Molecular and Cellular Endocrinology 570 (2023) 111933

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Genistein early in life modifies the arcuate nucleus of the hypothalamus morphology differentially in male and female rats

Jose Manuel Fernandez-Garcia [a,b], Beatriz Carrillo [a,b], Patricia Tezanos [c], Helena Pinos [a,b,*], Paloma Collado [a,b]

[a] Departamento de Psicobiología, Facultad Psicología, Universidad Nacional de Educación a Distancia (UNED), Madrid, Spain
[b] Instituto Mixto de Investigación Escuela Nacional de Sanidad-UNED (IMIENS), Madrid, Spain
[c] Departamento de Neurociencia Traslacional, Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, 28002, Spain

ARTICLE INFO

Keywords:
Genistein
Arcuate nucleus
GPER
Nissl-stained neurons
Sexual dimorphism

ABSTRACT

In the present work we analyzed the effects of postnatal exposure to two doses of genistein (10 μg/g or 50 μg/g) from postnatal (P) day 6 to P13, on the morphology of the arcuate nucleus (Arc). The analyses of Arc coronal brain sections at 90 days showed that the ArcMP had higher values in volume, Nissl-stained neurons and GPER-ir neurons in males than in females and the treatment with genistein abolished these sex differences in most of the parameters studied. Moreover, in males, but not in females, the GPER-ir neurons decreased in the ArcMP but increased in the ArcL with both doses of genistein. In the ArcLP, GPER-ir population increased with the lowest doses and decreased with the highest one in males. Our results confirm that the Arc subdivisions have differential vulnerability to the effects of genistein during development, depending on which neuromorphological parameters, dose and sex are analyzed.

1. Introduction

The arcuate nucleus (Arc) is a hypothalamic structure located at the base of the third ventricle with a privileged position, close to the median eminence, to directly receive signals about the body's metabolic status from the gastrointestinal and adipose systems (Morton et al., 2014; Vohra et al., 2022). It is widely demonstrated that the Arc has a core involvement in the regulation of food intake mainly through proopiomelanocortin (POMC) and neuropeptide Y/Agouti related protein (NPY/AgRP) neurons (Sohn et al., 2013; Heisler and Lam, 2017; Sternson and Eiselt, 2017). Furthermore, it has been recently reported that the Kisspeptin/Neurokinin B/Dynorphin (KNDy) neurons in the Arc regulate the negative feedback on GnRH secretion (Mittelman-Smith et al., 2012; Constantin, 2022; Xie et al., 2022) therefore, the Arc is also involved in the physiology of the reproductive system (Dudek et al., 2018; Harter et al., 2018; Wang and Moenter, 2020).

The Arc is vulnerable to the effects of endocrine disrupting compounds (EDCs), such as bisphenol A (BPA), Aroclor 1221, organophosphate flame retardants (OPFRs), diethylstilbestrol or tributyltin, most of which produce alterations in different systems both in adulthood (Vail and Roepke, 2020; Marraudino et al., 2021) and during development (Walker et al., 2013; 2014; Roepke et al., 2016). These compounds have estrogenic effects and are considered detrimental to health (Casals-Casas and Desvergne, 2011). Phytoestrogens are also considered EDCs, but unlike those previously mentioned, their effects have been observed to be beneficial in preventing cardiovascular diseases (Fusi et al., 2020); improving menopausal symptoms (Chen et al., 2019; Thangavel et al., 2019) and preventing ischemic strokes (Castelló-Ruiz et al., 2011). However, currently there is no agreement on these potential beneficial effects, because some other investigations have shown no clear improvement in clinical studies (Lephart et al., 2004; Crain et al., 2008; Patisaul and Jefferson, 2010), while others have reported a variety of alterations such as cytotoxicity in primary cortical neurons increasing cell death; disruption in the timing of ovarian development (Losa et al., 2011); sex differential changes in the number of POMC neurons in the Arc (Fernandez-Garcia et al., 2021) or an increase in neuritic arborization in primary hypothalamic neurons (Marraudino et al., 2019).

Genistein is a phytoestrogen belonging to the group of isoflavones present in high levels in soybeans, which are highly consumed by the Asian population and constitute a basic nutrient in their diet. In Western countries its consumption has increased significantly in recent decades, and it is used as a substitute for breast milk when newborns develop

* Corresponding author. Universidad Nacional de Educación a Distancia (UNED), Departamento de Psicobiología C/ Juan del Rosal 10, 28040, Madrid, Spain.
E-mail address: hpinos@psi.uned.es (H. Pinos).

https://doi.org/10.1016/j.mce.2023.111933
Received 22 November 2022; Received in revised form 16 March 2023; Accepted 16 April 2023
Available online 18 April 2023

some type of allergy to milk (Patisaul and Jefferson, 2010; Verduci et al., 2020). The estrogenic effects of genistein occur through the nuclear estradiol receptors (ER) α and ERβ, although a higher affinity of this compound for the ERβ has been described (Kuiper et al., 1998; Casanova et al., 1999; Patisaul et al., 2002). Apart from the effects through nuclear receptors, actions of genistein via G-coupled membrane receptor (GPER) have also been shown and some authors reported a similar binding affinity to both ERα and ERβ (Thomas and Dong, 2006). Specifically, genistein through GPER activation produces anti-inflammatory effects in microglial cells (Du et al., 2018) or an increase in glial cell migration (Ariyani et al., 2020). Since, of the three receptors, the GPER is the most recently discovered receptor, less data is available to link this receptor with the effects of genistein. Therefore, studying the neuronal population expressing GPER, as well as the effects that early administration of genistein has on this population in the long term, is of utmost interest.

The early stages of development are highly vulnerable to exposure to potentially harmful external agents, mainly because they interfere with the processes of establishment and maturing of neural networks. Specifically, the Arc neurogenesis occurs during the prenatal stage, but synaptogenesis takes place mainly during the early postnatal stage in rodents (Bouret et al., 2004; Rice and Barone, 2000; Ishii and Bouret, 2012). The vulnerability of the Arc to exposure to genistein has been demonstrated as the administration of genistein during the first three days of life decreased the number of kisspeptin immunoreactive fibers in Arc in female rats (Losa et al., 2011). Moreover, in a previous study we showed that genistein administration from P6 to P13 modified the number of POMC neurons in the Arc differentially in adult male and female rats (Fernandez-Garcia et al., 2021).

Considering the effects that genistein has when administered during the early stages of development, and our previous results in which we have also shown that during the treatment period, from P6 to P13, the arcuate melanocortin system is sensitive to the effects of estradiol, in the present work we will address four main objectives, 1) to analyze what effects genistein have on the neuronal population of all subdivisions of the Arc in a sensitive period of development, from P6 to P13, in the long term, when the animals are adult; 2) to examine whether the population of neurons expressing the membrane receptor GPER is altered by genistein during this same period, as it is increasingly being shown that this membrane receptor could be a relevant pathway in the action of phytoestrogens on the brain; 3) stemming from the two previous objectives, but no less important, our results will allow us to show a neuroanatomical study of the neuronal population and of the population expressing GPER receptors in male and female control animals in all subdivisions of the Arc; 4) finally, we will study males and females, since previous reports on the Arc have shown the existence of sex differences in several morphological parameters of this nucleus and also in the response to exposure to different EDCs.

2. Materials and methods

2.1. Animals

Wistar rats were maintained under controlled conditions of temperature, humidity, and light (22 ± 2 °C; 55 ± 10% humidity; 12 h light/12 h dark cycle, lights on from 08:00 to 20:00). Throughout the whole experiment, special attention was paid to minimize animal suffering and reduce the number of animals used to the minimum necessary, 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).

For mating, a male was placed in a cage with two females for one week. Pregnant females were housed individually in maternity cages with wood shavings as the nesting material. After delivery, on postnatal day 1 (P1), pups born on the same day were weighed, sexed, and randomly distributed (five females and five males/dam). Pups were treated with a daily subcutaneous injection of synthetic genistein (Genistein Synthetic, ≥98%, Sigma-Aldrich St. Luois, MO, USA) with one of two doses, 10 μg/g (low dose, G10) or 50 μg/g (high dose, G50), or vehicle (corn oil). The doses were selected after reviewing our previous results and those of other authors (Faber and Hughes, 1991; 1993; Bateman and Patisaul, 2008; Ponti et al., 2017; 2019; Fernandez-Garcia et al., 2021). The injections were administered between 8:00 and 10:00 a.m. All pups received eight injections between days P6 and P13, resulting in the following groups: control male (n = 10, CM), control female (n = 9, CF), G10 male (n = 10, G10M), G10 female (n = 9, G10F), G50 male (n = 10, G50M) and G50 female (n = 8, G50F) as can be seen in Fig. 1A.

2.2. Tissue preparation

On P90, animals were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Then, the animals were transcardially perfused with saline, followed by 4% paraformaldehyde (PAF) between 9:00 and 11:00 a.m. The speed of the perfusion pump was set at 45 revolutions per minute. All females were in the diestrous phase. The brains were removed, stored in a freshly prepared PAF solution for 2 h at 4 °C and then washed several times in phosphate-buffered saline (PBS). Next, the brains were stored in a 30% sucrose solution in PBS at 4 °C until they were examined. The brains were then frozen with Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and serially sectioned along the coronal plane at a thickness of 40 μm. Serial sections were collected in four series, of which two were used in this study for the histological analysis processed via the Nissl staining method for labelling neurons (Fig. 1B) and GPER immunostaining (Fig. 1C).

