Dietary withdrawal of phytoestrogens resulted in higher gene expression of 3-beta-HSD and ARO but lower 5-alpha-R-1 in male rats

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Abbreviations:

17β-HSD: 17β-hydroxysteroid dehydrogenase

3β-HSD: 3β-hydroxysteroid dehydrogenase

3α-HSD: 3α-hydroxysteroid dehydrogenase

5αR-1: 5α-reductase-1

AlloP: allopregnanolone

ARO: P450aromatase

E_2: estradiol

ER: estrogen receptor

HP: high phytoestrogen

HP-HF: high phytoestrogen – high fat

LP: low phytoestrogen

NS: neurosteroids

P450-17α: cytochrome P450 17α-hydroxylase

P450scc: cytochrome P450 side-chain cleavage

Pg: progesterone

PR: progesterone receptor

StAR: steroidogenic acute regulatory protein

Abstract

Removing dietary phytoestrogens causes obesity and diabetes in adult male rats. Based on the facts that hypothalamic food intake control is disrupted in phytoestrogen-deprived animals and that several steroids affect food intake, we hypothesized that phytoestrogen withdrawal alters the expression of hypothalamic steroidogenic enzymes. Male Wistar rats fed with a high-phytoestrogen diet from conception to adulthood were subjected to phytoestrogen withdrawal by feeding them a low-phytoestrogen diet, or a high phytoestrogen–high fat diet. Withdrawal of dietary phytoestrogens increased 3β–HSD and ARO gene expression and decreased those of 5αR-1. This is a direct effect of the lack of dietary phytoestrogens and not a consequence of obesity, as it was not observed in high-fat fed rats. Phytoestrogen withdrawal and high-fat diet intake reduced hypothalamic expression of ERα correlated with low levels of ERα-O, ERα-OS and ERα-OT transcripts. Variations in gene expression of steroidogenic enzymes may affect the content of neurosteroids. As neurosteroids are related to food intake control, the changes observed may be a novel mechanism in the regulation of energy balance in obese phytoestrogen-deprived animals. In rats, steroidogenesis and estrogen receptor signaling appear to be altered by phytoestrogen withdrawal in the rat. The ubiquity of phytoestrogens in the diet and changing intakes or withdrawal suggest that aspects of human health could be affected based on the rat and warrant further research.

Keywords: phytoestrogens – obesity – hypothalamus – steroidogenic enzymes - ERα

1. Introduction

Estrogens play a major role in the control of energy homeostasis and glucose metabolism. They act on hypothalamic nuclei controlling food intake, energy expenditure and body fat distribution [1]. Phytoestrogens are nonsteroidal compounds found in many legumes and particularly abundant in soy products. There is increasing evidence that phytoestrogens counteract certain cellular events involved in the development of metabolic syndrome [2]. Our group has recently reported that removing phytoestrogens from the diet in male adult rats increases body weight and energy intake through a hypothalamic response characterized by upregulation of orexigenic and downregulation of anorexigenic neuropeptides. [3]. However, data regarding the effects of phytoestrogens on brain development and function are scarce. This is an important issue because most commercially available laboratory rodent diets contain large amounts of soy that may influence several parameters in research studies.

The brain, like the adrenal glands, gonads and placenta, is a steroidogenic organ. Neurosteroids (NS) are defined as steroids present in the brain, which are synthesized from endogenous precursors by enzymes which are present *in situ* [4]. NS are endogenous modulators of neuronal function responsible for a broad spectrum of biological and pathophysiological effects.

NS are synthesized in various brain regions, but biosynthesis of NS in the hypothalamus has been scarcely studied [5]. The adult rat brain possesses the necessary enzymatic machinery to synthesize a variety of steroids using cholesterol as a substrate. Steroidogenic acute regulatory protein (StAR) mediates the transfer of cholesterol to the inner mitochondrial membrane, where cytochrome P450 side-chain cleavage (P450scc) catalyzes the conversion of cholesterol to pregnenolone. Pregnenolone is metabolized to

progesterone (Pg), and/or androstenedione by 3β-hydroxysteroid dehydrogenase (3β–HSD), whose presence has been demonstrated in various brain regions of rodents and humans together with Pg receptor (PR), suggesting a paracrine action of locally synthesized Pg [6]. Furthermore, Pg is converted by the enzyme 5α–reductase-1 (5αR-1) into dihydroprogesterone and subsequently, by the action of 3α-hydroxysteroid dehydrogenase (3α–HSD), into allopregnanolone (AlloP). Androstenedione is converted by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) into testosterone, and then P450 aromatase (ARO) is the main enzyme involved in the synthesis of estradiol (E_2) from testosterone.

Steroids have been closely related to the regulation of food intake. Chronic administration of Pg by subcutaneous patches increases energy intake in adult female rats [7]. Estrogens also regulate key aspects of metabolism, including food intake, body weight and insulin sensitivity [8]. Ovariectomized rats receiving E_2 show reduced food intake and body weight [9], effects that are also evident in intact rats [10]. This may be driven by the actions of E_2 at the central level, since its administration in hypothalamic nuclei reduces energy intake [11]. Besides, it has been reported that administration of AlloP results in increased food intake and weight gain [12, 13]. In view of these results, we can expect that the NS synthesized *de novo* in the brain, particularly in the hypothalamus, may have a significant impact on the control of food intake.

