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Blocking of Estradiol Receptors ERα, ERβ and GPER During Development, Differentially Alters Energy Metabolism in Male and Female Rats

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Abstract—Estradiol not only participates in the regulation of energy metabolism in adulthood, but also during the first stages of life as it modulates the alterations induced by under- and over-nutrition. The objectives of the present study were to determine: 1) If estradiol is involved in the normal programming of energy metabolism in rats; 2) If there is a specific window of time for this programming and 3) If males and females are differentially vulnerable to the action of this hormone. Estrogen receptors (ER) α, ERβ and GPER were blocked by their specific antagonists MPP, PHTPP and G15, respectively, from postnatal day (P) 1 (the day of birth) to P5 or from P5 to P13. Physiological parameters such as body weight, fat depots and caloric intake were then analysed at P90. Hypothalamic AgRP, POMC, MC4R, ERα, ERβ and GPER mRNA levels and plasma levels of estradiol, were also studied. We found that blocking ER receptors from P5 to P13 significantly decreases long-term body weight in males and hypothalamic POMC mRNA levels in females. The blocking of ERs from P1 to P5 only affected plasma estradiol levels in females. The present results indicate programming actions of estradiol from P5 to P13 on body weight in male and POMC expression in female rats and emphasize the importance of including both sexes in metabolic studies. It is necessary to unravel the mechanisms that underlie the actions of estradiol on food intake, both during development and in adulthood, and to determine how this programming differentially takes place in males and females.

Key words: estradiol receptor alpha (ERα), estradiol receptor beta (ERβ), G-protein-coupled estrogen receptor (GPER), energy metabolism programming, POMC, sex differences.

INTRODUCTION

Estradiol is involved in the regulation of energy metabolism and has been demonstrated to decrease food intake and inhibit weight gain (for review see Asarian and Geary, 2013; López and Tena-Sempere, 2015; Mauvais-Jarvis et al., 2013). Moreover, estradiol modulates adipose tissue by controlling fat distribution and storage and by regulating the lipogenesis/lipolysis balance in visceral and subcutaneous fat depots (Cooke and Naaz, 2004; Shi et al., 2009; Mauvais-Jarvis et al., 2013; Santosa and Jensen, 2013; McCarthy et al., 2017).

Hypothalamic control of the balance between anorexigenic and orexigenic peptides in the regulation of energy metabolism is essential and the participation of estradiol in the regulation of food intake appears to include modification of this balance. Specifically, up-regulation of anorexigenic peptides, such as α-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine- regulated transcript (CART), in the hypothalamus and down-regulation of orexigenic peptides such as neuropeptide Y (NPY), agouti-related peptide (AgRP) or orexin in this same structure are the mechanisms proposed for the regulatory actions of

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Abbreviations: AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CF, control female; ERB-F, estrogen blocked receptor female; CM, control male; ERB-M, estrogen receptor blocked male; NPY, neuropeptide Y; PCR, quantitative real-time polymerase chain reaction; POMC, proopiomelanocortin.

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estradiol on food intake (Silva et al., 2010; Santollo et al., 2012; Rebouças et al., 2016). Furthermore, the hypophagic effect of estradiol is suggested to be the result of enhanced leptin sensitivity in the hypothalamus (Rocha et al., 2004). Even though the mechanisms through which this hormone regulates food intake are not completely known, the strongest evidence points to a direct action of estradiol on the POMC neurons in the arcuate nucleus (Gao et al., 2007).

Estradiol's actions on energy metabolism are reported to be mainly conveyed through estrogen receptor (ER) α (Roepke, 2009; Meyer et al., 2011; Mauvais-Jarvis et al., 2013; Frank et al., 2014; Santollo and Daniels, 2015), but recent reports have shown that ERβ and G-protein coupled ER (GPER) are also involved in some aspects of energy metabolism regulation (Roepke, 2009; Meyer et al., 2011; Davis et al., 2014; Santollo and Daniels, 2015; Ponnusamy et al., 2017). Numerous studies have analyzed the specific actions of estradiol via ERα on energy metabolism and the neurohormonal regulation of food intake (Roepke, 2009; Meyer et al., 2011; Frank et al., 2014; Santollo and Daniels, 2015; Ponnusamy et al., 2017), but less is known regarding the roles of ERβ and GPER. Some authors report that ERβ participates in the control of peripheral fat accumulation (Roepke, 2009) and that the actions of estradiol through GPER underlie the anorectic effects of leptin and cholecystokinin (Davis et al., 2014) and regulate adiposity (Wang et al., 2016). Moreover, it has been suggested that the three receptors could interact to mediate the actions of estradiol on energy metabolism (Roepke, 2009; Meyer et al., 2011; Frank et al., 2014).