2.3. Nissl staining and morphometrical analysis

Sections were stained with a 0.1% solution of cresyl violet (C5042, Merck Chemicals, Darmstadt, Germany). Once the Nissl stain was performed, all sections were photographed with a magnification of X10 with a NIKON Digital Sight DS-Fi1 camera connected to a NIKON Eclipse 80i microscope with the Olympus CellA v1.1.6 program. All measurements of volume and number of Nissl-stained neurons from these images were manually taken by one of the authors and verified by another. The persons conducting the morphometrical analysis were blinded about the condition of the animals.

The limits of the Arc were established, according to the Atlas of the Rat Nervous System (Paxinos and Watson, 2014). The Bregma coordinates of each subdivision are shown in Table 1. The regions considered in this study were the medial (ArcM), the medial lateral (ArcL), the dorsal (ArcD), the medial posterior (ArcMP) and the lateral posterior (ArcLP) subdivisions of the nucleus following a rostro-caudal axis.

2.3.1. Volume

The volume of the Arc was estimated by applying the classic Cavalieri principle (Gundersen and Jensen, 1987). The right and left hemispheres were measured independently. In each section, the area of the coronal section of the Arc was estimated by superimposing a grid of crosses with an area per point of 3300 μm^2, using the ImageJ program version 2.0.0.0-rc-69/1,52n, as can be seen in Fig. 1D. All the crosses of the grid located entirely within the previously set delimitations in all sections of all subdivisions of the Arc were counted to make an estimate of the volume following the formula of the Cavalieri principle. Cavalieri's principle, a well-established stereological technique, uses interpolation between samples to estimate the volume of three-dimensional (3D) objects using the following formula:

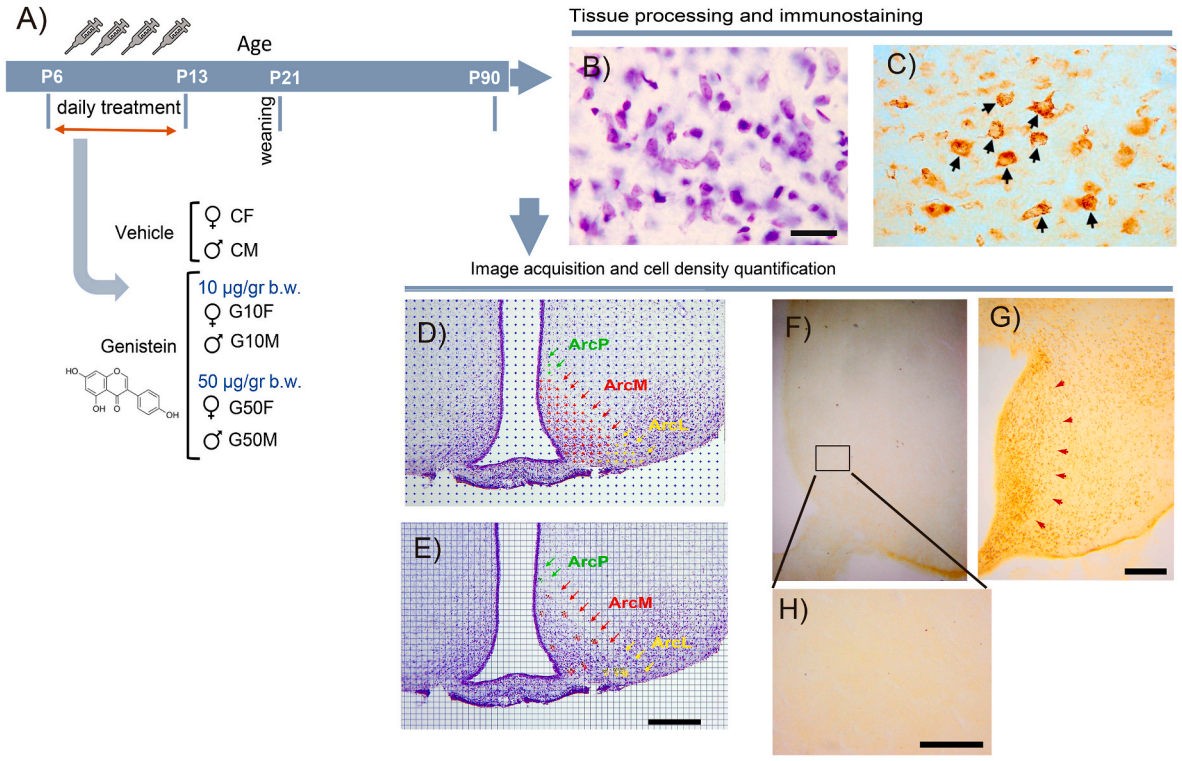


Fig. 1. Schematic representation of material and methods. A) From postnatal day 6 (P6) to P13 Pups were treated with a daily subcutaneous injection of synthetic genistein with 10 µg/g (low dose, G10) or 50 µg/g (high dose, G50), or vehicle (corn oil). The groups obtained were control male (CM), control female (CF), G10 male (G10M), G10 female (G10F), G50 male (G50M) and G50 female (G50F). After the sacrifice on P90, tissue was serially sectioned on the coronal plane and used for histological analysis processed via the Nissl staining method and GPER immunostaining. B) Photomicrograph (x40) showing a detail of Nissl-stained neurons. Nissl-positive neurons were considered to be all those in which the nucleolus was marked by the Nissl stain. C) Photomicrograph (x40) showing the detail of typical GPER-ir Arc neurons. All cells in which the morphology of the neuronal soma could be distinguished were considered. D) Photomicrograph (x10) showing the Cavallieri method used for the estimation of Arc volume in Nissl-stained sections. Briefly, a grid of red crosses of 3300 µm2 was superimposed on the photo. All crosses that were within the nucleus' boundaries were counted (marked with red crosses). E) Method applied for counting Nissl stained and GPER-ir neurons in the Arc section. A grid of blue lines (amplified in the photo for ease of identification) with an area of 1497.69 µm2 was superimposed over the section. As can be seen in the photo, all neurons (marked as red circles) included in each square's limits were taken into account. F) And G) Photomicrograph (x10) showing negative control and immunostaining of GPER in the Arc, marked with red arrows. In image F, the incubation with the first antibody was omitted. H) Photomicrograph (x40) showing a detail of negative control section without signal. ArcD = Arcuate dorsal; ArcL = Arcuate lateral; ArcM = Arcuate medial (Paxinos and Watson, 2014). Scale bars = B = 25 µm; E = 300 µm; G = 150 µm; H = 50 µm.

Table 1
Bregma coordinates of Arcuate nucleus regions studied according to Paxinos and Watson (2014).

Arcuate Nucleus (Bregma −1.92 mm to −4.36 mm)	
Anterior subdivision (Bregma −1.92 mm to −3.36 mm)	**Posterior subdivision** (Bregma −3.48 mm to −4.36 mm)
Dorsal (**ArcD**)	Medial Posterior (**ArcMP**)
Medial (**ArcM**)	
Lateral (**ArcL**)	Lateral Posterior (**ArcLP**)

$$V\ (mm^3) = f \times (SP \times a(p) \times t)$$

Where f is the fraction used, P is the number of points counted in each of the sections, a(p) is the area associated with each point and t is the thickness of the sections used.

2.3.2. Number of neurons

Nissl-positive neurons were considered to be all those in which the nucleolus was marked by the Nissl stain (Fig. 1 B). The right and left hemispheres were measured independently in each animal. The Arc was analyzed from −1.92 mm to −4.36 mm bregma, following Paxinos and Watson (2014). The Arc was observed in an average of 14 sections, 8 of which corresponded to the anterior region and 6 of them to the posterior region. These numbers could vary depending on the animal, but by no more than one or two sections. For the estimation of the number of neurons, a grid with quadrants with an area of 1497.69 μm^2 was placed on each section in which the Arc appeared, as represented in Fig. 1E. Two selection patterns were used due to the different size of the Arc subdivisions. In the ArcM, ArcD and ArcMP subdivisions, one in ten quadrants were randomly selected and in the more lateral subdivisions, ArcL and ArcLP, one in five quadrants were randomly selected. Only those quadrants whose total area was within the boundaries of the subdivisions of the nucleus were included. All neurons in which neuronal bodies were distinguished within the quadrant were considered, excluding those in which the grid lines overlapped with neuronal morphology.

2.4. GPER immunostaining and morphometrical analysis

First, the cryoprotectant was washed with 0.1% PBS, then the sections were incubated with a PBS solution containing 0.2% bovine serum albumin (BSA) and 0.2% Triton X-100 for 30 min and treated to block endogenous peroxidase activity (buffer containing 1:1 methanol and 0.3% hydrogen peroxide; stirring for 10 min, at room temperature). Next, 3 washes were performed on PBS for 5 min. Then, the sections were incubated for 48 h at 4 °C with a rabbit polyclonal antibody against

GPER diluted 1:250 in PBS and 0.2% de BSA, (Anti-G-protein coupled receptor 30 antibody ab39742, Abcam, Cambridge, UK). This step was omitted in the negative control sections (Fig. 1 F,H). Our group tested the positive control in a previous study in which the same antibody was used (Marraudino et al., 2021). After several brief washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG serum (Invitrogen by Thermo Fisher, 1:200, USA) for 90 min and then in avidin-peroxidase complex (ABC Peroxidade Staining Kit Thermo Scientific, USA) for 60 min at room temperature. Finally, the presence of peroxidase activity was revealed with a solution containing 0.02 g/mL diaminobenzidine (DAB, Aldrich, Madrid, Spain) and 0.025% hydrogen peroxidase in Tris–HCl, pH 7.6. The sections were mounted on gelatin-coated slides, dehydrated in ethanol, washed in xylene, coated with DPX and covered (Surgipath Europe Ltd., Peterborough, UK).