Aromatization by ARO will ultimately lead to the formation of estrogens, and most estrogen effects are mediated through specific nuclear estrogen receptors (ER). A significant characteristic of phytoestrogens is their ability to bind to both ERα and β [14]. However, only ERα has been reported to have a major influence on energy homeostasis [15]. For many genes, including ERα, it has been demonstrated that untranslated regions (UTRs) can differentially determine protein expression by influencing mRNA stability

[16, 17] and translational efficiency [18]. In the rat, it has been described that a system of untranslated first exons is associated with the promoter selection for ERα transcription initiation, and to date five active promoters called OS, ON, O, OT, and E1 have been identified [19, 20]. Little is known about the precise function of each ERα promoter. However, it has been reported that a mechanism of promoter selection is involved in the regulation of ERα gene expression in many organs, including the rat brain [20]. Since ERα mRNA transcription and maturation may be differentially regulated by estrogens [21], it is very interesting to know whether phytoestrogen withdrawal can modify the differential usage of ERα gene promoters in the rat hypothalamus.

Here we hypothesize that phytoestrogen withdrawal alters the expression of enzymes involved in the *de novo* synthesis of NS in the hypothalamus, a key area involved in food intake control. The NS synthesized *de novo* in this area may have a significant impact on the control of food intake. To test our hypothesis, we analyzed the levels of mRNAs from multiple genes involved in hypothalamic steroidogenesis. Thereby, our specific objective was to analyze the mRNA level of steroidogenic enzymes in a model of obesity and hyperphagia with disruption in hypothalamic energy intake control induced by phytoestrogen withdrawal achieved by the use of a diet free of soybean product. The expression of these enzymes in the rat hippocampus has been widely reported, but more information about their hypothalamic expression is needed. Also, the expression of steroid hormone receptors and the differential usage of gene promoters that control ERα transcription and translation in the hypothalamus may add information about the mechanisms of action of phytoestrogens in the brain.

2. Methods and Materials

2.1. Animals, diets, and dietary treatment

All procedures were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences [22] and were approved by the Ethical Committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral (UNL, Santa Fe, Argentina). Eighteen male adult Wistar rats were obtained at the Department of Human Physiology (UNL). From conception until the beginning of the experiment, the animals were housed under controlled conditions (23 ± 2ºC and 12-hour light-dark cycle) with free access to a high soy-containing regular animal chow diet [high phytoestrogen (HP) diet] (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina). The phytoestrogen content of the diets was analyzed at the National Institute of Industrial Technology (INTI, Buenos Aires, Argentina) by HPLC. HP diet contained 400 mg/kg of phytoestrogens. These phytoestrogens came from the soy contained in the feed. When the rats reached 250-300 g (approximately 3 months old) they were randomly divided into three weight-matched groups and subjected to the dietary treatment previously described by our group [3] for fifteen weeks. Briefly, one group was used as control and had *ad libitum* access to the same HP diet. Another group was fed a soy-free diet [low-phytoestrogen (LP)] (Zeigler Phytoestrogen Reduced Rodent Diet I; Zeigler Brothers, Gardner, PA, USA) to evaluate the effects of withdrawing phytoestrogens from the diet. The removal of phytoestrogens from the diet was achieved using this diet that is free of soybean products. This is a limitation of our study, as the diet is formed by a complex mixture of ingredients which is slightly different from the ingredients of HP diet, as can be seen in Table 1. However, despite of the ingredients, the final nutrient composition of HP and LP diets is similar, as seen in Table 1. The third group was fed the same HP diet supplemented with 21.3g of lard per 100 g of food as a source of dietary fat [high

phytoestrogen–high fat (HP-HF)]. This has been widely reported as a useful model to induce obesity and metabolic syndrome [23]. Phytoestrogens levels were not detectable by HPLC analysis in this diet. The composition of the diets can be seen in Table 1. Body weights were recorded weekly and food intake daily during the experimental period. Food intake was determined by the weight difference between offered and remaining food, adjusted to the waste by collecting food spillage, and energy intake was calculated by multiplying these amounts of ingested food with the respective energy contents, as previously reported by our group [24].

At the end of the dietary treatments, rats were euthanized by decapitation. Trunk blood was collected, samples were centrifuged and serum was frozen and stored at -80°C until analysis. The brain was removed from the skull immediately after decapitation and the mediobasal hypothalamus was dissected according to Paxinos atlas (coordinates -1.0 to -4.5 mm from Bregma and 3 mm in depth) [25], snap-frozen in liquid nitrogen, and stored at -80°C until RNA isolation.