The early stages of development are crucial for determining the adequate development of the neurophysiological systems that regulate energy metabolism (Simerly, 2006; Bouret, 2013; Dearden and Ozanne, 2015). Hormones, such as leptin and ghrelin, are involved in this programming, with leptin acting as a trophic factor in the development of hypothalamic circuits that control food intake, and ghrelin, on the contrary, playing an inhibitory role in the establishment of these same neural circuits (Bouret et al., 2004; Bouret, 2013; Steculorum et al., 2015). During early development, programming of the neurohormonal feeding circuit is especially vulnerable to nutritional conditions (Taylor and Poston, 2007; Chang et al., 2008; Patel and Srinivasan, 2010; Zeltser, 2018). Estradiol also appears to participate in programming of this system as, when administered from postnatal day (P) 6 until P13, it reverted the neurophysiological alterations produced by maternal overnutrition in males and the alterations in POMC mRNA produced by under and over-nutrition in female rats (Carrillo et al., 2016; Pinos et al., 2018; Carrillo et al., 2019). Therefore, estradiol during this specific time window can differentially modulate at least some of the alterations produced by malnutrition in male and female rats. The organizational role of estradiol was reported in the 80 s of the 20th century where estradiol, aromatized from testosterone, was shown to determine the differentiation of the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski, 1985; Döhler et al., 1986), the anteroventral periventricular area (AVPV) (Simerly, 1989) and the majority of the nuclei of the vomeronasal system (Guillamon and Segovia, 1997). Moreover, estradiol influences food intake acting through the neural signal transducer and activator of transcription 3 (STAT3), the same pathway through which leptin exerts its anorexigenic effects in adulthood and its trophic effects during development (Darnell, 1996; Vaisse et al., 1996; Elmquist et al., 1998; Björnström and Sjöberg, 2005). Taken together, these data might suggest that estradiol could be involved in programming the neurophysiology and neurohormonal systems that regulate energy metabolism.

There are numerous studies indicating differences in metabolic control between males and females. For example, in mice with ERα specifically knocked out in POMC neurons, female knock-outs eat more and gain more weight than the wild type reference group, but males do not (Xu et al., 2011). Sex differences in body weight in GPER knockout mice have also been reported (Mårtensson et al., 2009). It is highly likely that this sexual dimorphism reflects differences in the mechanisms that underlie all processes related to energy metabolism where estradiol is involved; therefore, it is particularly relevant to study both sexes to detect possible differences between males and females in the programming effects of estradiol.

Thus, the first objective of this study was to analyze the effects of estrogen signalling during early postnatal life on long-term metabolism. The second aim was to determine if the programming actions of estradiol are different in males and females, as there are sex differences in the regulation of body weight, adiposity distribution and insulin resistance (Shi et al., 2009; Asarian and Geary, 2013). Finally, we also explored the effects of estrogenic actions during two different periods of development to determine if the timing of susceptibility to estradiol's programming effects differs between males and females. To this end, the activities of ERα, ERβ and GPER were blocked from P5 to P13, a period during which estradiol has a differential modulatory role on the alterations produced by malnutrition in male and female rats (Carrillo et al., 2016; Pinos et al., 2018; Carrillo et al., 2019). The activities of these same receptors were also blocked from P1 to P5 to discern whether there is a differential period of sensitivity to estradiol in both sexes. The endpoints analysed included body weight, weight of fat depots, calorie intake and the expression of hypothalamic neuropeptides and receptors involved in regulation of food intake, as well as hypothalamic ER expression and plasma estradiol levels.

EXPERIMENTAL PROCEDURES

All experiments were designed 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 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. Wistar rats were reared under stable temperature, humidity and light conditions (22 ± 2 °C; 55 ± 10% humidity; 12 h light/12 h dark cycle, lights on from 08:00 to 20:00) with food and water ad libitum. For mating, a male was placed in a cage with two females for one week. Pregnant females were housed individually in plastic maternity cages with wood shavings as the nesting material.

Two different experiments were designed to better define the most susceptible period to the lack of estradiol activity on ERα, ERβ and GPER. In the first experiment ERα, ERβ and GPER inhibitors were administered from P1 to P5 and in the second experiment ERα, ERβ and GPER inhibitors were administered from P5 to P13. The treatment with estradiol inhibitors in this second experiment began at P5 to assure, at least to some extent, that the release of estradiol from the ovary, that begins to become functional in this period, had no effect on the ERs studied.

