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ORIGINAL ARTICLE

Soya bean rich diet is associated with adult male rat aggressive behavior: relation to RF amide-related peptide 3-aromatase-neuroestrogen pathway in the brain

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Abstract

Relation between soya bean (SB) consumption and aggressive behavior has not been elucidated yet. Thus, this study was conducted to investigate the effect of large amount of SB consumption on adult male rats' aggressive behavior through investigating changes in the expression of gonadotropin-inhibitory hormone/ RF amide-related peptide 3 (GnIH/RFRP3), neuropeptide FF receptor, cytochrome P450, family 19, subfamily A, polypeptide 1 (Cyp19A1), estrogen receptors α and β and the levels of neuroestrogen, dopamine, glutamate and testosterone as well as aromatase activity in the brain. Adult male rats were divided into three equal groups: group I, control group, received standard diet; group II and group III received 25% and 50% SB of their standard diet contents, respectively, for 12 weeks. The obtained results showed that feeding male rats with large amount of SB could induce aggressive behavior in a dose dependant manner possibly through inhibition of brain GnIH/RFRP-aromatase-neuroestrogen pathway. These effects may be through decreasing aromatase activity, neuroestrogen concentration, *Cyp19A1* and ER β mRNA levels and increasing ER α mRNA levels and immunostaining as well as testosterone, dopamine and glutamate levels in the brain. These findings also provide further support for the inhibitory role of RFRP3 on aggressive behavior of male rats. These data may open new avenues for the potential harmful effects of consumption large amounts of SB rich food on humans.

Keywords Soya beans · RF amide-related peptide-3 · Aromatase · Neuroestrogen · Aggressiveness

Introduction

The master regulator of the sexual and behavioral status in most vertebrates is the sex hormones; therefore, their signaling pathways may explain the neural circuits underlying these behaviors (Yang and Shah 2014). Gonadotropin-inhibitory hormone (GnIH) is a12-amino acid hypothalamic neuropeptide. It is the main inhibitor for some essential signals in the hypothalamic-pituitary-gonadal axis, especially gonadotropin releasing hormone (GnRH). GnIH and GnRH neurons exist in close proximity in the hypothalamus, which may enable the direct inhibition of GnRH neurons by GnIH (Kriegsfeld et al. 2006; Smith and Clarke 2010). This inhibitory effect is mediated by two G protein-coupled receptors, neuropeptide FF receptor (NPFFR) and G Protein–Coupled Receptor Gene74 (GPR74) (Liu et al. 2001). The GnIH mammalian ortholog is the RF amide-related peptide, (RFRP), it is expressed within the dorsomedial nucleus of the hypothalamus of rat's neurons, with its fibers extending to both the preoptic area and the median eminence, making contact inside the hypothalamus with GnRH neurons (Kriegsfeld et al. 2010; Tsutsui et al. 2012). RF amide-related peptides (RFRPs) possess a characteristic LPXRF-amide (X/L or Q) motif at their C-termini. RFRP1 and RFRP3 are the two major forms of RFRP that have been identified as major cleavage products of a single RFRP gene (Kriegsfeld et al. 2010).