The counting of the GPER-ir neurons in the Arc was carried out by means of black and white photographs 20X taken with the NIKON Digital Sight DS-Fi1 camera connected to the NIKON Eclipse 80i microscope with the Olympus CellA v1.1.6 program. These photographs were manually analyzed using the ImageJ program version 2.0.0.0-rc-69/1,52n by the same authors whose were blinded about the condition of the animals.

All sections of the brains were sorted according to Paxinos and Watson (2014) and all sections with positive marking in GPER in the Arc were selected.

Immunohistochemical marking of GPER has been characterized as predominantly cytoplasmic, with a wide distribution throughout the Arc. All cells in which the morphology of the neuronal soma could be distinguished were considered, excluding those showing a dendritic morphology characteristic of glial cells (Llorente et al., 2020). The right and left hemispheres were counted independently. To do this, a grid with quadrants with an area of 1497.69 μm2 were first put in place, as previously described for counting Nissl-stained neurons. The delimitations of all the subdivisions of the Arc were then established and the count was carried out, as mentioned above, with the exception of the ArcD and ArcM. The limits between these two subdivisions were difficult to establish in these GPER-ir stained sections, so they were considered as a continuum (ArcD-M).

Like in the Nissl-stained sections, in the ArcD-M and ArcMP subdivisions one in ten quadrants were randomly selected and in the more lateral subdivisions, ArcL and ArcLP, one in five quadrants were randomly selected. Only those quadrants entirely within the boundaries of the subdivisions of the nucleus were included. All cells within the quadrant were counted, excluding those in which the grid lines overlapped with the cells.

2.5. Plasma estradiol levels

Plasma estradiol levels were measured in duplicate by ELISA (CEA461Ge, Cloud-Clone Corp.) 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.

2.6. Statistical analysis

The data obtained were submitted to one-way ANOVA, with the hemisphere as a factor to determine the potential differences between the right and left hemispheres. Once the effect of the hemisphere was discarded, the mean value of the two hemispheres and that of each subdivision was used for the posterior statistical analyses performed. In order to know the possible differences between the anterior and posterior region of Arc, the sum of ArcM, ArcL, ArcD was considered to be the total anterior Arcuate and the sum of ArcMP and ArcLP was considered to be the total posterior Arcuate. Similarly, the sum of all subdivisions constituted the Total Arcuate. To determine sexual dimorphism, ANOVAs between males and females with treatment as a factor, were developed. The significance level was set at $p < 0.05$. To determine intra-sex differences, male and female groups were analyzed independently using a one-way ANOVA followed by S–N–K *post hoc* tests with a significance level at $p < 0.05$.

3. Results

3.1. Volume

In Table 2, the mean and SD volumes obtained in this study can be seen. With respect to sex differences in control groups, a significant difference was found between males and females in the total posterior Arcuate volume ($F1,10 = 7.864$; $p = 0.019$).

When each subdivision was examined, we also found a significant difference in the ArcMP ($F1,10 = 12.635$; $p = 0.005$). In both cases, males have a greater volume than females (see Fig. 2 A, B and * in Table 2).

In genistein treated groups this dimorphism is absent. When each sex was examined separately, no differences were detected between female or male groups.

3.2. Number of neurons

In the posterior division of the Arc (Fig. 3M) a significant difference in neurons was found in the ArcMP subdivision in the control groups ($F1,10 = 9.061$; $p = 0.013$) with males having a higher number of neurons than females, as can be seen in Fig. 3 (A,B,C). The same results were found between G10 males and females in the ArcMP ($F1,10 = 11.678$; $p = 0.008$) and, in the total posterior Arcuate, ($F1,10 = 5.962$; $p = 0.037$) males were seen to have a higher number of neurons than females in all cases (Fig. 3D and E,F,N).

In the anterior division of the Arc, significant differences between G10 groups can be observed in the ArcL ($F1,10 = 7.406$; $p = 0.024$) with females having a higher number of neurons than males (Fig. 3G and H,I). In the G50 group, a difference was found in the ArcD with females having a higher number of neurons than males ($F1,10 = 6.260$; $p = 0.034$) (Fig. 3J and K,L).

When each sex was analyzed separately, no differences were detected between the male groups. Between the female groups, a main effect in the ArcM was found ($F2,13 = 4.203$; $p = 0.039$). The *post hoc* analysis revealed a significant difference between the CF and G10F groups due to an increment in the number of neurons in the latter ($CF < G10F$; $p < 0.05$) as can be seen in Fig. 4.

3.3. GPER-ir

GPER-ir cells were widely distributed across the Arc and their distribution was similar to Nissl-stained cells in this nucleus. With respect to sex differences in the control groups, a significant difference was found between males and females in the total posterior Arcuate ($F1,10 = 17.802$; $p = 0.002$) (Fig. 5 D). When each subdivision was examined, we found a significant difference in the ArcMP between the control groups ($F1,10 = 14.072$; $p = 0.004$) (Fig. 5A and B,C). In both cases, males had more GPER-ir cells than females. In genistein treated groups this dimorphism was absent.

When each sex was analyzed separately, no differences were detected between female groups. In males, a main effect of treatment was seen in the ArcL ($F2,14 = 4.830$; $p = 0.025$), in the ArcMP subdivision ($F2,14 = 5.319$; $p = 0.019$), in the ArcLP subdivision ($F2,14 = 9.692$; $p = 0.002$) and in the total posterior Arcuate ($F2,14 = 7.590$; $p = 0.006$). A *post hoc* analysis showed significant differences between the CM and G50M groups in different subdivisions. As can be seen in Fig. 6, G50M have greater number of neurons than CM in the ArcL ($CM < G50M$; $p < 0.05$) but not in the ArcMP, the ArcLP and in the total posterior Arcuate ($CM > G50M$; $p < 0.05$ in all cases) (Fig. 6A and B,C,D). The comparison between CM and G10M showed significant differences between these

Table 2
Mean and SD values of volume (mm^3) in each subdivision, region and in the Total Arcuate nucleus.

	CM			CF			G10M			G10F			G50M			G50F		
ArcM	0.132	±	0.016	0.131	±	0.024	0.136	±	0.022	0.132	±	0.020	0.129	±	0.027	0.136	±	0.026
ArcL	0.045	±	0.006	0.049	±	0.003	0.046	±	0.006	0.045	±	0.007	0.044	±	0.008	0.044	±	0.009
ArcD	0.012	±	0.001	0.013	±	0.002	0.013	±	0.002	0.012	±	0.002	0.012	±	0.001	0.012	±	0.002
ArcMP	0.040	±	0.006	0.030*	±	0.004	0.034	±	0.008	0.030	±	0.005	0.034	±	0.008	0.035	±	0.007
ArcLP	0.020	±	0.004	0.018	±	0.003	0.020	±	0.003	0.019	±	0.002	0.020	±	0.003	0.020	±	0.003
Arc (Anterior)	0.189	±	0.018	0.192	±	0.025	0.195	±	0.025	0.189	±	0.026	0.185	±	0.025	0.192	±	0.036
Arc (Posterior)	0.060	±	0.009	0.048*	±	0.006	0.054	±	0.010	0.049	±	0.006	0.054	±	0.011	0.055	±	0.009
Arc Total	0.250	±	0.026	0.240	±	0.029	0.249	±	0.022	0.237	±	0.031	0.238	±	0.034	0.247	±	0.042