In experiment 1, on postnatal day 1 (P1) pups born on the same day were weighed, sexed, and randomly distributed (five females and five males/dam). From P1 to P5 pups received a daily s.c. injection of compounds to block ERα [MPP (1,3-bis (4-hydroxy-phenyl)-4-methy l-5-[4-(2-piperidine letoxy)phenol]-1 pyrazole diclorhydrate), 1 mg/kg], ERβ [PHTPP (4-[2-phenyl-5,7-bis (trifluoromethyl)pyrazole[1,5-a] pyrimidine-3-il] phenol), 1 mg/kg)] and GPER [G15 (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta-[c]quinoline, 7.4 nmol/animal, 0.277 mg/kg] dissolved in corn oil. Doses of ERα, ERβ and GPER ligands were based on previous *in vivo* studies (Waters et al., 2009; Grassi et al., 2013; Grassi et al., 2016). Control animals were treated s.c. with vehicle (corn oil). From weaning (P21) to P90 body weight and food intake were measured every 7 days. This experimental design resulted in the following four groups: control male (CM_{1-5} = 6); control female (CF_{1-5} = 6); estrogen receptor-blocked males (ERB-M_{1-5} = 8); and estrogen receptor-blocked females (ERB-F_{1-5} = 5).

In experiment 2, the procedure was the same as in experiment 1 except for the period of estradiol treatment; in this case injections were made from P5 to P13. The following four groups were studied in this second experiment: control male (CM_{5-13} = 8); control female (CF_{5-13} = 8); estrogen receptor-blocked males (ERB-M_{5-13} = 8); and estrogen receptor-blocked females (ERB-F_{5-13} = 8).

In both experiments, on P90, P91 or P92 rats were weighed and decapitated between 9:00 and 11:00 a.m. One day prior to sacrifice a vaginal smear was taken from all females to determine the estrous phase. On P90 females in the diestrous phase were sacrificed as well as the same number of animals of each group. Females were then sacrificed during the diestrous phase during the next couple of days, with the same number of rats from the other groups being sacrificed on the same days. Trunk blood was collected in ethylenediamine-tetra-acetic acid (EDTA) containing glass tubes, centrifuged for 15 minutes at 2000g at 4 °C and the plasma collected and stored in aliquots at −80 °C. The hypothalami were dissected, from the rostral edge of the optic chiasm to the anterior margin of the mammillary bodies, and rapidly frozen in dry ice and stored at −80 °C. Subcutaneous (abdominal), visceral (perigonadal), and brown fat pads were rapidly removed, weighed, and frozen at −80 °C.

Quantitative real-time polymerase chain reaction (PCR)

The Tri-Reagent protocol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the hypothalamic tissue. Two μg of total RNA were used to synthesize cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For quantitative real-time PCR, assay-on-demand kits (Applied Biosystems) were used: Agouti-related peptide (AgRP; Rn014311703_g1), proopiomelanocortin (POMC; Rn00595020_m1), melanocortin 4 receptor (MC4R; Rn01491866_s1), ERα (Rn01640372), ERβ (Rn00562610_m1), GPER (Rn01643280-s1) 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 7000 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. According to the manufacturer's guidelines, the ΔΔCT method was used to determine relative expression levels. Statistics were performed using ΔΔCT values.

Plasma estradiol levels

An ELISA kit (CEA461Ge, Cloud-Clone Corp.) was used to measure plasma estradiol levels. All samples were measured in duplicate by following the manufacturer's instructions. Absorbance in each well was measured with a Tecan Infinite M2000 (Grödig, Austria). The detection range of the method was 12.35–1000 pg/ml.

Statistical analysis

To analyse all parameters studied, data were submitted to a two-way ANOVA (sex and treatment) to determine the effect of each factor and the interaction of the studied variables followed by Student-Newman-Keuls test *post hoc* analyses when significant differences were found. When an interaction effect was detected in hypothalamic mRNA peptide levels or in estradiol receptors mRNA levels, data were normalized to control values (control as 100%) for each sex. Therefore, these data were submitted to a one-way ANOVA (treatment) for each sex independently to specifically determine differences intra-sex. The significance level was set at $p < 0.05$.

RESULTS

Experiment 1: Blocking of ERα, ERβ and GPER from P1 to P5

Body weight. A main effect of sex ($F1,24 = 92.65$, $P < 0.0001$) was found on body weight at P90. Data obtained of single effect analysis showed that CM_{1-5} were heavier than CF_{1-5} ($F1,13 = 24.368$, $P < 0.001$) and $ERB\text{-}M_{1-5}$ were also heavier than $ERB\text{-}F_{1-5}$ ($F1,13 = 57.866$, $P < 0.0001$). No effect of treatment or interaction of sex by treatment were detected on this parameter (Fig. 1A).

Kilocalorie intake. As for body weight, a main effect of sex ($F1,21 = 61.878$, $P < 0.0001$) was found regarding energy intake. Males ate significantly more than females in both control and blocked groups ($F1,13 = 36.81$, $P < 0.0001$ for control groups; $F1,13 = 93.92$, $P < 0.0001$ for blocked groups). No effects of treatment or interaction of sex by treatment were detected on this parameter (Table 1).

