. 3A, B). However, neither males nor females showed significant differences in subcutaneous fat with respect to their respective control groups.

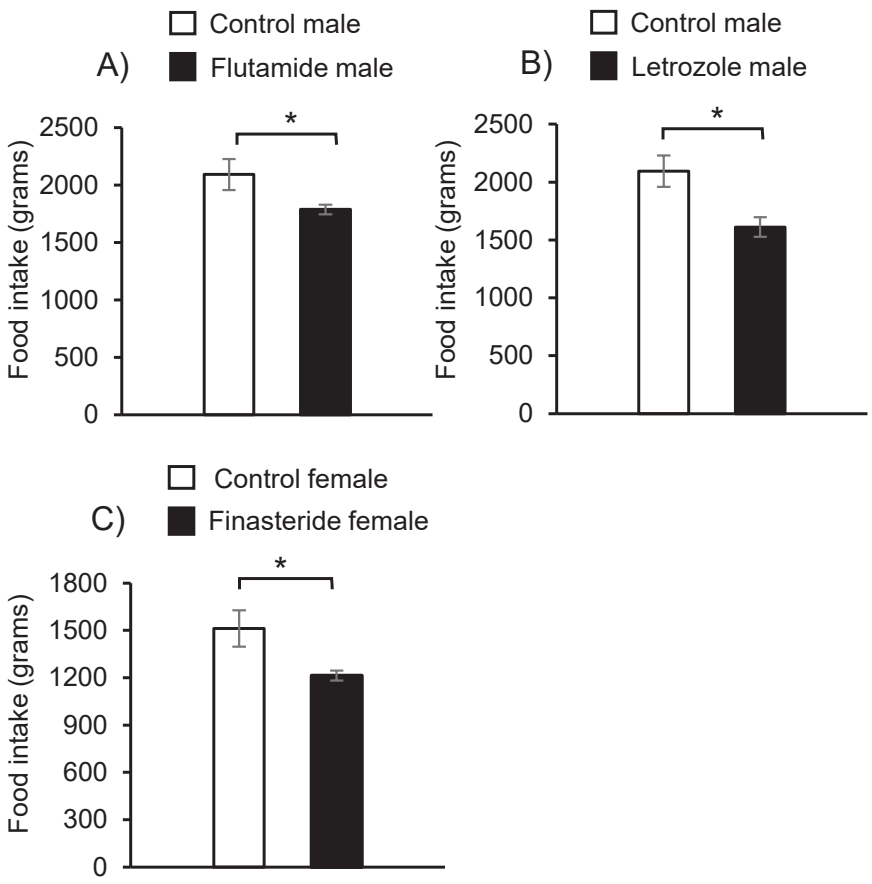

Fig. 2. Graphs show significant differences in food intake measured in grams among groups due to treatment administered in each sex. A) control male vs flutamide male; B) control male vs letrozole male; C) control female vs finasteride female; * shows statistically significant differences ($p<0.05$ in all cases). All values are expressed as mean ± S.D.

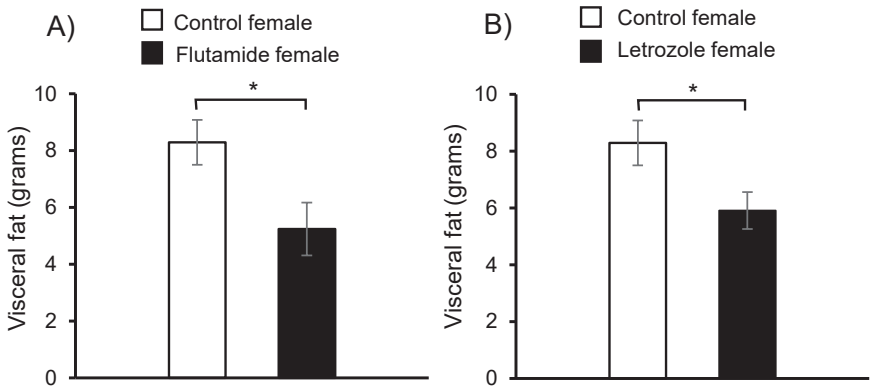

Fig. 3. Histograms show significant differences in visceral fat (in grams) in females since were not detected significant differences between male groups. A) control female vs flutamide female; B) control female vs letrozole female; * shows statistically significant differences ($p<0.05$ in all cases). All values are expressed as mean ± S.D.