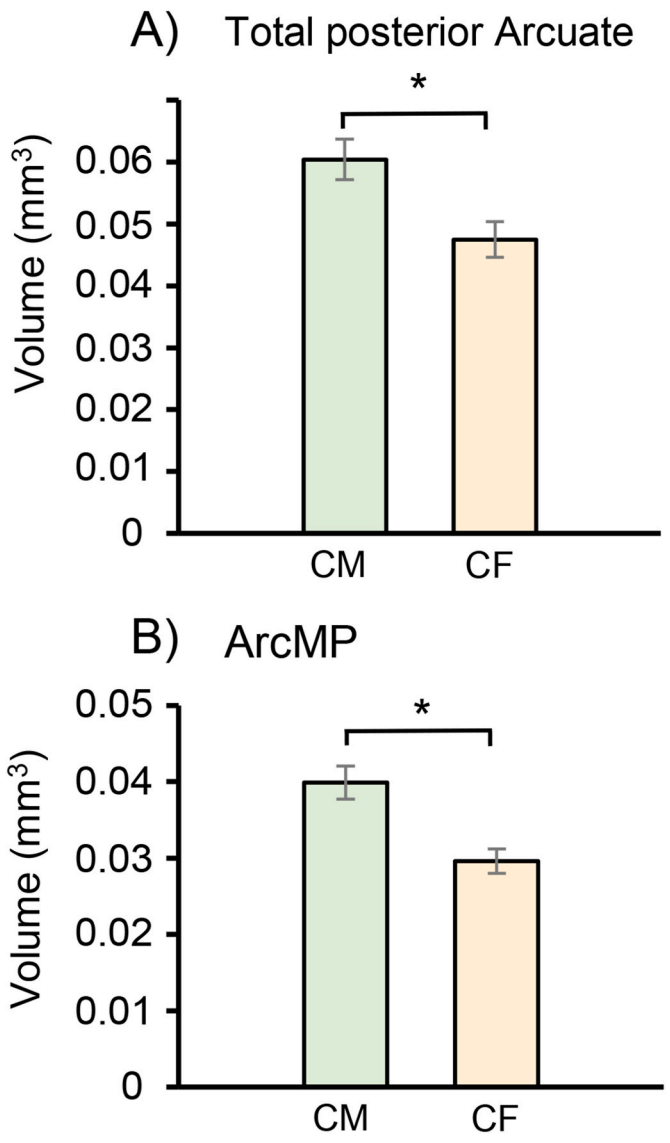


Fig. 2. Histogram shows sex differences in volume in A) Total posterior arcuate nucleus. B) Medial posterior arcuate subdivision ArcMP. CM = control male; CF = control female. *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

two groups in the ArcL and ArcLP, with G10M showing a greater number of neurons than CM in both subdivisions ($p < 0.05$ in both comparisons) (Fig. 6 A,C). Moreover, differences between G10M and G50M groups were seen in the ArcLP and in the total posterior Arcuate with the G10M groups showing more neurons than the G50M groups ($p < 0.05$ in both cases) as can be seen in Fig. 6 C,D.

3.4. Plasma estradiol levels

Estradiol showed a main effect of sex ($F1,33 = 5.77$; $p = 0.02$), treatment ($F2,13 = 15.125$; $p = 0.001$) and the interaction between the factors ($F2,13 = 7.825$; $p = 0.002$). The *post hoc* analysis showed sexual dimorphism between the G50M and G50F groups due to the increase in the values in males ($p < 0.05$) (Fig. 7A).

When the male groups were analyzed, we found a main effect of treatment ($F2,13 = 10.871$; $p = 0.001$). The *post hoc* analysis showed a difference between the G50M groups and the other two male groups, due to the increase in the levels of estradiol in the G50M group ($p < 0.05$ for all comparisons) (Fig. 7B). In females, a main effect for treatment was found ($F2,13 = 12.346$; $p = 0.001$). The *post hoc* test showed a significant difference between the CF group and the genistein treated females, with the CF group having the lowest values ($p < 0.05$ for all comparisons) (Fig. 7C).

With respect to testosterone values, no main effect of treatment was detected. When each sex was examined separately, no differences were detected between the female or male groups.

4. Discussion

The Arc is a crucial nucleus in the regulation of energy metabolism and its participation in the control of reproductive behaviors has also been demonstrated. The heterogeneity of the neural populations which make up this structure support the importance of distinguishing between subdivisions when studying it. In the present work we have shown that the Arc is a structure vulnerable to the action of genistein during the early postnatal stage because, depending on the subdivision analyzed and on the neuronal population considered, males and females responded differently to the two doses of genistein used in this study, administered from P6 to P13. The principal result of our study is that, of all the subdivisions of the Arc, the posterior region, and specifically its GPER-ir population, is the most vulnerable to the effects of genistein.

Neuroanatomical study of the Arc has shown that the ArcMP showed sexual dimorphism in volume and in the two populations studied, the Nissl-stained neurons and the GPER-ir neurons. The Arc is involved in the regulation of sexual dimorphic behaviors (Asarian and Geary, 2013) and sex differences in various morphological parameters of this structure have been reported, with most following a male greater than female pattern (Matsumoto and Arai, 1980; Leal et al., 1998). Although analysis of the volume, number of neurons and density of the Arc has been carried out in males or females (Peng and Hsu, 1982; Yang et al., 1993; Hsu and Peng, 1978), only one article has reported sex differences in these parameters (Leal et al., 1998). Given the heterogeneity of the neurons that make up the Arc and the diversity of functions in which they participate (Sohn et al., 2013; van den Pol et al., 2019; Campbell et al., 2017; Quarta et al., 2021), our findings provide added value by pointing out which specific subdivision of the Arc presents differences between males and females in the two neuronal populations analyzed. This might explain the differences in the physiology of the behaviors that this nucleus regulates, such as feeding (Asarian and Geary, 2013) and reproductive behaviors (Schafer et al., 2018; Wang and Moenter, 2020) and

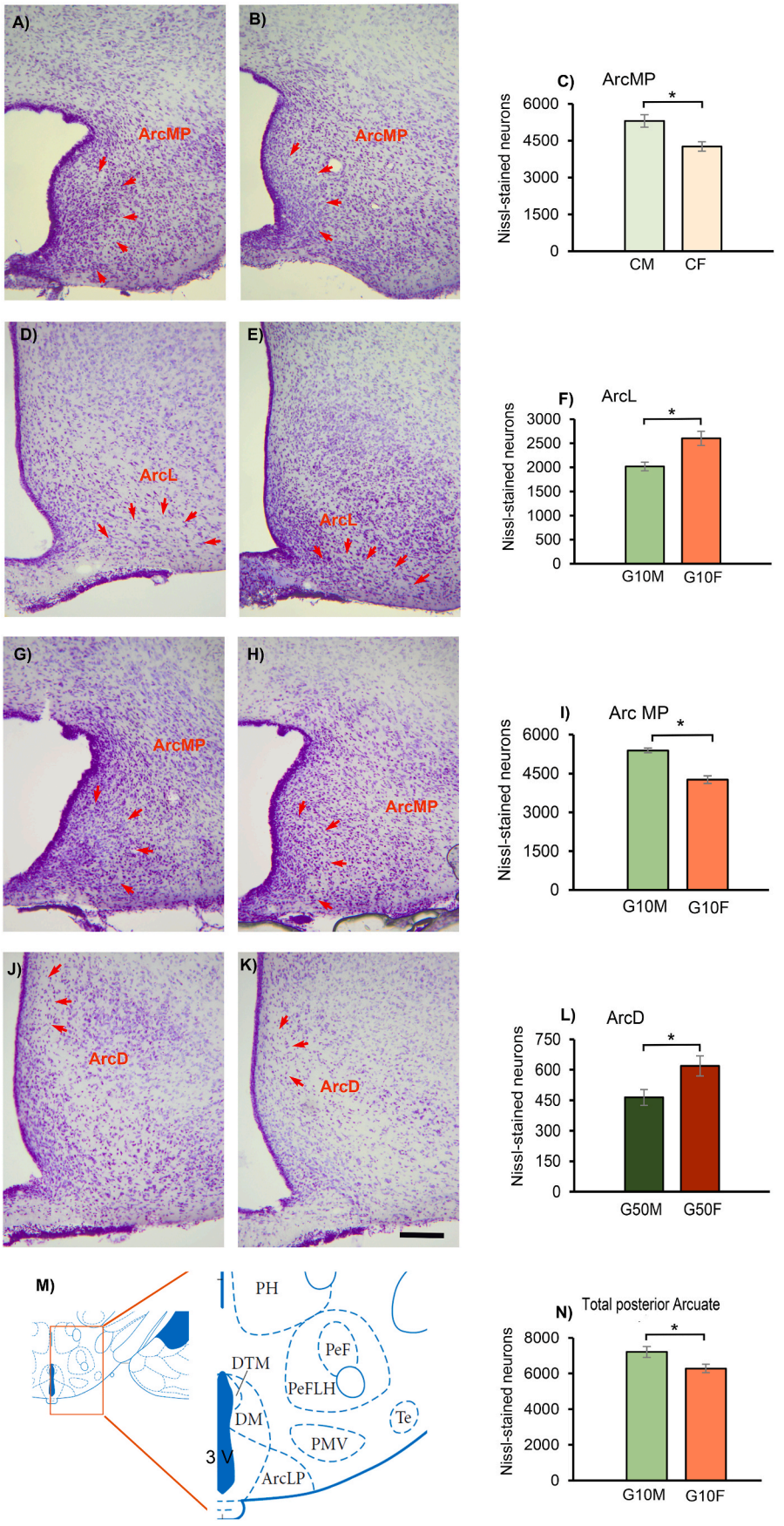


Fig. 3. Photomicrographs (x10) and graphs showing the sexual dimorphism found in different subdivisions between males and females in each treatment. Limits of the Arc are signalled by red arrows in each photomicrograph according to Paxinos and Watson (2014). In figures A,B,C: images representing the sexual dimorphism found in ArcMP between control groups: A) CM; B) CF; C) Number of ArcMP Nissl-stained neurons in control groups. In figures D,E,F sexual dimorphism found in the ArcMP between G10 groups is represented: D) G10M; E) G10F; F) Number of ArcMP Nissl-stained neurons in G10 groups. In figure G,H,I representative image of sex differences between G10 groups in the ArcL: G) G10M; H) G10F; I) Number of ArcL Nissl-stained neurons on G10 groups. In J,K,L figures: sex differences between G50 groups: J) G50M; K) G50F; L) Number of ArcD Nissl-stained neuron on G50 groups. Figures M and N: M) Schematic representation of posterior Arcuate nucleus; N) Number of total posterior Arcuate Nissl-stained neurons in G10 groups. CM = control male; CF = control female; G10M = G10 male; G10F = G10 female; G50M = G50 male; G50F = G50 female. ArcD = Arcuate dorsal; ArcL = Arcuate lateral; ArcMP = Arcuate medial posterior. *indicates significant differences between males and females ($p < 0.05$ in all cases). All values are expressed as means ± S.D. Bar = 150 μm.