Fat mass. No effect of sex, treatment or interaction was seen in visceral, brown or subcutaneous fat depots (Table 1).

Hypothalamic mRNA levels. No main effect of sex, treatment or interaction were found on hypothalamic AgRP, MC4R mRNA levels (Table 1), or POMC mRNA levels (Fig. 1E).

Hypothalamic estrogen receptor α, β and GPER mRNA levels. Blocking ERα, ERβ and GPER from P1 to P5 produced a main effect of sex ($F1,21 = 4.365$, $P = 0.05$) in hypothalamic ERα at P90. Posterior analysis did not show significant differences between groups. No main effects of the factors sex, treatment or interaction were detected in hypothalamic ERβ or GPER mRNA levels (Table 1).

Plasma estradiol levels. Plasma estradiol levels showed an effect of interaction of sex by treatment ($F1,21 = 10.68$, $P < 0.005$). *Post hoc* comparison showed significant differences between females, with control females having higher values than blocked females ($P < 0.05$). Moreover, sex differences were detected between blocked groups, with males having higher values than females ($P < 0.05$) (Fig. 1C).

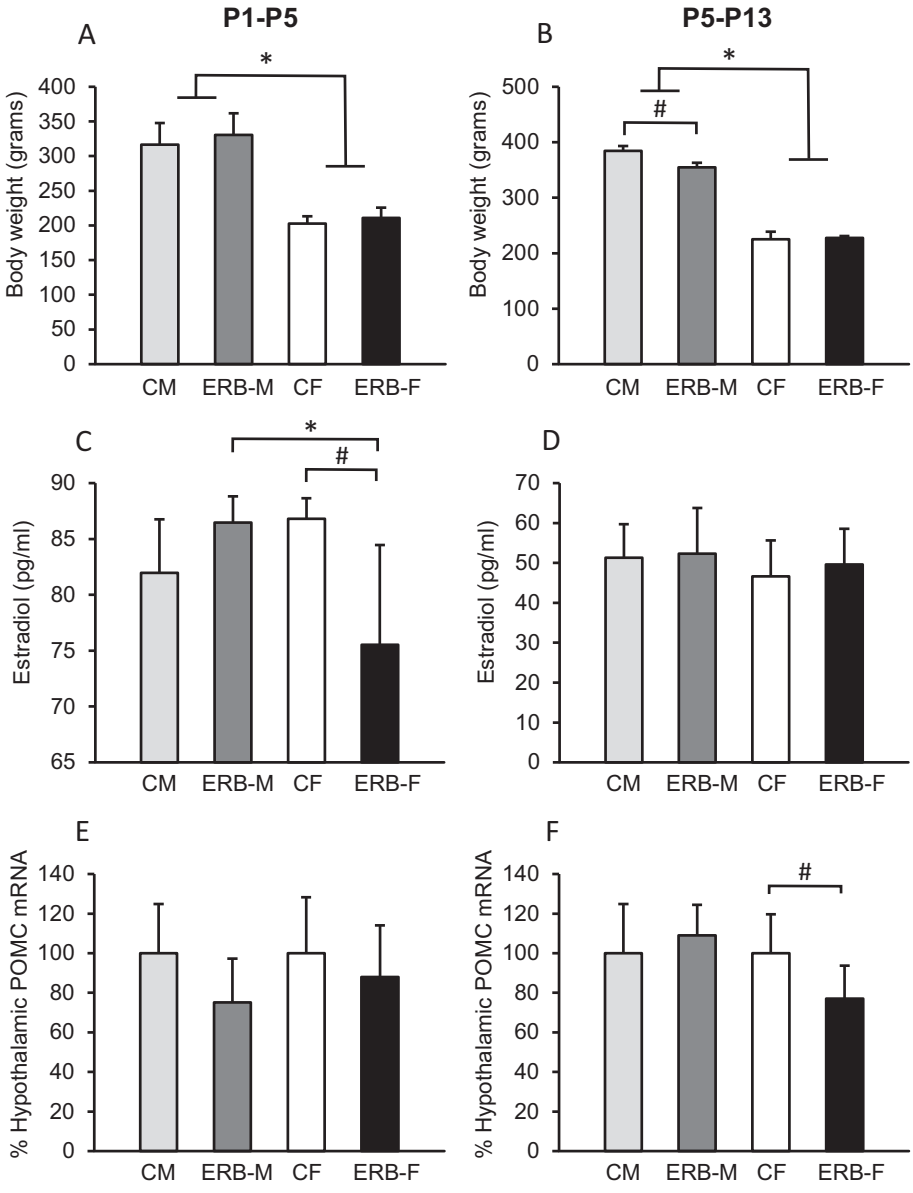

Fig. 1. Comparison of results of final body weight, plasma estradiol levels and POMC mRNA levels in the hypothalamus of the two experiments. Males weighed more than females but with no effect of blockage of estradiol receptors between P1–P5 (**A**), while blockage of estradiol receptors between P5–P13 reduced adult body weight in males (**B**), although both male groups continued to weigh more than females. In contrast, blockage of estrogen receptors from P1–P5 reduced adult plasma estradiol levels in females (**C**), with no effect of estrogen receptor blockage from P5–P13 (**D**). Hypothalamic POMC mRNA levels were not affected by estrogen receptor blockage at P1–P5 (**E**) but were significantly reduced in females when estrogen receptors were blocked from P5–P13 (**F**). Statistically significant differences are labeled as follows: *sex differences; #differences intra sex. CM = Control male; CF = Control female; ERB-M = ER-blocked males; ERB-F = ER-blocked females. All values are expressed as mean ± S.D.

Table 1. Sex differences due to the blocking of ERs from P1 to P5

	CM	ERB-M	CF	ERB-F
Energy intake (kcal)	721.61 ± 60.96	689.54 ± 20.10	553.07 ± 30.22 [a]	542.59 ± 26.72 [a]
Visceral fat (mg/kg b.w.)	18.8 ± 3	20.4 ± 6	23.1 ± 2.9	23.6 ± 10.0
Subcutaneus fat (mg/kg b.w.)	6.5 ± 1	7.2 ± 2	5.1 ± 1	6.3 ± 1
Brown fat (mg/kg b.w.)	0.7 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.6 ± 0.1
Estradiol receptor mRNA (%)				