2.2. RNA isolation, reverse transcription and real-time quantitative PCR for steroidogenic enzymes and proteins and sex steroid receptors

Total RNA was isolated from the hypothalamus by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription and real-time quantitative PCR were performed as previously described [3]. Briefly, two micrograms of total RNA were subjected to reverse transcription using 200 pmol of random primers (Promega, Madison, WI, USA) and Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega, Madison, WI, USA). Twenty units of ribonuclease inhibitor RNAout (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube at a final

volume of 30 µl of 1× reverse transcriptase buffer. Reverse transcription was performed at 37 °C for 90 min and at 42 °C for 15 min. Reactions were stopped by heating at 80 °C for 5 min and cooling on ice. Each reverse-transcribed product was diluted with RNAse free water to a final volume of 60 µl and further amplified in duplicate. For cDNA amplification, 5 µl of cDNA was combined with HOTFIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Buenos Aires, Argentina) to a final volume of 20 µl. Ribosomal protein L19 was used as housekeeping gene as it is highly stable and no change in the expression between the experimental groups was observed in studies from our group and others [3, 26, 27]. The primer pairs used for ribosomal protein L19, steroidogenic enzymes and proteins and sex steroid receptors are shown in Table 2. cDNA levels were determined by using a real-time PCR system StepOne Cycler (Applied Biosystems Inc., Life Technologies, Carlsbad, CA, USA). Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. The threshold cycles (Ct) and PCR efficiency were calculated using Step One software (Applied Biosystems, Life Technologies). Fold change from HP values was determined using the delta-delta Ct method [28]. No significant differences in Ct values were observed between the different experimental groups for L19.

2.3. Relative expression levels of ERα transcripts containing alternative 5´UTR exons

To determine whether ERα mRNA expression changes could be associated with modifications in transcriptional promoter usage, we used an optimized PCR protocol previously described by our group [27]. To analyze the relative expression levels of total ERα mRNA and ERα transcripts containing the untranslated first exons OS, ON, O, OT,

and E1, we designed specific sense primers for ERα 5´-UTRs OS, ON, O, OT, E1 and a common antisense primer specific for ERα exon 1 (Table 3 and Fig. 1). RNA isolation, reverse transcription and real-time quantitative PCR were performed as described above and ribosomal protein L19 was also used as housekeeping gene.

2.4. Circulating hormone measurements

The levels of E_2 and testosterone were measured using commercial RIA kits (Immunotech, Marseille, France) based on competitive binding with I_{125}-labeled hormone, according to the manufacturer's recommendations. Standard curve interpolation was used.

2.5. Statistical analyses

Sample size was determined using G Power software [29]. Data (expressed as means ± SEM) were statistically analyzed by one-way ANOVA using IBM SPSS Statistics 19 software (IBM Inc., Armonk, NY, USA). Post-hoc multiple comparisons were made using Tukey's critical range test. Differences were considered significant at $p<0.05$. Different letters indicate significant differences at $p<0.05$ by Tukey's test after one-way ANOVA.

3. Results

3.1. Phytoestrogen withdrawal increased body weight and energy intake.

We previously reported [3] that when the animals were fed with the phytoestrogen-free diet, their body weights significantly increased [Final body weights (g): HP: 454±6[a]; LP: 485±6[b]; HP-HF: 479±5[b]]. Food intakes were strikingly increased in LP, but not in HP-HF rats, demonstrating that phytoestrogen withdrawal induces hyperphagia [Food Intakes (g/d): HP: 15.68±0.22[a]; LP: 22.33±0.35[b]; HP-HF: 16.44±0.15[a]]. Given that the HP-HF diet was hyperenergetic, energy intakes were higher in the HP-HF rats compared to HP, but they were even greater in LP fed rats [Energy intake (KJ/d): HP: 195.3±3.5[a]; LP: 358.1±2.8[b]; HP-HF: 301.9±1.5[c]].

3.2. Phytoestrogen withdrawal affected mRNA levels of neurosteroidogenic enzymes.

The effect of phytoestrogen withdrawal on the expression of neurosteroidogenic enzymes and proteins in the hypothalamus was assessed by real-time quantitative PCR. P450scc, 17β-HSD and P450-17α could not be quantified by this methodology in any of the samples analyzed. As seen in Fig. 2, the levels of mRNAs for ARO and 3β-HSD were increased by phytoestrogen withdrawal, 5αR-1 expression was decreased and StAR and 3α-HSD remained unchanged. These data clearly show that phytoestrogen withdrawal affects the transcriptional activation of enzymes involved in hypothalamic NS synthesis.

3.3. Phytoestrogen withdrawal modified the expression of sex hormone receptors

The hypothalamic expressions of sex hormone receptors are shown in Fig. 3. Phytoestrogen withdrawal caused a two-fold reduction in PR expression, and both, LP

and HP-HF diets decreased ERα mRNA levels in the hypothalamus. Androgen receptor expressions remained unchanged (Fig. 3).

3.4. Phytoestrogen withdrawal changed the relative abundance of ERα transcripts with alternative 5´UTRs

To determine whether hypothalamic ERα mRNA expression changes were associated with fluctuations in transcriptional promoter usage, relative expression levels of exons encoding 5´UTR OS, ON, O, OT, and E1 of the rat ERα gene were studied by real-time PCR. Expression of transcripts containing the ON exon could not be detected by this methodology. The reduction in ERα mRNA levels observed in LP and HP-HF rats was associated with lower levels of OT, O and OS transcripts (Fig. 4). Besides, LP diet increased ERα-E1 transcript content.