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Data from previous studies suggest that GnIH/RFRP is an important mediator of reproductive function and behavior acting at the level of the brain, pituitary, and the gonad in birds and mammals (Ubuka et al. 2018). A previous study of lin et al. (2011) pinpointed a subnucleus in the medial hypothalamus, the ventromedial hypothalamus ventrolateral area (VMHvl), as a locus for aggressive behavior and attack in male mice which was confirmed by a further study of yang et al. (2017) who showed the reliability of VMHvl activation-induced aggression in animals under different testing environment and with different experience. Cytochrome P450 aromatase (an enzyme encoded by *Cyp19A1* gene) is a monooxygenase involving in steroidogenesis and its predominant sites of activity are within brain hypothalamus and amygdala regions which are involved in reproductive functions and behaviors (Lephart 1996). It is to be noted that, neurons close to motor output pathways encode multiple features of a behavioral program. However, the expressing neurons of progesterone receptor and estrogen receptor α in the ventromedial hypothalamus which are distant from sensory input are important for aggression and mating multiple components (Lee et al. 2014). Aromatase is responsible for the aromatization of brain androgens into estrogens which plays a critical role in the sexually dimorphic brain structures biogenesis during perinatal development by converting testicular-derived testosterone to estrogen (neuroestrogens) within certain brain structures (Roselli et al. 1984). Aromatase has been reported to have a potential role in modulation of mood, pain and social behavior such as aggressiveness (Stanić et al. 2014). Previous studies showed that intra-cerebro-ventricular injection of GnIH/RFRP inhibited male rat aggressive behavior through activation of aromatase which subsequently lead to elevation of neuroestrogen concentration above its optimal levels in brain (Johnson et al. 2007; Ubuka et al. 2013). Interestingly, GnIH normally inhibits and lower the release of gonadotropin hormones and testosterone, hence, increasing aromatase activity which by its role converts testosterone to neuroestrogen that stabilize both aggressive and sexual behavior. Phytoestrogen may act as allosteric inhibitor to aromatase thus inhibiting the conversion of testosterone to neuroestrogen The accumulated testosterone may worsen the aggressive behavior (Ubuka et al. 2014; Ubuka and Tsutsui 2014).

It was found that, estrogen-synthesizing enzyme aromatase activity is rapidly regulated by glutamatergic neurotransmission in intact neural systems (Balthazart et al. 2006). Unlike GnIH/RFRP, dopamine and glutamate stimulate male sociosexual behavior through inhibition of aromatase activity in the brain (Balthazart et al. 2003). Glutamate decrease aromatase activity by its phosphorylation in the medial perioptic area in rat brain, also, dopamine may compete with testosterone and prevent its transformation into estrogens by acting as an alternative substrate for aromatase. Thus, glutamate and dopamine may facilitate male aggressive behavior expression by decreasing aromatase activity and maintaining neuroestrogen optimum concentration (Dominguez et al. 2006; Kleitz-Nelson et al. 2010). Different types of molecules with a customary diphenolic structure are classified as nonsteroidal phytoestrogens in configuration. Physicochemical and physiological properties of estrogens are represented in phytoestrogens which are classified into: isoflavones, lignans and coumestans. The major class of phytoestrogens contained in soybean is isoflavones (Barnes 2010; Patisaul and Jefferson 2010). Phytoestrogens is associated with favorable health effects as their use in menopause therapy and prevention of cancer and cardiovascular diseases (Balthazart et al. 2003; Rietjens et al. 2017), while their potential adverse effects could not be ignored. Consumption of SB products was associated with sporadic cases of aggressive behavior in adult male monkeys (Adgent et al. 2011; Simon et al. 2004), poor semen quality in men (Chavarro et al. 2008) and low testosterone level in prostate cancer patients (Thelen et al. 2014). The impact of soya bean and its contents of phytoestrogens which are found in high amounts in most food products on brain and social behaviour is still a debating issue. Phytoestrogens were proved to reach the brain after intraperitoneal injection of genistein and daidzein to adult Sprague–Dawley rats (Soucy et al. 2006). Several studies have established a connection between the increased brain concentration of phytoestrogens in regions abundant with estrogen receptors (ER) and the relevance to their potential impact on learning, memory and behavior (Bakker and Baum 2008).

Thus, we hypothesized that consumption of large amount of SB (as a rich source for phytoestrogens) could induce male aggressive behavior possibly through a negative feedback mechanism on GnIH/RFRP3 and/or aromatase (which could decrease GnIH and aromatase, respectively) and/or via competition with neuroestrogen to bind ER (which could reduce neuroestrogen concentration). High doses of SB may also inhibit aromatase activity through induction of dopamine and glutamate. Thus, this study was conducted to investigate the effect of large amount of SB consumption on the aggressive behavior in adult male rats through investigating changes in the expression of some genes related to GnIH/RFRP3-aromatase-neuroestrogen pathway (*GnIH/RFRP3, NPFFR, Cyp19A1, ERα,* and *ERβ*), and the levels of neuroestrogen, dopamine, glutamate and testosterone as well as aromatase activity in the brain.