Alpha	100 ± 21.92	88.70 ± 7.89	130.48 ± 45.23	123.21 ± 34.36
Beta	100 ± 26.97	92.19 ± 17.71	99.92 ± 19.64	95.97 ± 13.07
GPER	100 ± 5.17	132.97 ± 52,41	100 ± 14.89	136.02 ± 68.23
AgRP mRNA (%)	100 ± 28.12	90.52 ± 26.06	86.41 ± 15.32	89.98 ± 33.89
MC4R mRNA (%)	100 ± 31.65	303.16 ± 212	170.56 ± 71.55	219.73 ± 155.34

Values are expressed in mean ± S.D.
[a] Significant differences with respect to the male of same treatment.

Experiment 2: Blocking of ERα, ERβ and GPER from P5 to P13

Body weight. The blocking of ERα, ERβ and GPER from P5 to P13 differentially altered body weight in male rats. Main effects of sex ($F1,28 = 1710.80$, $P < 0.0001$), treatment ($F1,28 = 15.706$, $P < 0.0001$) and interaction ($F1,28 = 22.044$, $P < 0.0001$) were found between these factors on this parameter. *Post hoc* analysis showed a significant difference between $CM_{5\text{-}13}$ and $CF_{5\text{-}13}$ and between ERB-$M_{5\text{-}13}$ and ERB-$F_{5\text{-}13}$ ($P < 0.05$ in all comparisons) with males being heavier than females. Moreover, a significant decrease in body weight due to the blocking of ERs was detected in males ($CM_{5\text{-}13}$ > ERB-$M_{5\text{-}13}$; $P < 0.05$) (Table 2, Fig. 1B).

Kilocalorie intake. A main effect of sex ($F1,28 = 237.93$, $P < 0.0001$) was found regarding energy intake. Males showed a higher energy intake than females of both groups ($F1,14 = 156.589$, $P < 0.0001$ for control groups; $F1,14 = 102.569$, $P < 0.0001$ for blocked groups) (Table 2). No effect of treatment or interaction was found.

Fat mass. No effect of sex, treatment or interaction of treatments was found on brown or subcutaneous fat depots. In contrast, a main effect of sex ($F1,28 = 20.373$, $P < 0.0001$) was found on visceral fat mass. Sex differences were seen in both conditions, control and ERs blocked ($F1,14 = 18.160$, $P < 0.001$ and $F1,14 = 103.435$, $P < 0.0001$, respectively). In both cases, males had more visceral fat mass than females (Table 2).

Hypothalamic mRNA levels. A main effect of sex ($F1,21 = 17.212$, $P < 0.0001$) and treatment ($F1,21 = 9.188$, $P < 0.006$), but no interaction was shown on hypothalamic AgRP mRNA levels. Analysis between groups showed significant sex differences between $ERB_{5\text{-}13}$ males and $ERB_{5\text{-}13}$ females ($F1,13 = 5.176$, $P < 0.039$), with males having higher levels than females (Table 2). When each sex was analyzed independently, there were no differences between control and ER-blocked groups in either sex in the mRNA levels of this neuropeptide.