3.4. Hypothalamic peptides mRNA levels

3.4.1. Hypothalamic AgRP mRNA levels

Analysis of hypothalamic AgRP mRNA levels showed significant differences between males and females in control ($F1,8=13.387$; $p=0.008$), flutamide ($F1,8=18.743$; $p=0.003$) letrozole ($F1,8=21.745$; $p=0.002$) and finasteride ($F1,8=19.321$; $p=0.003$) groups, with male groups having higher values in all cases (Table 2).

When each sex was analysed separately, the results showed that males and females responded differentially depending on the treatment. In males, significant differences were seen between CM and LetM ($F1,8=14.392$; $p=0.007$), with CM having higher hypothalamic AgRP mRNA levels than LetM (Fig. 4A). However, in females, the significant differences were observed between CF and FinF ($F1,8=8.887$; $p=0.018$) with CF having higher hypothalamic AgRP mRNA levels than FinF (Fig. 4B).

3.4.2. Hypothalamic NPY mRNA levels

Almost sex differences between CM and CF were detected in this parameter ($F1,7=5.516$; $p=0.051$) with CF having higher values than

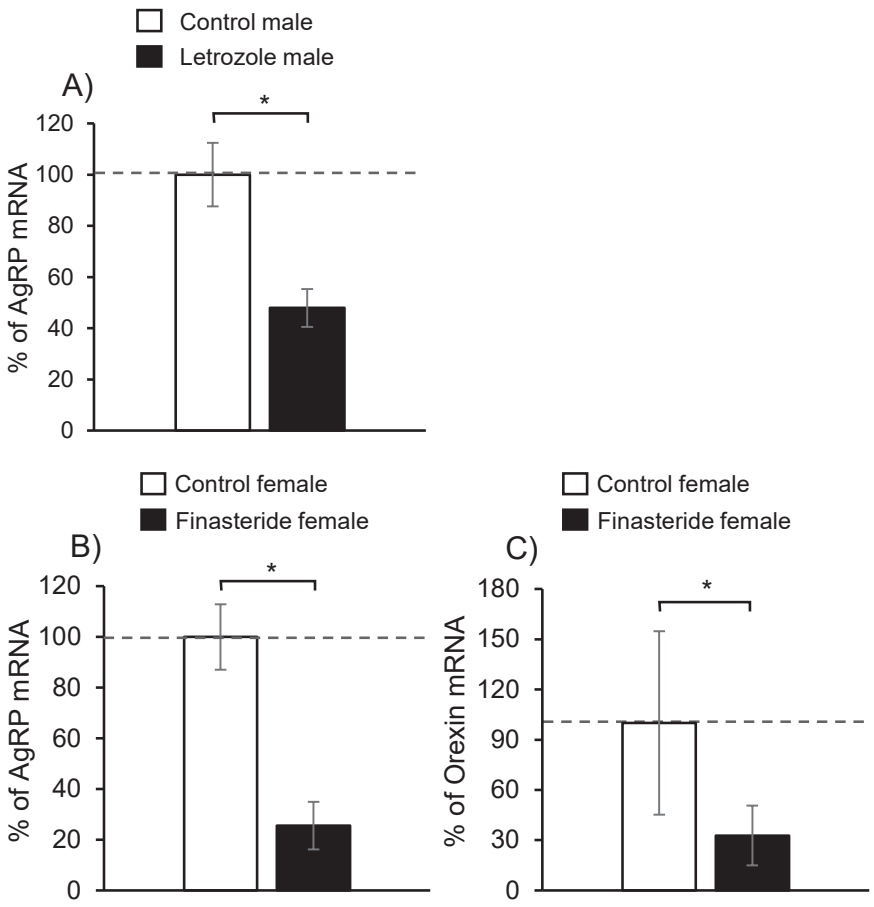

Fig. 4. Histograms show significant differences in hypothalamic peptides related to food intake among groups due to treatment administered in each sex: A) hypothalamic AgRP mRNA levels (%) between control male vs letrozole male; B) hypothalamic AgRP mRNA levels (%) between control female vs finasteride female; C) hypothalamic orexin mRNA levels (%) between control female vs finasteride female. * shows statistically significant differences ($p<0.05$ in all cases). All values are expressed as mean ± S.D.