Materials and methods

Animals

Adult male Sprague–Dawley albino rats ($n = 45$, weight = 180–210 g), aged from 12 to 14 weeks, obtained from the Animal House, Faculty of Medicine, Tanta University, had access to standard chow diet (Table 1) and water ad libitum. They were

Table 1 Diet composition in the studied groups

Component	Control group (g/kg diet) N = 15	Group II (25% SB, g/kg diet) N = 15	Group III (50% SB, g/kg diet) N = 15
Corn starch	495.2	310.9	105.7
Maltodextrin	141.3	120.4	100.4
Sucrose	95	85	85
Cellulose	45	45	45
Soya bean	NA	250	500
Brain	20	20	20
Casein, high nitrogen	145	110.2	100.4
L-cysteine	1.5	1.5	1.5
Minerals	40	40	30
Vitamin mix	15	15	10
Tartaric acid	2	2	2
Total calories from 1000 g diet	3580.7	3685.9	3790.8

N number. NA: not applicable

left to acclimatize for 1 week before the experiment and were maintained at 22 ± 20 C, 50 ± 5% humidity and 12 h light-12 h dark cycle. They were kept in cages with female rats (every male rat was housed with a female rat) to reduce sexual behavioral induced stress as housing each resident with a female for at least one week before the start of the experiments, facilitates territoriality development, as territoriality is enhanced strongly in the presence of females and/or sexual experience (Albert et al. 1988). As a consequence of territoriality, unfamiliar males' intruders will be attacked by the resident in its home cage which enables the study of offensive aggression by using the resident as the experimental animal. As well, it would prevent social isolation, which is stressful for social animals and may cause reduced welfare and social behavior aberrant forms (Koolhaas et al. 2013). Animal handling and experimental protocols were approved by the Ethical Committee of the Faculty of Medicine, Tanta University (number# 32969/2) and were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.85–23, revised in 1996).

Chemicals

All chemicals used unless otherwise noted were purchased from Sigma (St. Louis, MO, USA). Chemicals and solvents were of high analytical grade.

Soya beans preparation

SB was purchased from local market, sun-dried and fairly roasted for 10 min at 85°C. The roasted SB was then grinded into coarse particles with average diameter of 1.5 mm for disposal anti-nutritive substances and reducing urease activity. Diets of all groups of animals comply with the requirements and accepted standards.

Experimental design

The rats were randomly divided into three groups (15 rats/group): Group I (the control group) received standard chow diet. Group II: received 25% SB of their standard chow diet contents for 12 weeks [every kg of diet is a mix of 750 mg of chow diets and 250 mg of the prepared soya bean, which were mixed with a high speed blinder (Toshiba)]. Group III: received 50%SB of their standard chow diet contents for 12 weeks [every kg of diet is a mix of 500 mg of chow diets and 500 mg of the prepared soya bean, which were mixed by blinder]. The diet content of SB was tried at different percentages. Our preliminary results from dose-response pilot study with the following concentrations at different percentages (5%, 15%, 25%, 35%, and 50%). At 25%, the aggressive behavior was noticed on the animals and at 50% percentages of soya bean, prominent and vigorous aggressive behaviors and marked effects on the studied biochemical markers were noticed. The composition of diets is shown in Table 1.The amount of diet ingested by the rats was determined by regular weighing of the troughs every other day.