POMC mRNA levels were altered in females due to the blocking of ERα, ERβ and GPER. A main effect of sex ($F1,21 = 14.905$, $P < 0.001$), with an interaction between sex and treatment ($F1,21 = 6.69$, $P < 0.01$) was found. No sex differences were detected in this parameter in the control groups, but a slight increase in hypothalamic POMC mRNA levels in ERB-$M_{5\text{-}13}$ and a significant decrease in hypothalamic POMC mRNA levels in ERB-$F_{5\text{-}13}$ resulted in a sex difference in the ER blocked groups ($P < 0.05$; Fig. 1F). In males no differences between groups were detected. However, a significant difference ($F1,14 = 6.311$, $P < 0.02$) was found in females, with the treatment provoking a decrease in POMC mRNA levels (Fig. 1F).

Hypothalamic estrogen receptor α, β and GPER mRNA levels. A main effect of sex was detected with females showing higher hypothalamic ERα mRNA levels than their respective male groups ($F1,21 = 20.097$, $P < 0.001$ for control groups and $F1,14 = 14.057$, $P < 0.002$ for ER-blocked groups). No effect of treatment or interaction was found on ERα mRNA levels (Table 2).

There was no effect of sex or treatment on hypothalamic ERβ mRNA levels, but there was an interaction between these factors ($F1,21 = 5.713$, $P < 0,026$). *Post hoc* analyses did not show sex differences between male and female control groups. However, a slight decrease in males and a slight increase in females of the ER blocked groups resulted in a significant difference between these male and female blocked groups in this parameter (Table 2).

Finally, neither main effects of the factors sex or treatment nor interaction were detected in hypothalamic GPER mRNA levels.

Plasma estradiol levels. Plasma estradiol levels were not different between the groups studied with no main effect of sex, treatment or interaction in this parameter (Fig. 1D).

DISCUSSION

Numerous studies have shown that the neural systems that regulate energy metabolism are programmed during early development and that hormones such as leptin and ghrelin can influence this process in rats (Bouret

Table 2. Sex differences due to the blocking of ERs from P5 to P13

	CM	ERB-M	CF	ERB-F
Energy intake (kcal)	845.50 ± 43.73	821.40 ± 35.98	591.38 ± 55.89[a]	606.52 ± 32.62[a]
Visceral fat (mg/kg b.w.)	22.6 ± 3	13.4 ± 2	23.1 ± 2[a]	15.1 ± 4[a]
Subcutaneous fat (mg/kg b.w.)	7.7 ± 1	7.8 ± 1	6.9 ± 2	7.2 ± 0.8
Brown fat (mg/kg b.w.)	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.4 ± 0.2
Estradiol receptor mRNA (%)				
Alpha	100 ± 8.61	103.32 ± 22.62	136.09 ± 25.82[a]	143.44 ± 30.91[a]
Beta	100 ± 13.13	87.14 ± 16.08	95.81 ± 11.42	110.04 ± 19.41[a]
GPER	100 ± 8.9	100.73 ± 12.17	96.08 ± 9.94	97.60 ± 15.47
AgRP mRNA (%)	100 ± 24.15	120.92 ± 23.37	74,14 ± 21.20	83.44 ± 22.38[a]
MC4R mRNA (%)	100 ± 52.60	82.55 ± 17.74	65.23 ± 13.31	103.14 ± 65.21

Values are expressed in mean ± S.D.
[a] Significant differences with respect to the male of same treatment.

et al., 2004; Taylor and Poston, 2007; Dearden and Ozanne, 2015; Steculorum et al., 2015). Here we show that blocking ERα, ERβ and GPER from P5 to P13 induces long-term alterations in energy metabolism, specifically body weight and hypothalamic POMC mRNA levels, and that this effect is sexually dimorphic. Indeed, body weight was decreased in males but not in females, while hypothalamic POMC mRNA levels where only modified in females. This suggests that estradiol might participate in metabolic programming during early development, and that males and females differ in their response to the blocking of estradiol receptors during the first stages of postnatal life. Moreover, we demonstrate that from P5 to P13 energy metabolism is more susceptible to the actions of estradiol than during the first days of postnatal life because when ERs are blocked from P1 to P5, the above described modifications were not detected.