3.5. Phytoestrogen withdrawal altered blood hormones

The obese phenotype induced by the LP and HP-HF diets was concomitant with a reduction in serum testosterone levels vs HP group, as can be seen in Table 3. Remarkably, only phytoestrogen withdrawal reduced circulating E_2 levels vs the HP diet.

4. Discussion

We have recently reported that that if male rats consume phytoestrogens until they reach adult life, when the feed is replaced by a low phytoestrogen diet, rats develop an obese phenotype with marked body weight gain, diabetes and alterations in energy intake. Increased energy intake is caused by a hypothalamic orexigenic neuropeptide response mediated by POMC and AgRP [3]. However, other hypothalamic processes such as steroidogenesis and ERα signaling via E_2 may be an alternative mechanism by which phytoestrogen withdrawal exerts some of the observed effects. Steroidogenesis has been extensively reported in the hippocampus, but the neuroanatomical distribution and biological activity of neurosteroidogenic enzymes in different areas of the mammalian brain has been barely studied [5]. The expression of several enzymes in the cortex, cerebellum, and hypothalamus has been recently reported [30], but an association between these enzymes and the metabolic status of the animals remains unknown. To our knowledge, this study is the first to demonstrate a relationship between dietary phytoestrogen withdrawal and hypothalamic expression of steroidogenic enzymes. The current study using real-time PCR analysis provides a clue to understanding the effect of removal of dietary phytoestrogens from the standpoint of the steroidogenic pathway in this brain area.

Here we analyzed the effect of phytoestrogen withdrawal on the levels of mRNAs for several steroidogenic molecules. Among mRNAs encoding steroidogenic enzymes, our data indicate that phytoestrogen withdrawal increases the levels of mRNAs for ARO (2.9-fold) and 3β-HSD (3.8-fold), while it reduces 5αR-1 (2-fold) expression in the hypothalamus. Hence, phytoestrogen withdrawal appears to affect E_2, Pg and AlloP synthesis. This effect seems to be directly exerted by the absence of phytoestrogens in the

diet and not an indirect consequence of obesity, as the expression of all the enzymes and proteins evaluated remained unchanged in high-fat fed obese rats. This question would be addressed more accurately with the use of a pair-feeding paradigm, in which the ability of the animals to consume food in excess of that eaten by nonobese controls is prevented [31].

Pg is produced by 3β–HSD, and the increased expression observed could lead to a high hypothalamic Pg content, as previously reported [32]. These authors showed that when the 3β–HSD gene is over-expressed in the spinal cord, this change is concomitant with an increase in enzyme activity and hyper-production of Pg. In our experiment, the high expression of 3β–HSD may be producing increased hypothalamic Pg that could inhibit PR expression, as colocalization of 3β–HSD and PR has been previously reported [6]. Besides, a direct effect of phytoestrogens on this enzyme has been previously reported: genistein and daidzein specifically inhibit 3β-HSD activity in porcine adrenocortical cells [33, 34].

AlloP is synthesized from Pg by the activities of 5αR-1 and 3α-HSD in the rat hypothalamus. It has been reported that a decrease in 5αR-1 mRNA levels leads to attenuation of AlloP synthesis followed by a decrease in AlloP brain content [35]. In our experiment, the reduction in 5αR-1 mRNA level was significant, suggesting that phytoestrogen withdrawal might reduce hypothalamic AlloP synthesis by modulating 5αR-1 mRNA levels.

The high expression of ARO could lead to increased aromatization of testosterone into E_2 in phytoestrogen-deprived rats. Phytoestrogens have been reported to affect estrogen synthesis, but the results are conflicting [36, 37]. Therefore, we cannot assign a direct effect of phytoestrogens on ARO enzyme activity or gene expression.

Brain-derived E_2 may be generated using circulating testosterone as a precursor, and also by *de novo* synthesis. Thus, the actual E_2 levels in the brain will depend on the amount of circulating testosterone (predominantly in males), the circulating levels of E_2 (predominantly in females) and the steroidogenic pathway mainly represented by ARO [38]. The regulation of *Cyp19a* gene expression in the brain has been widely studied. E_2 and Pg have been reported to reduce *Cyp19a* gene expression while circulating testosterone is known to increase it [37, 39, 40]. However, our results are not in agreement with these data. Circulating testosterone was reduced in LP- and HP-HF-fed rats, but this did not affect androgen receptor or ARO gene expression in the expected manner. The observed reduction in circulating testosterone was expected to decrease (and not increase) ARO expression. Also, phytoestrogen withdrawal reduced serum E_2; however as circulating E_2 levels are not as physiologically significant in males than in females, its impact on ARO expression could be less important. However, circulating LH and FSH levels as well as hypothalamic GnRH gene expression remained unchanged (data not shown). These results may suggest that in this animal model the regulation of *Cyp19a* gene expression could be independent of the hypothalamic-pituitary-gonadal axis, and governed by other pathways. The data stated above lead us to believe that several mechanisms may play a role in the changes observed in ARO, 3β–HSD and 5αR-1 gene expression in the hypothalamus. Epigenetic mechanisms associated with differential promoter methylation could be involved. Recent data from our group shows that lifestyle factors such as environmental enrichment affect mRNA expression and the DNA methylation state of steroidogenic enzymes in the hippocampus [41]. However, we did not study methylation state of ARO, 5αR-1 or 3β–HSD in this experimental model and that is one of the limitations of the present study. Therefore, dietary factors such as presence/absence of phytoestrogens could also exert epigenetic modifications.