Resident-intruder test of aggression

Every male animal was housed in a cage with a floor space of half a square meter to give sufficient space for full expression of the highly active aggression with a female for 12 weeks and only males which impregnated the females were used in the present study to ensure the male's sexual maturity and to exclude any abnormal socio-sexual behavior. The special diets for the 3 studied groups (control, 25% SB and 50% SB groups, respectively) were offered throughout this housing

period. To assess the level of aggression, females were removed and the experimental male rats were kept on their diets and were housed individually for 24 h. Then, during the dark phase which is the rats' main activity phase, they were confronted in their home cage with a male intruder that had been kept on a standard chow diet. The intruder was of the same species and continuously housed with a female. The intruder and resident had no previous contact with each other. During a 10 min test period, the aggressive behavior of the resident rats was monitored and rats were selected when reaching the criterion for aggression and exhibited consistent levels of aggressive behaviors. During this behavior, the resident rat defeated the intruder rat as revealed by surrendering the intruder (acquiring a supine position at least 3 times and for at least 3 s). The number of attacks and the duration of various aggressive acts, including lateral threat, tail rattle, biting, clinch attacks, biting and fighting exhibited by the resident rat were also recorded.

Blood and tissue sampling

At the end of the experiment, three days after the resident-intruder test, rats were anesthetized by ether and sacrificed. Blood samples were collected directly from the animals by heart puncturing and sera were separated and stored in aliquots at −70 °C till used for the biochemical assay. The brain tissue samples (thalamus and hypothalamus) were excised together due to difficulty in dissection from each other, rinsed in ice cold isotonic saline to remove extraneous materials, and divided into 3 parts; the first part was used for preparation of tissue homogenates for different biochemical assays, the second part was stored at −70 °C for molecular investigation. The third part was preserved in 10% buffered paraformaldehyde for immunohistochemical studies. Briefly, the bilaterally dissected thalamus/hypothalamus was first divided transversally into anterior (4/5) and posterior (1/5) and then the anterior segment (4/5) was divided longitudinally into equal two halves (2/5 each). The posterior segment (1/5) was used for immunohistochemistry, while one half of the anterior segment was used for biochemical assay and the second for real time PCR.

The hypothalamus was chosen as a target region for the experiment because, the hypothalamic area is one of the best-studied areas in relation to aggression of all brain areas since 1955 (Hess and Akert 1955). Also, it contains the preoptic area which is responsible for sexual behavior and aggressive behavior (Tsutsui et al. 2012; Angoa-Pérez and Kuhn, 2015) and it is the site of release of both GnRH and GnIH (Tsutsui et al. 2012).

Preparation of brain tissue homogenates

The brain was homogenized in 20% ice-cold phosphate buffer using Potter-Elvehjam-type glass homogenizer. Then, to remove nuclei and unbroken cells, the homogenate was centrifuged using a cooling centrifuge (Eppendorf, R 5804) at 1500 g for 10 min at 4 °C. The supernatants were frozen at −80C until analysis of different biochemical markers. The protein concentrations of brain tissue were determined by the Lowery method (Lowry et al. 1952).

Biochemical assays

The following biochemical parameters were estimated in brain tissues homogenates:

Dopamine level was quantitatively assayed by an enzyme linked immunosorbant assay (ELISA) kit (Catalog# CEA851Ge, Cloud Clone Corp, USA) according to the manufacturer's instructions. The intensity of color developed was reversely proportional to the concentration of dopamine in samples. The color intensity developed was read on ELISA reader (Stat Fax 2000) at 450 nm. The results were expressed as pg/mg protein.

Glutamate levels by an enzymatic assay kit (Sigma Aldrich, Catalog # MAK004) which resulted in a colorimetric product proportional to the glutamate present and was read on Spectrophotometric multiwell plate reader at 450 nm (Labomed, Spector 23, CA, USA). The results of glutamate were expressed as ng/mg protein.

Neuroestrogen levels by rat ELISA kit (MyBioSource, USA, Catalog# MBS2607338), according to the manufacturer's instructions. The color intensity developed was read on ELISA reader at 450 nm. The results were expressed as pg/mg protein.

Aromatase enzyme activity by flurometric assay kit (Catalog # K983–100, Biovision, USA) according to the manufacturers's instructions. Briefly, aromatase assay utilizes a fluorogenic substrate that is converted into a highly fluorescent metabolite detected on GloMax® (Catalog: GM3000) in the visible range (Ex/Em = 488/527 nm). A highly selective aromatase inhibitor (Letrozole) was used for determination of aromatase activity which displays greater than 100-fold selectivity for aromatase over other enzymes, ensuring targeted inhibition. Aromatase specific activity was calculated by running parallel reactions in the presence and absence of the inhibitor and subtracting any residual activity detected with the inhibitor present. Aromatase (CYP19A) Specific Activity was expressed as pmole/min/mg protein, where one unit of aromatase activity is the amount of enzyme that generates 1 μmole of fluorescent metabolite per min by hydrolysis of 1 μmole Aromatase Substrate at 37 °C and pH 8.