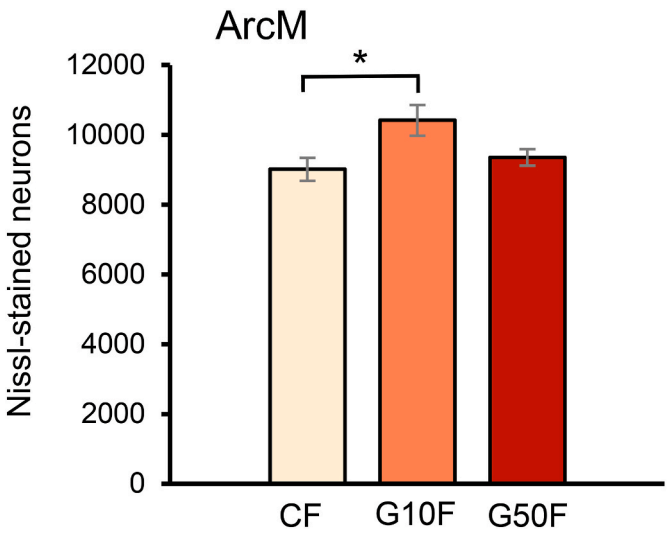


Fig. 4. Histogram shows differences in Nissl-stained neurons among female groups in the ArcM subdivision CF = control female; G10F = G10 female; G50F = G50 female. ArcM = Arcuate medial (Paxinos and Watson, 2014). *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

highlights the importance of analyzing the Arc considering all subdivisions.

Treatment with genistein from P6 to P13 altered the normal development of several subdivisions of the Arc depending on the dose. The posterior region of the Arc was the most affected by the administration of genistein. On the one hand, the sex differences found in the Nissl population remain unchanged with the low dose of genistein, but with the high dose the dimorphism disappeared in this region. While for the GPER-ir population, the sex differences found in the control groups were abolished with both of the genistein doses used in the study. On the other hand, the GPER-ir neurons in the posterior subdivisions were also affected in males although not in females. It is also important to note that except for the low dose of genistein in the ArcLP, which increased the GPER-ir neurons, the high and/or low dose of genistein in the ArcMP subdivision resulted in a significant decrease of the GPER-ir population. It might be surprising that the population of GPER neurons was affected by genistein treatment in males but the total number of Nissl-stained neurons including the former was not. This could be explained by the fact that the population of GPER neurons constitutes only a proportion of the total number of neurons in this subdivision and, therefore, differences found in a specific population are not reflected in the total number of neurons.

Although the most consistent results of our study were shown in the posterior region of the Arc, other effects of genistein were detected in the anterior region of the nucleus. The only effect on the ArcM was an increase in the population of Nissl-stained neurons with the low dose of genistein in females. In addition, both doses increased the number of GPER-ir neurons in males in the ArcL. It has been shown that in the posterior region of the Arc, but not in the anterior one, excitatory glutamate neurons abound and participate in the control of satiety projecting axonal connections to local neurons containing POMC (van den Pol et al., 2019). Moreover, higher concentrations of GH-RH-ir were reported in the caudal with respect to anterior Arc (King et al., 1975). Considering the reported differences in the neurophysiology of the different subdivisions of the Arc, it could be suggested that genistein during the early postnatal stage differentially affects the various neuronal populations that comprise the Arc depending on the sex of the animals and the dose of genistein administered.

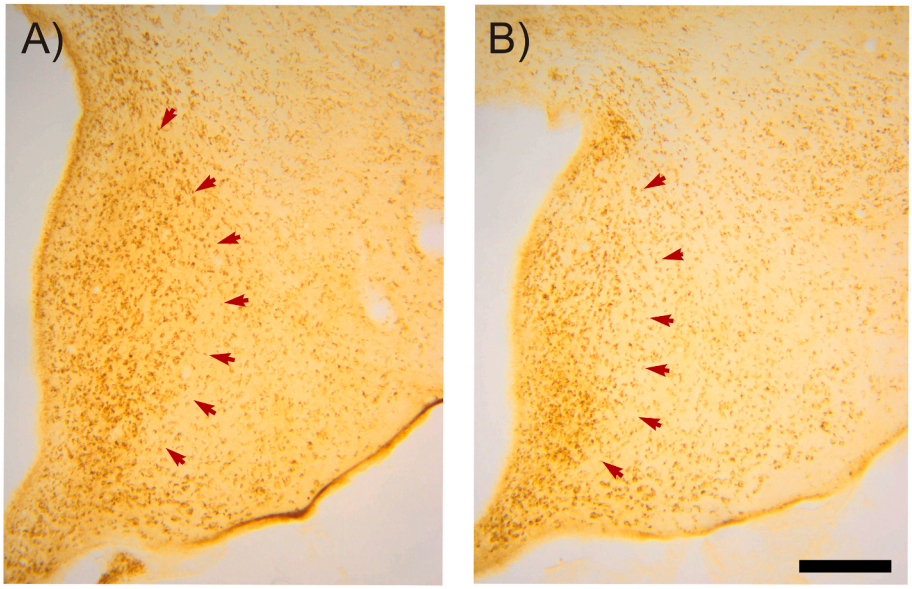
It is worth highlighting the differential effect that dose had in some subdivisions, both on Nissl population and on GPER-ir neurons. It could be surprising that the lower dose had greater effect than the higher one, as in the case of the number of Nissl neurons in the ArcM or the GPER-ir population in the ArcLP. However, considering the different sensitivity of genistein to the E2 receptors and the diversity of neuronal populations and their connection patterns throughout the Arc subdivisions (Sohn et al., 2013; van den Pol et al., 2019; Campbell et al., 2017; Quarta et al., 2021), together with the capability of genistein to exert estrogenic and/or antiestrogenic effects (Mueller, 2002), a combination of these factors could be an explanation for the greater effect on these two populations with the lowest dose.

Few studies have researched the differential effect of genistein in males and females and even fewer its influence during development. Some data has shown that in neonates, genistein produces a decrease in the size of oxytocin neurons in the supraoptic nucleus in males but not in females (Yoshimura et al., 2011). When the effects of exposure to genistein during development were analyzed in adulthood, results showed that genistein abolished sexual dimorphism in a specific region of the hypothalamus (Ponti et al., 2019) and that the number of nitric oxide synthase (NOS) cells decreased in the amygdala in males but not in females (Ponti et al., 2017). Specifically in the Arc, exposure to genistein in the first three days of life was found to decrease kisspeptin fiber density on postnatal day 21 (Losa et al., 2011). Furthermore, our group has previously found a differential response in the Arc in males and females via the same genistein protocol used in the present study, showing that the population of proopiomelanocortin (POMC) neurons in the ArcM subdivision was significantly decreased by genistein treatment in females but not in males (Fernandez-Garcia et al., 2021). All these results support the importance of including males and females in all studies dealing with the effects of genistein on the different neural systems.

It must be taken into account that exposure to genistein occurred during a specific period of the early postnatal stage and that the effects were detected when the animals were adults. During that specific stage of development, the hypothalamic circuits are being programmed (Bouret et al., 2004; Rice and Barone, 2000; Ishii and Bouret, 2012). It should come as no surprise that the administration of a phytoestrogen during this period may alter the normal development of different aspects of the Arc because this period has been demonstrated to be sensitive to the effects of estradiol. Previous research from our group has shown that estradiol treatment during this same period modulated the physiological and brain alterations produced by a high-fat diet. We have also shown that the inhibition of ERα, ERβ and GPER had differential long-term effects on body weight and mRNA POMC levels in both sexes (Carrillo et al., 2016; 2019; Pinos et al., 2018). Furthermore, the specific action of GPER in response to genistein in different functions and processes has been demonstrated (Du et al., 2018; Ariyani et al., 2020). Our data shows that the GPER expression in the Arc is susceptible to the effects of genistein differentially in males and females during development, depending on the doses and the subdivisions considered.

It could be thought that the alterations in the expression of GPER are due to circulating levels of sex hormones, since estradiol activates this receptor. It has been demonstrated that estradiol stimulates GPER profiles and glycogen content in female but not in male astrocytes (Ibrahim et al., 2020). Moreover, GPER fluctuates along the estrous cycle in different structures in a sexually dimorphic pattern (Marraudino et al., 2021). Therefore, it appears that the population of neurons expressing GPER responds differentially depending on estradiol levels. But, at this point, it is necessary to emphasize that the changes found in adult animals in the present work do not seem to be due to an effect of plasma gonadal hormone levels, since no significant changes in circulating testosterone were observed between groups and those detected in estradiol occurred in both males and females at the two doses studied. What could be suggested is that early exposure to the phytoestrogen could condition circulating levels of estradiol in adult animals.