Many of the effects of E_2 are mediated by ERα. Our results show that obesity, regardless of whether it is caused by phytoestrogen withdrawal or by high-fat diet intake, reduces hypothalamic expression of ERα. However, only phytoestrogen withdrawal reduced circulating E_2 levels. We have previously reported that the decreased hypothalamic ERα signaling via a reduction of circulating E_2 levels may be an alternative mechanism generating some of the effects observed in this animal model [3]. A possible mechanism involved in ERα disruption could include differential promoter usage, as evidenced by changes in the relative abundance of alternative spliced 5´UTR transcripts [19, 20]. Our results show that the reduction in ERα mRNA expression observed in obese animals is correlated with low levels of ERα-O, ERα-OS and ERα-OT transcripts.

We have previously reported that phytoestrogen withdrawal in adult male rats induces insulin resistance and diabetes [3]. It is well demonstrated that diabetes impacts on the nervous system, leading to cognitive deficiency and risk of stroke [42-44]. Diabetes modulates *in situ* the steroidogenic pathways to generate a protective response mediated by NS, which are widely known by their neuroprotective and reparative properties [45]. In animals subjected to chronic hyperglycemia induced by streptozotocin treatment, 3β–HSD gene expression and enzymatic activity are up-regulated in the spinal cord, followed by an increase in Pg production. Besides, it has also been reported that the induction of ARO in astroglia after brain injury and the local formation of E_2 by these cells may represent an adaptive response of the injured neural tissue to limit neurodegenerative damage [38, 46]. Therefore, given our current results, the increased 3β–HSD and ARO gene expression, and low 5αR-1 mRNA levels may lead to accumulation of Pg and E_2, which is part of an overall mechanism used by the hypothalamus to cope with diabetic neurodegenerative damage.

The obese phenotype observed in phytoestrogen-deprived animals is mediated by hyperphagia and high energy intake related to a hypothalamic orexigenic neuropeptide response [3]. Steroids are closely related to food intake control and the activity of key steroidogenic enzymes may be finely tuned by various neurotransmitters and neuropeptides, suggesting that some of the central effects of these neuromodulators can be mediated through the regulation of NS production [5]. The increased 3β–HSD gene expression leading to Pg accumulation may be in line with the orexigenic neuropeptide response, as Pg is widely known to increase food intake [7]. However, increased ARO gene expression and low 5αR-1 mRNA levels may lead to accumulation of E_2 and low levels of AlloP respectively, which could represent an anorectic signal. The possible reduction in the orexigenic signal of AlloP together with the increase in E_2 production may represent a homeostatic hypothalamic response to reduce energy intake and counterbalance the orexigenic signal mediated by hypothalamic neuropeptides. However, further studies are still needed to clarify a cause-effect relationship between the synthesis of the different NS and the control of energy intake.

In conclusion, the present study demonstrated a relationship between phytoestrogen withdrawal and gene expression of steroidogenic enzymes. The absence of dietary phytoestrogens increased 3β–HSD and ARO gene expression and decreased 5αR-1 mRNA levels, a fact that may lead to increased Pg and E_2 and reduced AlloP synthesis. These findings support our hypothesis that phytoestrogen withdrawal alters the expression of hypothalamic steroidogenic enzymes. Whether the observed effects are due exclusively to phytoestrogens or other soy micro or macronutrients remain unclear, as we used a diet free of soybean ingredient, and not estrogen withdrawal specifically. NS synthesis enhanced by phytoestrogen withdrawal may be an endogenous neuroprotective mechanism to cope with diabetic neurodegenerative damage. Besides, the changes

observed may play a role in energy balance given the influence of E_2, Pg and AlloP on food intake control. The ubiquity of phytoestrogens in the diet of humans and laboratory animals emphasizes the necessity of further research in this field.

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Figures

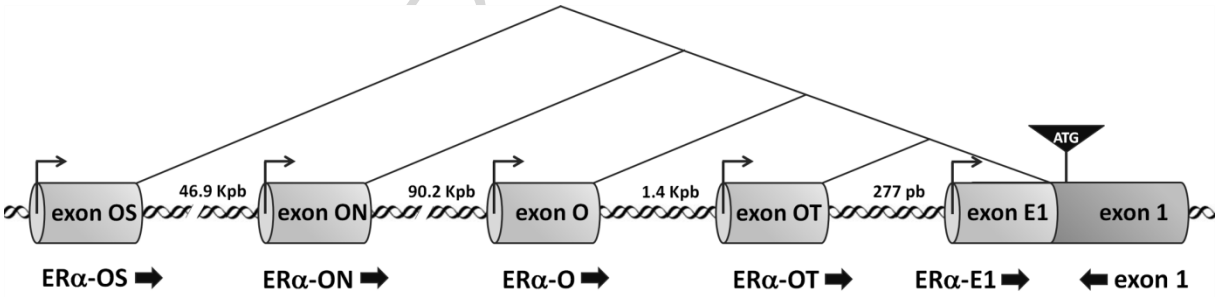

Fig. 1. Genomic organization of the promoter region of the rat estrogen receptor alpha (ERα) gene. Relative positions and orientations of promoter-specific real-time PCR primers are indicated by black arrows. The region common to all the alternative 5´UTRs of ERα is indicated by a dark gray box.