CM. No sex differences were observed in the rest of the experimental groups (Table 2). When each sex was analysed separately, no differences were found between male or female groups.

3.4.3. *Hypothalamic POMC mRNA levels*

No sex differences were observed between the control groups; however, sex differences were seen between LetM and LetF ($F1,8=21.745$; $p=0.002$) with LetM having higher hypothalamic POMC mRNA levels than LetF. Separate analyses by sex did not show significant differences between male or female groups (Table 2).

3.4.4. *Hypothalamic Orexin A mRNA levels*

Sexual dimorphism between control groups was not observed in this parameter, however, sex differences were seen between LetM and LetF ($F1,8=6.716$; $p=0.03$) with LetM having higher hypothalamic orexin mRNA levels than LetF (Table 2). The treatments did not affect the males since no differences were found in this parameter. However, in the females significant differences were found between CF and FinF ($F1,8=15.37$; $p=0.004$) with CF showing higher hypothalamic orexin mRNA levels than FinF (Fig. 4C).

4. Discussion

Inhibition of androgen receptors, as well as of the two main metabolic pathways of testosterone, aromatization and reduction, during the first five days of life produced differential effects in several metabolic parameters in male and female rats in adulthood. While body weight was altered in females, but not in males due to the lack of androgenic activity, food intake and visceral fat were affected in both males and females, depending on which testosterone metabolic pathway was blocked. As for the effects on the brain, the inhibition of aromatization in males and that of 5α-reductase in females, produced alterations in peptides related to the initiation of feeding: AgRP in the former and AgRP and orexin in the latter. In addition, sex differences in body weight and the tendency for sex differences in the hypothalamic mRNA NPY levels were altered when testosterone activity was inhibited after birth.

Numerous sex differences in energy metabolism due to the effects of androgens during development have previously been described (Asarian and Geary, 2013; Morford and Mauvais-Jarvis, 2016; Chowen et al., 2019). The present results show that treatment with inhibitors of AR, aromatase or 5α-reductase, early after birth do not alter the normal sex differences in body weight or food intake in the long term but do show some delay in the onset of sex differences in body weight occurs when the metabolic aromatization pathway of testosterone is inhibited.

Our results also confirm that androgen receptors or the two metabolic pathways of testosterone during the first five days of life do not play a relevant role in the regulation of body weight in males in the long term. However, the body weight in females significantly decreased during development and in adulthood independent of which metabolic route of testosterone was inhibited. It may be surprising that early postnatal androgen activity participates in body weight programming in females but not in males, since androgens are related to the masculinization of many neurophysiological parameters in males (Movérare-Skrtic et al., 2006; Mauvais-Jarvis, 2011). However, in relation to body weight, the modulatory (Carrillo et al., 2016; 2019; Pinos et al., 2018) and programming action of estrogenic activity on the

body weight of the male rats has been observed previously, because the inhibition of ERα, ERβ and GPER from day P6 to P13 produced a significant decrease in body weight in adult males but had no effect in females (Carrillo et al., 2020). Therefore, estradiol during the second week of life, but not testosterone early in life, seems to be an important factor to programme body weight in males (Carrillo et al., 2020). Regarding females, the influence of postnatal androgen activity has already been demonstrated, since postnatal testosterone or DHT treatment increased body weight and insulin resistance and caused changes in adipose tissue distribution in female rats (Nilsson et al., 1998; Alexanderson et al., 2007) and mice (Kanaya et al., 2013; Nohara et al., 2013) in adulthood. The present results demonstrate that the activity of androgens during the first five days of life is a relevant factor to programme body weight in female rats.