The involvement of estradiol in energy metabolism (Lopez and Tena-Sempere, 2015), its specific action on the activity of POMC



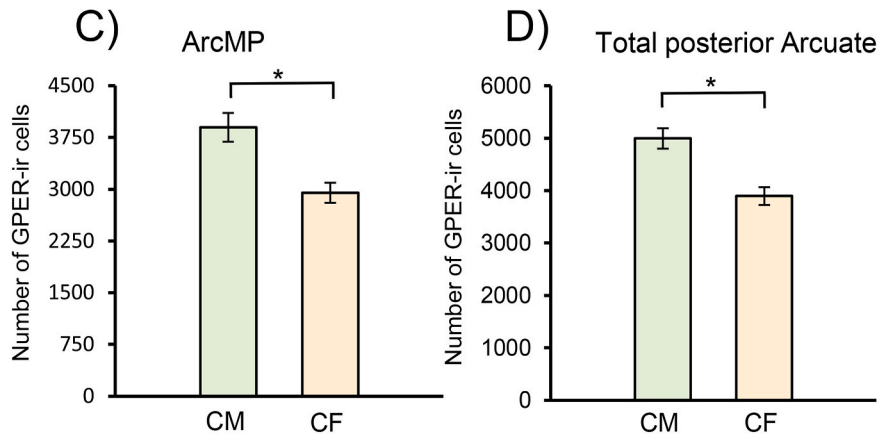


Fig. 5. Photomicrographs (x10) and graphs showing the sexual dimorphism found in GPER-ir cells in the ArcMP (A,B,C) and total posterior Arcuate (D) between males and females in control groups. A) Control male; B) Control female. CM = control male; CF = control female; red arrows show the limits of the ArcMP. ArcMP = Arcuate medial posterior subdivision (Paxinos and Watson, 2014). *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D. Bar = 150 μm.

neurons (Gao et al., 2007), and its programming during development (Carrillo et al., 2020) has already been demonstrated. Our data show that genistein, a phytoestrogen that acts via estrogen receptors, has a considerable impact on Arc morphology during development. However, our data cannot explain how genistein itself can alter the programming of Arc morphology. This would require the inclusion of a new group in which genistein was co-administered with an estradiol inhibitor (e.g. ICI compound), which would allow us to accurately conclude the role of genistein in Arc programming.

It is well-established that the Arc plays a relevant role in the regulation of energy metabolism and feeding behavior and just over two decades ago the presence of kisspeptin in this nucleus and its involvement in the regulation of reproductive physiology was observed (Gottsch et al., 2004; Harter et al., 2018). Both processes are influenced by estrogens and the early stages of development are crucial for both to ensure the correct establishment of the circuits that regulate these behaviors (Smith, 2006; Roepke, 2009; Frank et al., 2014; Santollo and Daniels, 2015; Harter et al., 2018). Our results show that genistein treatment during the second week of life can interfere with the correct development of the population expressing GPER and, therefore, the functions in which this receptor is involved may be altered. We have highlighted the most susceptible region to the effects of genistein, the ArcMP, but further research will be necessary to know the participation of this receptor in the mechanisms underlying feeding, reproduction, and the possible interaction between them.

5. Conclusions

Our results confirm that the Arc is a complex structure whose different subdivisions have a differential vulnerability to the effects of genistein during development depending on the neuromorphological parameters studied, since different doses of genistein alter the population of GPER neurons, specifically in the posterior medial subdivision of the Arc, in male but not in female rats. Although the participation of the Arc in the regulation of food intake has been well demonstrated, this nucleus has also been involved in the regulation of reproductive physiology due to its control over GnRH release. For this reason, studying the effects that agents such as genistein may have during development are crucial to furthering our understanding of the morphology and function of this nucleus. The fact that certain specific populations, such as glutamatergic, are located mainly in the posterior region of the Arc, whose GPER neuronal population seems to be sensitive to the genistein action during the developmental stages in males, opens the door to future research that unravels the possible role of the population that express GPER in the functions that the Arc controls.

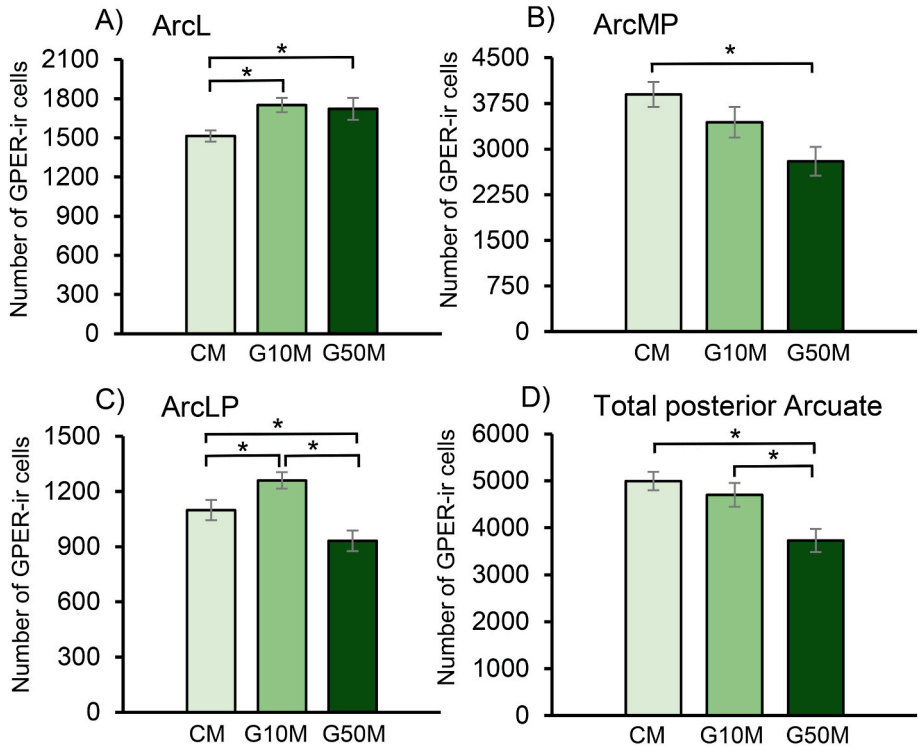


Fig. 6. Histogram shows differences in GPER-stained neurons among male groups in ArcL (A), ArcMP (B), ArcLP (C), total posterior Arcuate (D). CF = control female; G10M = G10 male; G50M = G50 male. ArcL = Arcuate Lateral subdivision; ArcMP = Arcuate medial posterior subdivision; ArcLP = Arcuate lateral posterior subdivision (Paxinos and Watson, 2014). *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

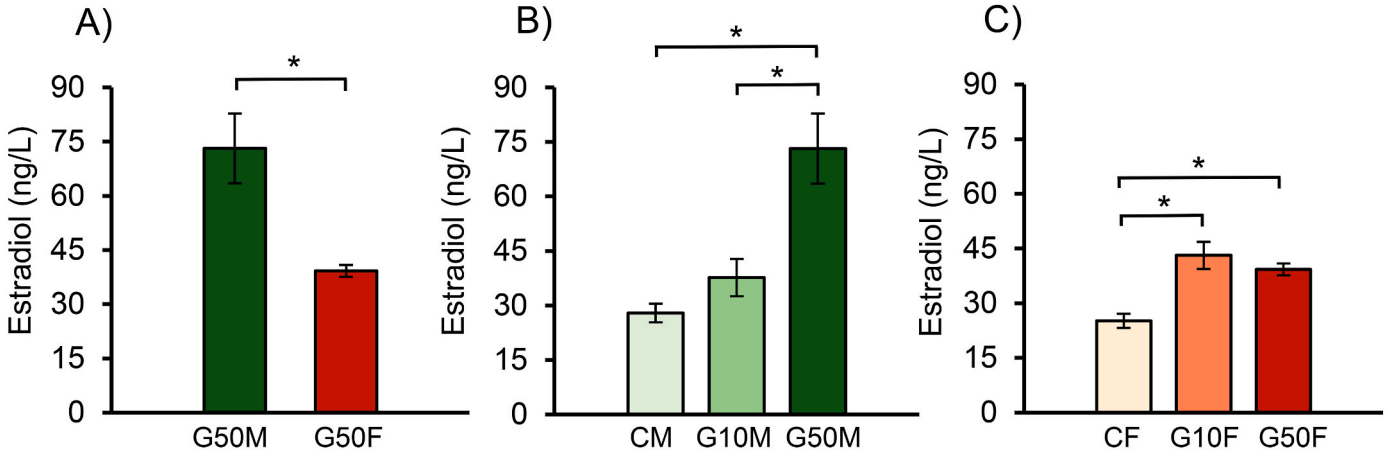


Fig. 7. Histogram shows differences in estradiol plasma levels: A) Between G50M vs G50F; B) Male groups; C) Female groups. CM = control male; CF = control female; G10M = G10male; G10F = G10 female; G50M = G50 male; G50F = G50 female. *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

Funding

The present work was supported by grants PSI 2017-86396-P, IMIENS and PID 2020-115829 GB-I00.

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.

Acknowledgments

We are grateful to Mr. A. Marcos, Mr. G. Moreno and Ms. Rosa Ferrado for their technical assistance. We are grateful to Ms. Ruth Craven for her language and editorial help.

References

Ariyani, W., Miyazaki, W., Amano, I., Hanamura, K., Shirao, T., Koibuchi, N., 2020. Soy isoflavones accelerate glial cell migration via GPER-mediated signal transduction pathway. Front. Endocrinol. 11, 854. https://doi.org/10.3389/fendo.2020.554941.