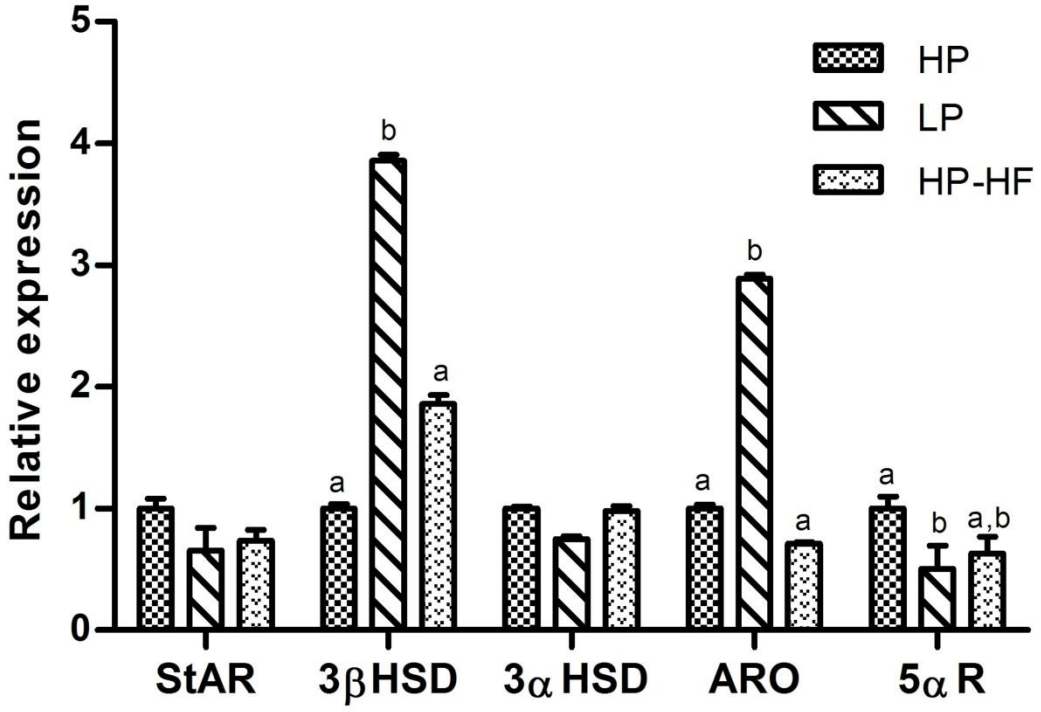

Fig. 2. Hypothalamic expression of neurosteroidogenic enzymes and proteins in rats fed a high-phytoestrogen (HP), low-phytoestrogen (LP) or high phytoestrogen–high fat (HP-HF) diet. StAR: steroidogenic acute regulatory protein; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 3α-HSD: 3α-hydroxysteroid dehydrogenase; ARO: P450aromatase, 5αR-1: 5α-reductase-1. Relative RNA expression was measured by quantitative real-time RT-PCR and fold change from HP values was calculated by the Pfaffl method (Pfaffl, 2001). Data shown are presented as means ± SEM (n=6). Different letters indicate significant differences at $p<0.05$ by Tukey's test after one-way ANOVA.