Adult males weighed more than females in both control and ER-blocked rats in both experiments. However, the blocking of ERα, ERβ and GPER from P5 to P13, was sex specific as this resulted in a significant body weight decrease in males, with no effect on body weight in females. This differential response of males and females to estradiol was reported in a previous study where estradiol during this same period impeded the high fat diet-induced increase in body weight in males while in females there was no effect of either high fat diet or estradiol on body weight (Carrillo et al., 2019). In agreement with these results, being raised in a small litter, resulting in increased food availability, increased body weight in males but not in females (Argente-Arizón et al., 2016). On the other hand, when ERs were blocked from P1 to P5 no variation in body weight was detected in males. Together these results suggest that body weight is more vulnerable to challenges during a specific time window, from P5 to P13, in males than in females and it is possible that the mechanisms that regulate this parameter might be programmed during this period, at least in males. Nevertheless, an initial programming of body weight, in both males and females, should not be discarded, since overweight and obesity in adulthood has been related, among other possible factors, to obesity, diabetes or malnutrition of the mothers during the gestational period (Taylor and Poston, 2007; Dearden and Ozanne, 2015). Moreover, it has been reported that estrogen exposure prenatally also has long-term effects on other neuroendocrine systems such as those controlling reproductive function and behaviors (Hines and Goy, 1985; Yamamoto et al., 2005; Horan et al., 2017).

While the vulnerability of body weight in males from P5 to P13 is consistent with previous results, why the blocking of ERs affects weight gain in males but not in females is less easily explained. No differences in caloric intake were found, indicating that the decrease in body weight in the ER-blocked males is not due to a decrease in energy consumption. Blocking ERs during this specific period had no significant effect in either sex on any of the fat depots analyzed. This contrasts with previous studies reporting that the lack of estradiol activity, mainly through ERα receptor (Shi et al., 2009), increases fat depots. However, these previous studies were performed in postpubertal animals and not during development as reported here. Moreover, in the present study ERβ and GPER, in addition to ERα, were also blocked and both, ERβ and GPER, have been implicated in some aspects of fat accumulation. Specifically, ERβ is reported to inhibit actions of ERα on food intake (Matthews and Gustafsson, 2003) and GPER promotes adipogenesis *in vitro* (Wang et al., 2016); thus, our results might be explained by a differential action of estradiol during development and/or a possible interaction between different receptor subtypes in regulating fat depots (Roepke, 2009; Meyer et al., 2011). Moreover, studies are necessary to determine if energy expenditure is affected by this manipulation.

The body weight decrease in ER-blocked males does not appear to be due to a decrease in fat depots, although the slight decrease in visceral adipose most likely contributes to this phenomenon. Indeed, studies point to a direct action of estradiol through ERα, as well as an influence of ERβ and GPER, on the regulation of adipose tissue (Heine et al., 2000; Musatov et al., 2007; Shi et al., 2009; Meyer et al., 2011). In addition, visceral fat is metabolically more active and is the first depot reduced by weight lost (Shi et al., 2009). The blockage of ERs could also affect other tissues as estradiol deficiency is the main cause of bone loss, not only in females as evident after menopause (Sirola et al., 2003; Shea et al., 2015), but also in males (Khosla et al., 1998;

Aguirre et al., 2015). Estradiol also correlates positively with lean mass in men (Vandenput et al., 2002; Vandenput et al., 2010). Thus, the significant decrease in body weight in males due to the blocking of ERs could involve a loss of lean mass and bone mass in addition to a decrease of visceral fat. Finally, it is important to note that, in males, androgens acting on their receptors, as well as estrogens from the aromatization of testosterone, acting on estradiol receptors, participate in the regulation of energy metabolism and, therefore, of body weight (Mauvais-Jarvis, 2011). Treatment with estradiol increases androgen receptors in various brain regions (Handa et al., 1987; Handa et al., 1996), which suggests a synergistic action of these steroids and their receptors in the control of body weight in male rats. Thus, it is possible that blocking estradiol receptors from P5 to P13 could alter the balance of estradiol and testosterone effects by upregulating androgen receptors, activation of which decreases body weight (Mauvais-Jarvis, 2011). Although more research is needed to disentangle the mechanism by which estradiol's effects during this specific period of development regulate body weight of males in adulthood, our results suggest that these mechanisms are different in males and females. It is possible that the programming of these mechanisms in females occurs at different periods from those studied here; therefore, vulnerability to changes in the activity of hormones relevant to this developmental programming might vary in both sexes, resulting in greater or lesser efficiency in the regulation of energy metabolism.

In contrast to the results of body weight, the lack of activity of estradiol on its receptors from P5 to P13 affected POMC mRNA levels in females but not in males. This is consistent with previous studies that directly relate the modulating effect of estradiol on POMC levels to metabolic control in female rats. An increase in estradiol levels during development can revert the undernutrition-induced decrease or overnutrition-induced increase of hypothalamic POMC mRNA levels to control levels (Carrillo et al., 2016; Carrillo et al., 2019). Thus, hypothalamic POMC mRNA levels in females, but not in males, have been consistently shown to be vulnerable to estradiol variations during development, at least from P6 to P13. Considering that the changes in POMC in females appear during the period when the ovary begins to become functional, the release of estradiol during this critical period might be considered crucial for the programming of the melanocortin system in female rats.