Serum and brain tissue concentrations of testosterone were measured by rat ELISA kit (MyBioSource, USA, Catalog#: MBS282195) according to the manufacturer's instructions. This assay was a competitive enzyme immunoassay. The color development was stopped. The color intensity developed was read on ELISA reader at 450 nm.

Molecular analysis

Total RNA was isolated from brain tissues (hypothalamus and thalamus) using RNeasy Mini kit (Qiagen, #74104). The cDNA was synthesized by reverse transcription of RNA using Quantiscript reverse transcriptase according to the manufacturer's instructions (Qiagen, #205310). Specific primers for RFRP3: (F: 5' CCAAAGGTTTGGGAGAACAA3′ and reverse: 5'GGGTCATGGCATAGAGCAAT 3′ (Gene bank accession number #AB040288.1) [22], *NPFFR1* (F: 5'ACAACTTTGGCATTGTGGAA-3' and R: 5'-GATGCAGGGATGATGTTCTG-3′) (Gene bank accession number # NM_022291.2) *Cyp19A1* (F:5' CTGCTGAT CATGGGCCTCC 3′ and R: 5' CTCCACAGGCTCGG GTTGTT 3′) (Gene bank accession number # NM_017085.2), *ERa* (F: 5' TGAAGCACAAGCGT CAGAGAGAT 3′ and R: 5' AGACCAGACCAATC ATCAGGAT 3′) (Gene bank accession number # NM_012689.1), *ERβ* (F:5' TCTGTGTGAAGGCCATGATC 3′ and R: 5' GCAGATGTTCCATGCCCTTG 3′) (Gene bank accession number # NM_012754.1) and the internal control *β actin* (F:5' AAGTCCCTCACCCTCCCAAAAG 3′ and R: 5'AAGCAATGCTGTCACCTTCCC 3′) (Gene bank accession number # NM_031144.3) which was used as a reference to calculate fold change in target gene expression. A 2 μL of cDNA template, 12.5 μL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 1 μL forward primer, 1 μL reverse primer, and 8.5 μL of nuclease free water were used to prepare 25-μL of the PCR mix. The thermal cycling conditions were as follows: 10 min initial denaturation at 95 °C, followed by, 15 s of 40 cycles of denaturation at 95 °C then, 30 s annealing at 60 °C and finally, 30 s extension at 72 °C. At the end of the last cycle, the temperature was increased from 63 to 95 °C for melting curve analysis. The determination of the relative levels of gene expression was performed using the comparative cycle threshold ΔΔCt method and normalized to *β actin* gene. The qPCR was performed using QuantiTect SYBR Green Master Mix with reaction cycles, melting curve condition and fold change calculation as previously described (El-Magd et al. 2017).

Immunohistochemistry analysis

The brain tissues were divided antero-posterior parallel to bregma, embedded in paraffin, sectioned coronal (4 mm thickness) by adult brain slicer matrix, into anterior (4/5) and posterior (1/5). The posterior segment (1/5) was used for immunohistochemistry, deparaffinized and rehydrated. Slides were incubated in 3% H2O2 for 10 min to reduce nonspecific background staining arising due to endogenous peroxides. For antigen retrieval, specimens were heated for 20 min in 10 mmol/l citrate buffer (pH 6.0). Following incubation with Ultra V Block (Lab Vision Corporation, USA) for 7 min at room temperature to block background staining, slides were incubated with ERα (rabbit polyclonal, ab37438) (dilution 1: 100). Antibody binding was detected using the Ultra Vision LP Detection System according to the manufacturer's recommendations. Color development was performed with 3, 30-diaminobenzidine. ERα expression was nuclear immune-positive reaction, and the nuclei showed brownish punctuate. The mean counts of ERα expressing cells were assessed by image analysis using LEICA Q WIN MICROSOFT under high-power fields (HPF) at 400 magnifications by counting a total of 20HPF/slide areas containing positive cells and expressed as percentage of positive cells to the total area examined. Values were expressed as the means of percent of immunoreactive cells present in 20 HPF areas ± the standard error of mean (SEM). In order to detect unintended background staining, the regular immunohistochemical staining protocol was processed on rat brain specimens but omitting the step of primary antibody (negative control).