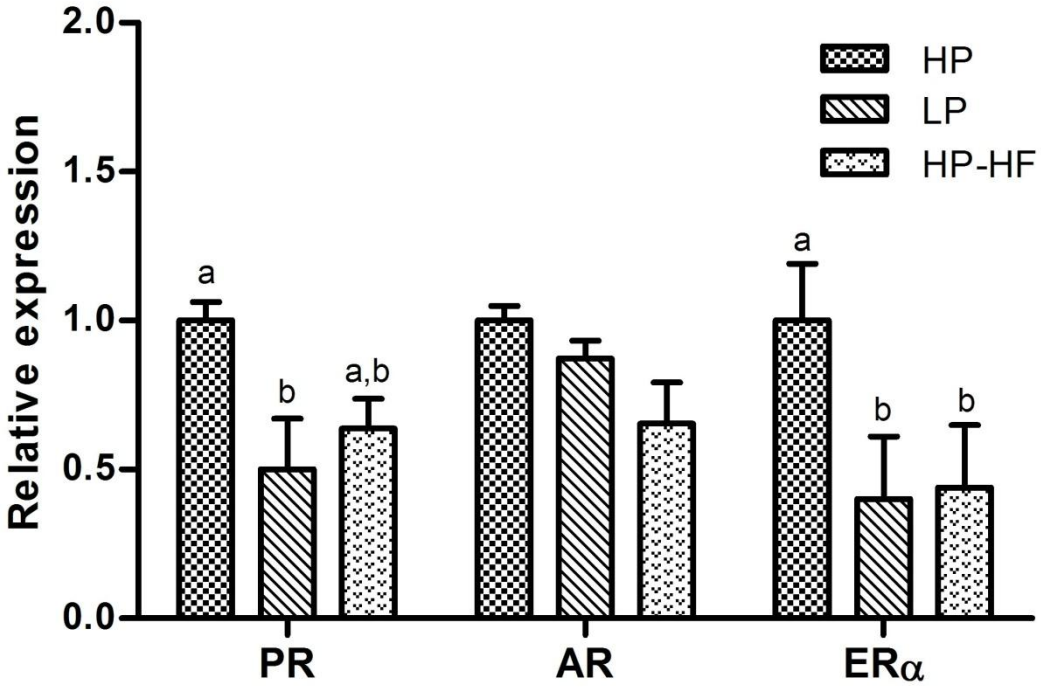

Fig. 3. Hypothalamic expression of hormone receptors in rats fed a high-phytoestrogen (HP), low-phytoestrogen (LP) or high phytoestrogen–high fat (HP-HF) diet. AR: Androgen receptor, PR: Progesterone receptor; ERα: Estrogen receptor alpha. Relative RNA expression was measured by quantitative real-time RT-PCR and fold change from HP values was calculated by the Pfaffl method (Pfaffl, 2001). Data shown are presented as means ± SEM (n=6). Different letters indicate significant differences at $p<0.05$ by Tukey's test after one-way ANOVA.

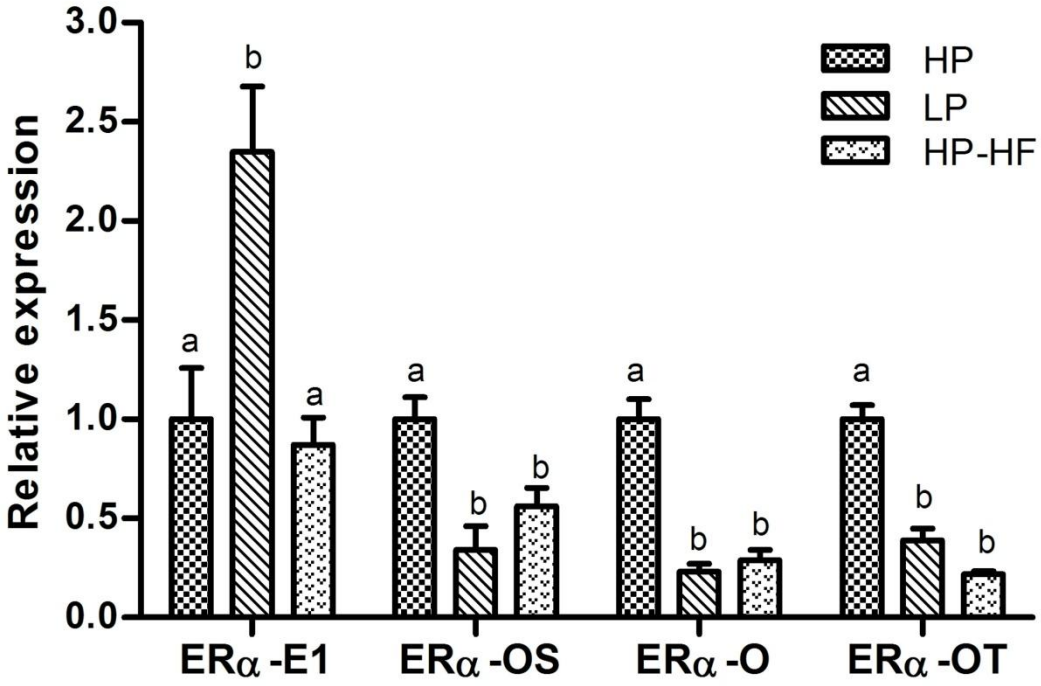

Fig. 4. Hypothalamic expression of ERα transcripts with alternative 5´UTRs in rats fed a high-phytoestrogen (HP), low-phytoestrogen (LP) or high phytoestrogen–high fat (HP-HF) diet. ERα-OS: Estrogen receptor alpha OS promoter; ERα-O: Estrogen receptor alpha O promoter; ERα-OT: Estrogen receptor alpha OT promoter; ERα-E1: Estrogen receptor alpha E1 promoter. Relative RNA expression was measured by quantitative real-time RT-PCR and fold change from HP values was calculated by the Pfaffl method (Pfaffl, 2001). Data shown are presented as means ± SEM (n=6). Different letters indicate significant differences at $p<0.05$ by Tukey's test after one-way ANOVA.

Table 1: Composition of the diets

	HP	LP	HP-HF
Ingredients (g/kg of diet)			
Corn	200	245	157
Wheat	150	350	118
Soy flour	230	-	181
Lard	-	-	213
Fish meal	200	100	157
Wheat middlings	100	100	78
Oat	-	100	213
Soy oil	20	-	16
Corn oil	20	-	16
Corn gluten meal	20	20	16
Sunflower flour	20	-	16
Brewers dried yeast	-	10	-
Limestone	-	15	-
Vitamin mix	10	10	8
Mineral mix	30	30	24
Nutrient composition			
Protein, g/kg	230	239	181
Total fat, g/kg	51	59	253
Carbohydrate, g/kg	508	525	400
Crude fiber, g/kg	60	35	47
Ash, g/kg	100	75	79
Genistein, mg/kg	190	ND	150
Daidzein, mg/kg	210	ND	165
Energy density (kcal/kg)	3411	3587	4599