Studies of the influences of estradiol on the development of neurohormonal feeding circuit are sparse; however, there are reports that point to some modulating actions during early development. For example, estradiol levels and POMC mRNA expression in the hypothalamus correlate and increase during development in both males and females, while this was not observed for steroidogenic factor-1 or ERα mRNA expression (Iwasa et al., 2017). Moreover, while most estrogen-responsive neurons involved in metabolic control maintain their responsivity to this hormone at least through middle age, POMC neurons do not (Santollo et al., 2012). Together these data could point to a specific role of estradiol on POMC neurons during development, and that the responsivity to this hormone in the establishment of the metabolic regulatory system in female rats is defined to a specific period that is included in the P5 to P13 interval. The observed decrease in hypothalamic POMC mRNA levels in females points to a possible imbalance in the neuropeptide regulatory system and might result in an increased vulnerability to eating disorders and metabolic challenges such as in high fat diets, high sugar diets, or low proteins diets.

The only direct mechanism reported through which estradiol clearly regulates energy metabolism at the hypothalamic level is, precisely, through POMC neurons (Gao et al., 2007). Here we show that in female rats, POMC neurons are also sensitive during development to the lack of estradiol, at least from P5 to P13, with this hormone being involved in determining POMC expression in adulthood. Although no effect was found here, hypothalamic POMC neurons in males could be vulnerable to the modulatory effects of estradiol during a different time window from those used in the present study. Indeed, the developmental effects of other hormones on metabolic circuits have been shown to have different effective time periods (Bouret et al., 2004; Steculorum et al., 2015). Leptin and ghrelin likely interact through the LepRb-pSTAT3 signaling pathway (Bouret et al., 2004; Bouret, 2013; Steculorum et al., 2015). Since estradiol and leptin share the neural signal transducer and activator of transcription 3 (STAT3) pathway to regulate feeding behavior (Gao and Horvath, 2008; Clegg, 2012), as well as possibly ghrelin, a synergistic action, at least among these three hormones, leptin, ghrelin and estradiol, on programming of feeding neural system cannot be discarded.

Apart from the alterations found in body weight in males and in hypothalamic POMC mRNA levels in females, no other significant differences were observed due to the blocking of ERs from P5 to P13. However, sex differences between male and female rats were detected in visceral fat, ERα, ERβ and AgRP. There are few reports about sex differences in ERs, but the levels and distribution of ERα and ERβ are reported to vary during development, with sex differences in different brain regions during the early stages of development and in adulthood (Wilson et al., 2002; Amateau et al., 2004; McCarthy et al., 2008; Wilson et al., 2011). However, the physiological repercussions of these differences are not known, and more research is necessary.

Blockage of the activity of ERs from P5 to P13, did not affect plasma estradiol levels during adulthood. On the other hand, when ERs are blocked from P1 to P5 plasma estradiol levels were decreased in adult females. Hence, adult POMC mRNA levels were affected in the first experiment, with no change in estradiol levels, but no effect on POMC was found in the second experiment, with changes in estradiol levels, suggesting that the alteration in the hypothalamic feeding circuit was programmed during development and is not the result of changes in plasma estradiol levels in adulthood.

Estradiol is known to participate in the development of other neural systems, such as the SDN-POA (Gorski, 1985; Döhler et al., 1986), the anteroventral periventricular nucleus of the hypothalamus (AVPV) (Simerly, 1989), the vomeronasal network (Guillamon and Segovia, 1997) and the cerebral cortex (Denley et al., 2018). Neonatal testosterone, via DHT and androgen receptors, is reported to be involved in programming POMC neurons in female mice, but neonatal estrogens were shown to predispose the animal to leptin resistance and obesity (Nohara et al., 2011).

Estradiol regulates energy balance through multiple mechanisms, many of which remain unknown. The present results indicate that the actions of estradiol on ERα, ERβ and GPER during a specific time window during development, from P5 to P13, are relevant for long-term metabolic control and emphasize the importance of studying both sexes. It is important to emphasize that these changes produced by the lack of estrogen activation of its receptors entail alterations of the metabolic control system that seems to be compensated, mainly in females, since in adulthood the alteration of POMC does not have effect on physiological parameters such as body weight or fat depots. Some authors have suggested that, instead of alterations, these changes during development should be considered 'adaptations' of the systems to cope with environmental challenges (Dearden and Ozanne, 2015).

It is essential to unravel the mechanisms that underlie the actions of estradiol on food intake, both during development and in adulthood, and how this programming differentially takes place in males and females. Such knowledge would help to produce tools that could facilitate the prevention, as well as the treatment, of metabolic disorders in a sex specific manner.

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