Statistical analysis

Normal distribution of data was tested by the Kolmogorov-Smirnov normality test, followed by the Levene test for variance equivalent. Comparisons between parameters obtained from more than 2 groups were assessed by one way- analysis of variance (ANOVA) using Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) followed by comparison of means with Tukey's Honestly Significant Difference (Tukey's HSD) post hoc test. Data were presented as mean ± SEM and significance was declared at $P < 0.01$.

Results

Effect of soya beans on rat body weight and aggressive behavior

There were no significant differences in rat weight were observed between the studied groups (Table 2). However, rats received 50% diet rich in SB (group III) required less time to socially defeat the intruder rat, compared to rats in group II and control rats, and demonstrated significantly higher number of attacks compared to group II and control rats ($p < 0.01$). Also, rats in group III spent more time in aggressive behavior than rats of group II and controls ($p < 0.01$).

Effect of soya beans on genes related to RFRP3-aromatase-neuroestrogen pathway

The effect of consumption of large amount of SB on the relative expression of genes involved in the GAN pathway, including *GnIH/RFRP3, NPFFR, Cyp19A1, ER a,* and *ER β*, in rat brain is shown in (Table 3 & Fig. 1). The results of qPCR

Table 2 Effects of SB-rich diet on body weight and aggressive behavior of adult male rats

	GI (normal control) N = 15	GII (25% SB) N = 15	GIII (50% SB) N = 15	F test	*P* value
Body weight (g)	412 ± 22.4	422 ± 16.5	427 ± 12.5	1.574	0.394
Time spent in aggressive behavior (seconds)	31 ± 12 [c]	130 ± 17[b]	170 ± 18 [a]	74.208	0.001*
Number of attacks	0.07 ± 0.00 [c]	2.89 ± 0.97[b]	7.81 ± 1.03 [a]	68.423	0.001*

Data are presented as mean ± SEM. Values in the same raw with different superscript letters are significantly different at $p < 0.01$

N number

exhibited a significant downregulation in the gene expression of *RFRP3, NPFFR, Cyp19A1*, and *ER β* in brains of rats received diets rich in SB (groups II and III) as compared to the control rats ($p < 0.01$). In contrast, the same treatment significantly increased the gene expression of *ER a* in brains of groups II and III as compared to group I (control) ($p < 0.01$). In general, the change in the expression of these genes was dose dependent, with notable changes in group III than group II. These results suggest that the stimulatory aggressive effect of SB is dose dependant and accompanied by significant decreased expression of *RFRP3, NPFFR, Cyp19A1*, and *ER β* and significant upregulated expression of *ER α* in the brain.

Effect of soya beans on ERα immunostaining

Examination of brain tissue (thalamus and hypothalamus) sections immune-stained with ERα antibody in rats fed SB-rich diets (group II and III) showed apparent increase in the positive immunoreaction in the nuclei, with higher immunostaining in group III (Fig. 2 c) than group II (Fig. 2 b), as compared to the control group (Fig. 2a). In support, quantification results also revealed a significant increase in the mean area percentage of ERα immunostaining in groups II and III, with higher immunostaining in group III than group II, as compared to group I (Fig. 2d). In general, these findings indicate that the stimulatory aggressive effect of SB is associated with significant increased ERα immunostaining in the brain. Figure 2e represents the negative control sample.