ND: not detectable by HPLC analysis

Table 2.: Sequences of primers used for real time quantitative PCR for steroidogenic enzymes and proteins and sex steroid receptors

Enzyme/ protein name	Gene name	Accesion number	Primer Sequences	Size
L19	*L19*	NM_031103	F: 5´- AGCCTGTGACTGTCCATTCC -3´ R: 5´- TGGCAGTACCCTTCCTCTTC -3´	99 pb
StAR	*Star*	NM_031558.3	F: 5´- GCAAAGCGGTGTCATCAG -3´ R: 5´- GGCGAACTCTATCTGGGTCT -3´	172 pb
P450scc	*Cyp11a1*	NM_017286.2	F: 5´- AGGGAGAACGGCACACACAG -3´ R: 5´- TCGCAGGAGAAGAGAGTCGC -3´	143 pb
3β-HSD	*Hsd3b*	NM_001007719.3	F: 5´- AGGCCTGTGTCCAAGCTAGTGT -3´ R: 5´- CTCGGCCATCTTTTTGCTGTAT -3´	161 pb
P450-17α	*Cyp17a1*	NM_012753.2	F: 5´- GGTGATAAAGGGTTATGCCA -3´ R: 5´- GCTTGAATCAGAATGTCCGT -3´	117 pb
3α-HSD	*Hsd3a*	M64393.1	F: 5´- GCACTCAACTGGACTATGTGGA -3´ R: 5´- GCTCATCTCGTGGGAAAAAT -3´	87 pb
17β-HSD-3	*Hsd17b3*	NM_054007.1	F: 5´- CAACCTGCTCCCAAGTCATT -3´ R: 5´- AACCCCTACTCCCGAAGAAA -3´	160 pb
ARO	*Cyp19a1*	NM_017085	F: 5´- TGGCAGATTCTTGTGGATGG -3´ R: 5´- CGAGGACTTGCTGATGATGAGT -3´	118 pb
5αR-1	*Srd5a1*	NM_017070	F: 5´- CACCTTCAACGGCTATGTAC -3´ R: 5´- AGGATGTGGTCTGAGTGGAT -3´	144 pb
AR	*Ar*	NM_012502.1	F: 5´- AGGGAGGTTACGCCAAAG-3´ R: 5´- AGACAGTGAGGACGGGAT-3´	101 pb
PR	*Pgr*	NM_022847.1	F: 5´- GACCAGTCTCAACCAACTAGGC-3´ R: 5´- ACACCATCAGGCTCATCCAG-3´	137 pb

L19: ribosomal protein (housekeeping gene); StAR: steroidogenic acute regulatory protein; P450scc: cytochrome P450 side chain cleavage; 3β-HSD: 3β-hydroxysteroid dehydrogenase, P450-17α: cytochrome P450 17α-hydroxylase; 3α-HSD: 3α-hydroxysteroid dehydrogenase; 17β-HSD-3: 17β-hydroxysteroid dehydrogenase type 3; ARO: P450aromatase, 5αR-1: 5α-reductase type 1; AR: Androgen receptor, PR: Progesterone receptor

Table 3.: Primers used for real time quantitative PCR for total ERα and transcripts containing alternative 5´UTR exons

Enzyme/protein name	Accesion number	Primer Sequences	Size
ERα	NM_012689	F: 5´- ACTACCTGGAGAACGAGCCC -3´ R: 5´- CCTTGGCAGACTCCATGATC -3´	153 pb
ERα-OT	X98236	F: 5´- CAGCAGGTTTGCGATGTCTAA-3´	275 pb
ERα-OS	NW_047550	F: 5´- CCGAAAACACAAGGCTCCATGCT-3´	336 pb
ERα-O	NM_012689	F: 5´- GACTTCTACAAACCCATGGA-3´	272 pb
ERα-ON	NW_047550	F: 5´- CTGGGGCATCTCCTTCAATATG-3´	336 pb
ERα-E1	X98236	F: 5´- TTTAACCTCGGGCTCTACTC-3´	247 pb
Exon 1	NM_012689	R: 5´- GGGCTTGCTGTTGTCCACGTAC-3´	-

ERα: Estrogen receptor alpha; ERα-OT: Estrogen receptor alpha OT promoter; ERα-OS: Estrogen receptor alpha OS promoter; ERα-O: Estrogen receptor alpha O promoter; ERα-ON: Estrogen receptor alpha ON promoter; ERα-E1: Estrogen receptor alpha E1 promoter

Table 4: Sex hormones profile

	HP	LP	HP-HF	p value
Testosterone (ng/mL)	1.10±0.28[a]	0.45±0.15[b]	0.48±0.08[b]	0.032
Estradiol (pg/mL)	5.45±0.43[a]	2.15±0.63[b]	5.22±0.34[a]	0.028

HP: High Phytoestrogen, LP: Low Phytoestrogen, HP-HF: High Phytoestrogen – High Fat.
Data shown are presented as means ± SEM (n=6). Different superscript letters indicate significant differences at $p<0.05$ by Tukey's test after one-way ANOVA.