In the current study the results from food intake and visceral fat could partially explain the body weight data. Unlike body weight, food intake was altered in males and females, although not with the same treatments. Food intake decreased in males when aromatase or androgen receptor activity was blocked, but this decrease was not reflected in body weight. Nevertheless, it has been shown that these two parameters are not always related, especially when these changes are produced during development (Asarian and Geary, 2013). On the other hand, the significant decrease in food intake in the female letrozole and finasteride groups seems to correlate with the body weight loss in these animals. These results in females are in line with those obtained in mice where treatment with neonatal testosterone propionate increased food intake at 6 weeks of age (Nohara et al., 2011), which, together with our results suggest that early postnatal androgenic activity participates in the programming of body weight and food intake in females.

The organizational hypothesis of gonadal steroids early after birth is well established (Phoenix et al., 1959; Arnold, 2009). Estradiol aromatized from testosterone organizes different characteristics of the morphology and function of the structures and neuronal networks that regulate reproductive behaviors (Gorski, 1985; Guillamón and Segovia, 1997). However, there is little data on the effects of gonadal steroids on the programming of the neurohormonal circuit of food intake (Arnold, 2009). In previous work we demonstrated that estradiol during the second week of postnatal life programmed POMC mRNA levels in female rats (Carrillo et al., 2020) and Nohara et al., (2011) determined that neonatal administration of testosterone programmed the sexual differentiation of POMC neurons in female mice. A recent study also showed that the specification of hypothalamic neuronal subtypes in a sex-dependent manner is controlled by the epigenetic modifier Kdm6a/Utx (Cabrera Zapata et al., 2022), whose expression is higher in XX hypothalamic neurons regardless of gonadal sex (Cabrera Zapata et al., 2021), indicating the contribution of both chromosome complement and gonadal hormones to the sexual differentiation of hypothalamic neurons. The present study shows that a lack of aromatase activity produces a decrease in orexigenic peptide AgRP in males, while the inhibition of 5α-reductase lead to a decrease in AgRP and orexin in female rats.

Aromatase appears around gestational day 16 in rats and mice, it has a peak in the perinatal period and then decreases, presenting low levels in adulthood (Lephart et al., 1992; Colciago et al., 2005; Cisternas et al., 2017). On the other hand, the organizational effects of testosterone have mainly been attributed to the effect of aromatized estradiol from testosterone (Döhler et al., 1984; Collado et al., 1993: Pérez-Laso et al., 1997). Considering that some parameters of feeding behavior, including the hypothalamic AgRP mRNA levels (present results) are sexually differentiated (Asarian and Geary, 2013), it is not surprising that aromatase may have an important implication in the early postnatal programming of feeding circuits as it does in other systems (Gorski, 1985; Guillamón and Segovia, 1997). Our results suggest that aromatase activity has a role in the programming of the hypothalamic AgRP mRNA levels in males, since the lack of activity of this hormone during early postnatal period impeded the adequate expression of AgRP in adulthood.

In the case of females, a recent report has shown that orexin diminishes in adulthood in female rats treated with testosterone on postnatal day 1, but the action of aromatized estradiol from testosterone cannot be ruled out (Cataldi et al., 2018). A direct action of androgens on the programming of POMC has been reported in mice (Nohara et al., 2011) and our results showed that DHT is involved in the programming of hypothalamic mRNA orexin and AgRP levels in female rats. Thus, our data supports a significant participation of DHT during the first five days of life in the programming of orexigenic peptides involved in the regulation of food intake specifically in female rats.

Considering the results obtained in males and females, it could be suggested that during the first five days of life androgens have more influence in the programming of the feeding circuits of females than of males, since the programming of AgRP mRNA hypothalamic levels are dependent on the aromatisation of testosterone to estradiol in males while AgRP and orexin levels are dependent on DHT in females. Furthermore, NPY and POMC mRNA hypothalamic levels do not appear to be influenced by androgen action early in life, because neither males nor females showed changes in these levels when androgen receptors or both metabolic pathways of testosterone were inhibited. At this point it should be kept in mind that not only could AR be involved in the actions of DHT, because it has been demonstrated that the metabolite of DHT, 3β-Diol, preferentially binds ERβ (Kuiper et al., 1997; Pak et al., 2005; Handa et al., 2008), which might, when activity of AR is inhibited, potentiate the action of estradiol through its ERβ. Thus, a synergistic action of both estrogen and androgen receptors cannot be ruled out.