Effect of soya beans on aromatase, neuroestrogen, dopamine and glutamate levels

To further confirm our hypothesis that SB induces aggressive behavior in adult male rats through inhibition of aromatase and neuroestrogen, we measured the change in aromatase activity and neuroestrogen concentration in the brain following feeding rats with diet rich in SB. Expectedly, brains of rats fed SB-rich diets showed a significant lower aromatase activity and neuroestrogen level, with lowest levels in group III than group II, relative to the control group ($p < 0.01$) (Table 3 & Fig. 3).

For their stimulatory role on aggressive behavior, dopamine and glutamate levels were also measured in the brain

Table 3 Effect of soya bean consumption on the studied biochemical markers

	GI (normal control) N = 15	GII (25% SB) N = 15	GIII (50% SB) N = 15	F test	P value
Brain aromatase (μU/mg protein)	100 ± 7.04 [a]	46.58 ± 4.28 [b]	28.44 ± 2.21 [c]	65.541	0.001*
Brain dopamine (pg/mg protein)	30.22 ± 6.32 [c]	100.65 ± 11.12 [b]	189.12 ± 15.5 [a]	61.251	0.001*
Brain glutamate (ng/mg protein)	15.32 ± 2.24 [b]	43.98 ± 9.6 [a]	57.77 ± 8.5 [a]	25.362	0.001*
Brain neuroestrogen (pg/mg protein)	100 ± 7.45 [c]	57.22 ± 9.89 [b]	19.28 ± 6.5 [c]	70.584	0.001*
Serum testosterone (ng/ml)	6.7 ± 0.82	7.52 ± 1.15	7.86 ± 1.34	1.205	0.421
Brain testosterone (pg/mg protein)	12.12 ± 0.76 [c]	17.34 ± 0.92 [b]	20.9 ± 0.85 [a]	8.543	0.008*
Brain Cyp19A1 mRNA relative expression	1.1 ± 0.06 [a]	0.42 ± 0.08 [b]	0.15 ± 0.04 [c]	31.264	0.001*
Brain ER-α mRNA relative expression	1.1 ± 0.09 [a]	3.32 ± 0.36 [b]	4.9 ± 0.57 [a]	27.521	0.001*
Brain ER-β mRNA relative expression	1.1 ± 0.09 [a]	0.51 ± 0.09 [b]	0.2 ± 0.05 [c]	13.541	0.001*
Brain RFRP3 mRNA relative expression	1.1 ± 0.04 [a]	0.5 ± 0.09 [b]	0.22 ± 0.07 [c]	14.085	0.001*
Brain NPFFR mRNA relative expression	1.1 ± 0.05 [a]	0.63 ± 0.07 [b]	0.4 ± 0.057 [c]	12.847	0.001*

Data are presented as mean ± SEM. Values in the same raw with different superscript letters are significantly different at $p < 0.01$. N: number. SB: soya bean, Cyp19A1: cytochrome P450, family 19, subfamily A, polypeptide 1, ER-α: estrogen receptor alpha, ER-β: estrogen receptor beta, RFRP3: RF amide-related peptide 3, NPFFR: neuropeptide FF receptor