Asarian, L., Geary, N., 2013. Sex differences in the physiology of eating. Am. J. Physiol. Regul. Integr. Comp. Physiol. https://doi.org/10.1152/ajpregu.00446.2012.

Bateman, H.L., Patisaul, H.B., 2008. Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus. Neurotoxicology 29 (6), 988–997. https://doi.org/10.1016/j.neuro.2008.06.008. Nov.

Bouret, S.G., Draper, S.J., Simerly, R.B., 2004. Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. J. Neurosci. 24 (11), 2797–2805. https://doi.org/10.1523/JNEUROSCI.5369-03.2004.

Campbell, J.N., Macosko, E.Z., Fenselau, H., Pers, T.H., Lyubetskaya, A., Tenen, D., et al., 2017. A molecular census of arcuate hypothalamus and median eminence cell types. Nat. Neurosci. 20 (3), 484–496. https://doi.org/10.1038/nn.4495.

Carrillo, B., Collado, P., Díaz, F., Chowen, J.A., Pinos, H., 2016. Exposure to increased levels of estradiol during development can have long-term effects on the response to undernutrition in female rats. Nutr. Neurosci. 19 (9), 414–422. https://doi.org/10.1179/1476830515Y.0000000012.

Carrillo, B., Collado, P., Díaz, F., Chowen, J.A., Pérez-Izquierdo, M.Á., Pinos, H., 2019. Physiological and brain alterations produced by high-fat diet in male and female rats can be modulated by increased levels of estradiol during critical periods of development. Nutr. Neurosci. 22 (1), 29–39. https://doi.org/10.1080/1028415X.2017.1349574.

Carrillo, B., Collado, P., Díaz, F., Chowen, J.A., Grassi, D., Pinos, H., 2020. Blocking of estradiol receptors ERα, ERβ and GPER during development, differentially alters energy metabolism in male and female rats. Neuroscience 426, 59–68. https://doi.org/10.1016/j.neuroscience.2019.11.008.

Casals-Casas, C., Desvergne, B., 2011. Endocrine disruptors: from endocrine to metabolic disruption. Annu. Rev. Physiol. 73, 135–162. https://doi.org/10.1146/annurev-physiol-012110-142200.

Casanova, M., You, L., Gaido, K.W., Archibeque-Engle, S., Janszen, D.B., Heck, H.D.A., 1999. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. Toxicol. Sci.: Off. J. Soc. Toxicol. 51 (2), 236–244. https://doi.org/10.1093/toxsci/51.2.236.

Castelló-Ruiz, M., Torregrosa, G., Burguete, M.C., Salom, J.B., Gil, J.V., Miranda, F.J., et al., 2011. Soy-derived phytoestrogens as preventive and acute neuroprotectors in experimental ischemic stroke: influence of rat strain. Phytomedicine 18 (6), 513–515. https://doi.org/10.1016/j.phymed.2011.02.001.

Chen, L.R., Ko, N.Y., Chen, K.H., 2019. Isoflavone supplements for menopausal women: a systematic review. Nutrients 11 (11), 2649. https://doi.org/10.3390/nu11112649.

Constantin, S., 2022. Targeting KNDy neurons to control GnRH pulses. Curr. Opin. Pharmacol. 67, 102316 https://doi.org/10.1016/j.coph.2022.102316.

Crain, D.A., Janssen, S.J., Edwards, T.M., Heindel, J., Ho, S.M., Hunt, P., et al., 2008. Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. Fertil. Steril. 90 (4), 911–940. https://doi.org/10.1016/j.fertnstert.2008.08.067.

Du, Z.R., Feng, X.Q., Li, N., Qu, J.X., Feng, L., Chen, L., Chen, W.F., 2018. G protein-coupled estrogen receptor is involved in the anti-inflammatory effects of genistein in microglia. Phytomedicine 43, 11–20. https://doi.org/10.1016/j.phymed.2018.03.039.

Dudek, M., Ziarniak, K., Sliwowska, J.H., 2018. Kisspeptin and metabolism: the brain and beyond. Front. Endocrinol. 9, 145. https://doi.org/10.3389/fendo.2018.00145.

Faber, K.A., Hughes Jr., C.L., 1991. The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat. Biol. Reprod. 45 (4), 649–653. https://doi.org/10.1095/biolreprod45.4.649. Oct.

Faber, K.A., Hughes Jr., C.L., 1993. Dose-response characteristics of neonatal exposure to genistein on pituitary responsiveness to gonadotropin releasing hormone and volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in postpubertal castrated female rats. Reprod. Toxicol. 7 (1), 35–39. https://doi.org/10.1016/0890-6238(93)90007-t. Jan, 1993.

Fernandez-Garcia, J.M., Carrillo, B., Tezanos, P., Collado, P., Pinos, H., 2021. Genistein during development alters differentially the expression of POMC in male and female rats. Metabolites 11 (5), 293. https://doi.org/10.3390/metabo11050293.

Frank, A., Brown, L.M., Clegg, D.J., 2014. The role of hypothalamic estrogen receptors in metabolic regulation. Front. Neuroendocrinol. 35 https://doi.org/10.1016/j.yfrne.2014.05.002.

Fusi, F., Trezza, A., Tramaglino, M., Sgaragli, G., Saponara, S., Spiga, O., 2020. The beneficial health effects of flavonoids on the cardiovascular system: focus on K+ channels. Pharmacol. Res. 152, 104625 https://doi.org/10.1016/j.phrs.2019.104625.

Gao, Q., Mezei, G., Nie, Y., Rao, Y., Choi, C.S., Bechmann, I., et al., 2007. Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. Nat. Med. 13 (1), 89–94.

Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F., Steiner, R.A., 2004. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145 (9), 4073–4077. https://doi.org/10.1210/en.2004-0431.

Gundersen, H.J.G., Jensen, E.B., 1987. The efficiency of systematic sampling in Stereology and its prediction. J. Microsc. 147, 229–263. https://doi.org/10.1111/j.1365-2818.1987.tb02837.x.

Harter, C.J., Kavanagh, G.S., Smith, J.T., 2018. The role of kisspeptin neurons in reproduction and metabolism. J. Endocrinol. 238 (3), R173–R183. https://doi.org/10.1530/JOE-18-0108.

Heisler, L.K., Lam, D.D., 2017. An appetite for life: brain regulation of hunger and satiety. Curr. Opin. Pharmacol. 37, 100–106. https://doi.org/10.1016/j.coph.2017.09.002.

Hsu, H.K., Peng, M.T., 1978. Hypothalamic neuron number of old female rats. Gerontology 24 (6), 434–440. https://doi.org/10.1159/000212283.

Ibrahim, M.M., Bheemanapally, K., Sylvester, P.W., Briski, K.P., 2020. Sex-specific estrogen regulation of hypothalamic astrocyte estrogen receptor expression and glycogen metabolism in rats. Mol. Cell. Endocrinol. 504, 110703 https://doi.org/10.1016/j.mce.2020.110703.

Ishii, Y., Bouret, S.G., 2012. Embryonic birthdate of hypothalamic leptin-activated neurons in mice. Endocrinology 153 (8), 3657–3667. https://doi.org/10.1210/en.2012-1328.

King, J.C., Gerall, A.A., Fishbaek, J.B., Elkind, K.E., 1975. Growth Hormone-Release Inhibiting Hormone (GH-RIH) pathway of the rat hypothalamus revealed by the unlabeled antibody peroxidase-antiperoxidase method. Cell Tissue Res. 160, 423–430. https://doi.org/10.1007/BF00225761.

Kuiper, G.G., Lemmen, J.G., Carlsson, B.O., Corton, J.C., Safe, S.H., Van Der Saag, P.T., et al., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139 (10), 4252–4263. https://doi.org/10.1210/endo.139.10.6216.

Leal, S., Andrade, J.P., Paula-Barbosa, M.M., Madeira, M.D., 1998. Arcuate nucleus of the hypothalamus: effects of age and sex. J. Comp. Neurol. 401 (1), 65–88. https://doi.org/10.1002/(sici)1096-9861(19981109)401:1<65:aid-cne5>3.0.co;2-d.

Lephart, E.D., Porter, J.P., Lund, T.D., Bu, L., Setchell, K.D., Ramoz, G., Crowley, W.R., 2004. Dietary isoflavones alter regulatory behaviors, metabolic hormones and neuroendocrine function in Long-Evans male rats. Nutr. Metabol. 1 (1), 1–14. https://doi.org/10.1186/1743-7075-1-16.

Llorente, R., Marraudino, M., Carrillo, B., Bonaldo, B., Simon-Areces, J., Abellanas-Pérez, P., et al., 2020. G Protein-Coupled estrogen receptor immunoreactivity fluctuates during the estrous cycle and show sex differences in the amygdala and dorsal hppocampus. Front. Endocrinol. 11, 537. https://doi.org/10.3389/fendo.2020.00537.

Lopez, M., Tena-Sempere, M., 2015. Estrogens and the control of energy homeostasis: a brain perspective. Trends Endocrinol. Metabol. 26 (8), 411–421.

Losa, S.M., Todd, K.L., Sullivan, A.W., Cao, J., Mickens, J.A., Patisaul, H.B., 2011. Neonatal exposure to genistein adversely impacts the ontogeny of hypothalamic kisspeptin signaling pathways and ovarian development in the peripubertal female rat. Reprod. Toxicol. 31 (3), 280–289. https://doi.org/10.1016/j.reprotox.2010.10.002.