Sexual dimorphism in the hypothalamic circuits that regulate food intake and energy metabolism has previously been described (Chowen et al., 2019; Wang and Xu, 2019). Our results showed that males had higher AgRP and lower NPY mRNA hypothalamic levels than females. Other authors have reported a different pattern of sex differences in the distribution of NPY mRNA-containing cells in the Arc (Urban et al., 1993) while other data in mice showed that females also had higher expression of hypothalamic AgRP mRNA (Lensing et al., 2016). These dissimilar results could be explained by differences in the species, or the experimental protocols used. Since very few studies compare both sexes in the expression of feeding neuropeptides, more research is needed to confirm the differences between males and females in the expression of neuropeptides that regulate energy metabolism.

The prominent role of feeding-related hormones, such as ghrelin or leptin in the programming of the intake circuitry, was shown almost two decades ago (Bouret et al., 2004; Steculorum et al., 2015) and more recently the involvement of gonadal hormones in this programming has also been demonstrated (Nohara et al., 2011; Carrillo et al., 2020). Our results highlight the involvement of androgens in this process in females and establish the importance of aromatization in the programming of AgRP in male rats. Moreover, androgenic activity during the first five days of life plays a vital role in regulating body weight and food intake in females in the long term. Considering that both androgens and estrogens are involved in different aspects of the programming of energy metabolism and feeding, that testosterone can be metabolised to estradiol and DHT and that DHT can exert part of its actions through ERβ, it is essential to study the effect of the gonadal hormones during development from a synergistic point of view. Moreover, it is crucial to take into account the differential effects in both sexes in order to have a more accurate picture of the role of all these hormones during development in the constitution of the feeding regulatory circuits.

5. Conclusions

The present work has highlighted two relevant aspects of the regulation of energy metabolism and the hypothalamic circuits that regulate feeding behaviour. On the one hand, the fundamental role those gonadal hormones, in this case androgens, play in the programming of body weight and food intake as well as of the orexigenic peptides AgRP and

orexin during development. The alteration of androgen activity during the first five days of life can produce long-term changes that could influence imbalances in energy metabolism and in the functioning of the hypothalamic circuits that regulate feeding. On the other hand, the differential effect that the action of androgens has on males and females early after birth, which results in a different vulnerability to the action of gonadal hormones. The involvement of androgens and estrogens in the regulation of physiology and feeding behaviour during adulthood is well established, but our results support the importance of the activity of these hormones during development. Hence, further research is needed to unravel the role that androgens and estrogens, either by themselves or through synergistic actions, play in the regulation of physiology and feeding behaviour during development in males and females.

Ethical guidelines

All experiments were designed according to the guidelines published in the "NIH Guide for the care and use of laboratory animals", the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, the European Union legislation [Council Directives 86/609/EEC and 2010/63/UE] and the Spanish Government Directive [R.D. 1201/2005]. Experimental procedures were approved by our Institutional Bioethical Committee [UNED, Madrid]. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary Moreover, no portion other than the abstract has been published or posted on the Internet. The corresponding author can provide all original data for review.

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Author statement

We have read and have abide by the statement of ethical standards for manuscript submitted to Brain Research Bulletin.

CRediT authorship contribution statement

Arévalo Maria Angeles: Investigation, Conceptualization. **Collado Paloma:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Pinos Helena:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carrillo Beatriz:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Fernandez-Garcia Jose:** Methodology, Investigation, Data curation. **Garcia-Ubeda Rocio:** Investigation, Data curation. **Grassi Daniel:** Methodology, Conceptualization. **Primo Ulises:** Investigation, Data curation. **Blanco Noemi:** Investigation, Data curation. **Ballesta Antonio:** Investigation, Data curation.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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