Marraudino, M., Farinetti, A., Arevalo, M.A., Gotti, S., Panzica, G., Garcia-Segura, L.M., 2019. Sexually dimorphic effect of genistein on hypothalamic neuronal differentiation in vitro. Int. J. Mol. Sci. 20 (10), 2465. https://doi.org/10.3390/ijms20102465.

Marraudino, M., Carrillo, B., Bonaldo, B., Llorente, R., Campioli, E., Garate, I., Pinos, H., Garcia-Segura, L.M., Collado, P., Grassi, D., 2021. G Protein-Coupled estrogen receptor immunoreactivity in the rat hypothalamus is widely distributed in neurons, astrocytes, and oligodendrocytes, fluctuates during the estrous cycle, and is sexually dimorphic. Neuroendocrinology 111 (7), 660–677. https://doi.org/10.1159/00050958.

Matsumoto, A., Arai, Y., 1980. Sexual dimorphism in 'wiring pattern' in the hypothalamic arcuate nucleus and its modification by neonatal hormonal environment. Brain Res. 190, 238–242. https://doi.org/10.1016/0006-8993(80)91173-7.

Mittelman-Smith, M.A., Williams, H., Krajewski-Hall, S.J., Lai, J., Ciofi, P., McMullen, N. T., Rance, N.E., 2012. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology 153 (6), 2800–2812. https://doi.org/10.1210/en.2012-1045.

Morton, G.J., Meek, T.H., Schwartz, M.W., 2014. Neurobiology of food intake in health and disease. Nat. Rev. Neurosci. 15 (6), 367–378. https://doi.org/10.1038/nrn3745.

Mueller, S.O., 2002. Overview of in vitro tools to assess the estrogenic and antiestrogenic activity of phytoestrogens. J. Chromatogr. B 777 (1–2), 155–165. https://doi.org/10.1016/s1570-0232(02)00282-9.

Patisaul, H.B., Melby, M., Whitten, P.L., Young, L.J., 2002. Genistein affects ERβ-but not ERα-dependent gene expression in the hypothalamus. Endocrinology 143 (6), 2189–2197. https://doi.org/10.1210/endo.143.6.8843.

Patisaul, H.B., Jefferson, W., 2010. The pros and cons of phytoestrogens. Front. Neuroendocrinol. 31 (4), 400–419. https://doi.org/10.1016/j.yfrne.2010.03.003.

Paxinos, G., Watson, C., 2014. The Rat Brain in Stereotaxic Coordinates, fourth ed. Academic Press, San Diego, CA, USA.

Peng, M.T., Hsu, H.K., 1982. No neuron loss from hypothalamic nuclei of old male rats. Gerontology 28 (1), 19–22. https://doi.org/10.1159/000212507.

Pinos, H., Carrillo, B., Díaz, F., Chowen, J.A., Collado, P., 2018. Differential vulnerability to adverse nutritional conditions in male and female rats: modulatory role of estradiol during development. Front. Neuroendocrinol. 48, 13–22. https://doi.org/10.1016/j.yfrne.2017.07.009.

Ponti, G., Rodriguez-Gomez, A., Farinetti, A., Marraudino, M., Filice, F., Foglio, B., et al., 2017. Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way. Neuroscience 346, 203–215. https://doi.org/10.1016/j.neuroscience.2017.01.024.

Ponti, G., Farinetti, A., Marraudino, M., Panzica, G., Gotti, S., 2019. Postnatal genistein administration selectively abolishes sexual dimorphism in specific hypothalamic

dopaminergic system in mice. Brain Res. 1724, 146434 https://doi.org/10.1016/j.brainres.2019.146434.
Quarta, C., Claret, M., Zeltser, L.M., Williams, K.W., Yeo, G., Tschöp, M.H., Diano, S., Brüning, J.C., Cota, D., 2021. POMC neuronal heterogeneity in energy balance and beyond: an integrated view. Nat. Metabol. 3 (3), 299–308. https://doi.org/10.1038/s42255-021-00345-3.
Rice, D., Barone Jr., S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108 (Suppl. 3), 511–533. https://doi.org/10.1289/ehp.00108s3511.
Roepke, T.A., 2009. Oestrogen modulates hypothalamic control of energy homeostasis through multiple mechanisms. J. Neuroendocrinol. 21 (2), 141–150. https://doi.org/10.1111/j.1365-2826.2008.01814.x.
Roepke, T.A., Yang, J.A., Yasrebi, A., Mamounis, K.J., Oruc, E., Zama, A.M., Uzumcu, M., 2016. Regulation of arcuate genes by developmental exposures to endocrine-disrupting compounds in female rats. Reprod. Toxicol. 62, 18–26. https://doi.org/10.1016/j.reprotox.2016.04.014.
Santollo, J., Daniels, D., 2015. Multiple estrogen receptor subtypes influence ingestive behavior in female rodents. Physiol. Behav. 152, 431–437. https://doi.org/10.1016/j.physbeh.2015.05.032.
Schafer, D., Kane, G., Colledge, W.H., Piet, R., Herbison, A.E., 2018. Sex-and sub region-dependent modulation of arcuate kisspeptin neurones by vasopressin and vasoactive intestinal peptide. J. Neuroendocrinol. 30 (12), e12660 https://doi.org/10.1111/jne.12660.
Smith, G.P., 2006. Ontogeny of ingestive behavior. Dev. Psychobiol. 48 (5), 345–359. https://doi.org/10.1002/dev.20145.
Sohn, J.W., Elmquist, J.K., Williams, K.W., 2013. Neuronal circuits that regulate feeding behavior and metabolism. Trends Neurosci. 36 (9), 504–512. https://doi.org/10.1016/j.tins.2013.05.00.
Sternson, S.M., Eiselt, A.K., 2017. Three pillars for the neural control of appetite. Annu. Rev. Physiol. 79, 401–423. https://doi.org/10.1146/annurev-physiol-021115-104948.
Thangavel, P., Puga-Olguín, A., Rodríguez-Landa, J.F., Zepeda, R.C., 2019. Genistein as potential therapeutic candidate for menopausal symptoms and other related diseases. Molecules 24 (21), 3892. https://doi.org/10.3390/molecules24213892.
Thomas, P., Dong, J., 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J. Steroid Biochem. Mol. Biol. 102 (1–5), 175–179. https://doi.org/10.1016/j.jsbmb.2006.09.017.
Vail, G.M., Roepke, T.A., 2020. Organophosphate flame retardants excite arcuate melanocortin circuitry and increase neuronal sensitivity to ghrelin in adult mice. Endocrinology 161 (11). https://doi.org/10.1210/endocr/bqaa168.
van den Pol, A.N., Acuna, C., Davis, J.N., Huang, H., Zhang, X., 2019. Defining the caudal hypothalamic arcuate nucleus with a focus on anorexic excitatory neurons. J. Physiol. 597 (6), 1605–1625. https://doi.org/10.1113/JP277152.
Verduci, E., Di Profio, E., Cerrato, L., Nuzzi, G., Riva, L., Vizzari, G., et al., 2020. Use of soy-based formulas and cow's milk allergy: lights and shadows. Front. Pediatr. 736 https://doi.org/10.3389/fped.2020.591988.
Vohra, M.S., Benchoula, K., Serpell, C.J., Hwa, W.E., 2022. AgRP/NPY and POMC neurons in the arcuate nucleus and their potential role in treatment of obesity. Eur. J. Pharmacol., 174611 https://doi.org/10.1016/j.ejphar.2021.174611.
Walker, D.M., Kermath, B.A., Woller, M.J., Gore, A.C., 2013. Disruption of reproductive aging in female and male rats by gestational exposure to estrogenic endocrine disruptors. Endocrinology 154 (6), 2129–2143. https://doi.org/10.1210/en.2012-2123.
Walker, D.M., Goetz, B.M., Gore, A.C., 2014. Dynamic postnatal developmental and sex-specific neuroendocrine effects of prenatal polychlorinated biphenyls in rats. Mol. Endocrinol. 28 (1), 99–115. https://doi.org/10.1210/me.2013-1270.
Wang, L., Moenter, S.M., 2020. Differential roles of hypothalamic AVPV and arcuate kisspeptin neurons in estradiol feedback regulation of female reproduction. Neuroendocrinology 110 (3–4), 172–184. https://doi.org/10.1159/000503006.
Xie, Q., Kang, Y., Zhang, C., Xie, Y., Wang, C., Liu, J., et al., 2022. The role of kisspeptin in the control of the hypothalamic-pituitary-gonadal axis and reproduction. Front. Endocrinol. 1353 https://doi.org/10.3389/fendo.2022.925206 eCollection 2022.
Yang, S.L., Hsu, C., Hsu, H.K., Liu, K.M., Peng, M.T., 1993. Effects of long-term estradiol exposure on the hypothalamic neuron number. Acta Endocrinol. 129 (6), 543–547. https://doi.org/10.1530/acta.0.1290543.
Yoshimura, R., Yamamoto, E., Endo, Y., 2011. Morphological effects of isoflavones (daidzein and genistein) on hypothalamic oxytocin neurons in the neonatal mouse brain slice cultures. Neurosci. Lett. 505 (2), 87–92. https://doi.org/10.1016/j.neulet.2011.09.067.