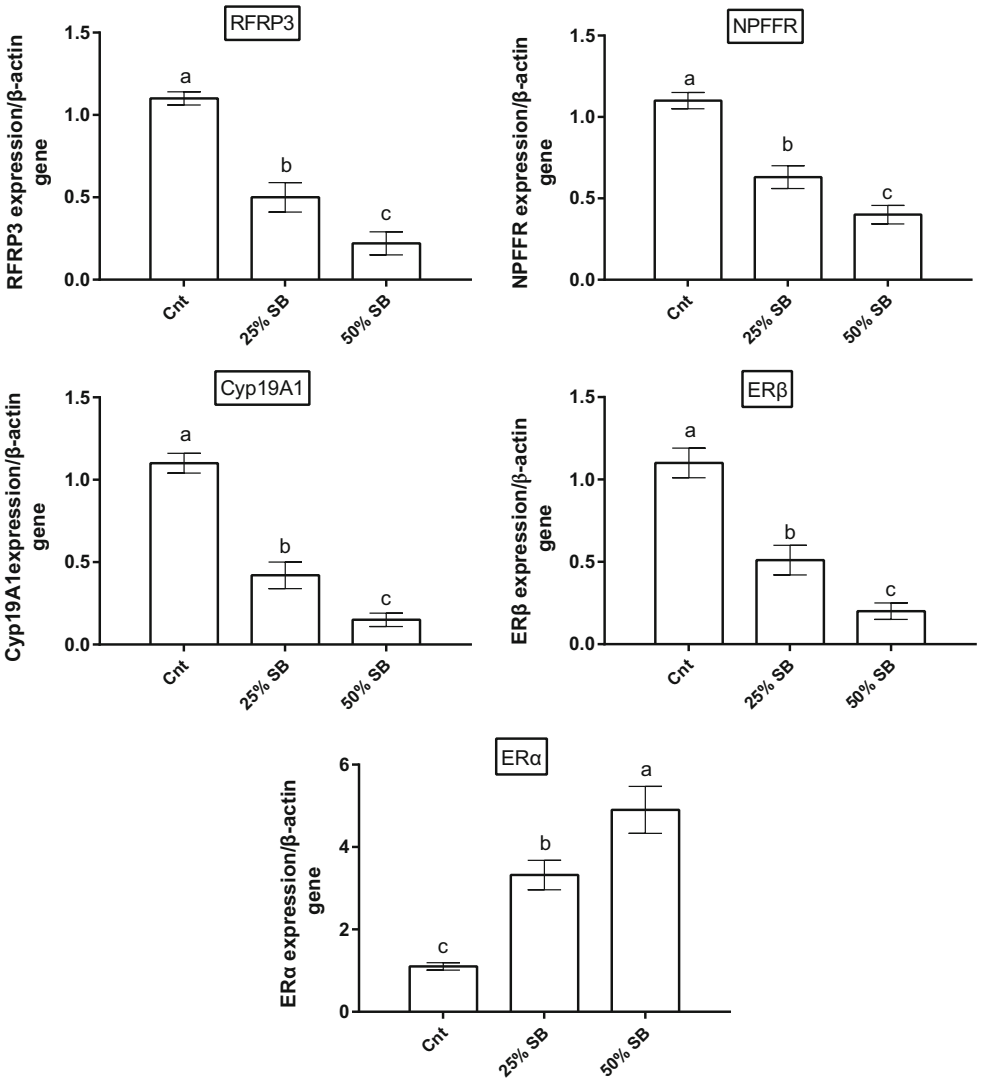

Fig. 1 Effect of feeding on SB-rich diet on relative expression genes involved in the GnIH/RFRP-aromatase-neuroestrogen pathway, including *RFRP3 (*RF amide-related peptide 3), *NPFFR (*neuropeptide FF receptor*), Cyp19A1(,*cytochrome P450, family 19, subfamily A, polypeptide 1) *ER-β* (estrogen receptor beta), *and ER-α* (estrogen receptor alpha), in the brain of adult male rats. Values are expressed as mean ± standard error of mean (SEM) and means in columns with different letters are significantly different at $p < 0.05$. Cnt, control group (group I) (15 rats); 25% SB, group II (15 rats); 50% SB, group III (15 rats)

tissues following consumption of SB-rich diet and the results showed a significant increase in their levels, with a significant higher dopamine levels in group III than group II, relative to the control group ($p < 0.01$) (Table 3 & Fig. 3). However, no significant difference in glutamate level was observed between group II and group III.

Effect of soya beans on serum and brain levels of testosterone

For its significant role in induction of male aggressiveness, testosterone levels were measures in serum and brain following feeding rats on SB-rich diet. No significant changes were noticed in the serum of animals fed SB-rich dietas compared to the control group ($p > 0.05$) (Table 3 & Fig. 4a). However, in the brain, there was a significant higher testosterone level in animals fed SB-rich diets (groups II and III) than the control group ($p < 0.01$) (Table 3 & Fig. 4b). Again, this change was dose dependant, with higher brain testosterone level in group III than in group II. These results indicate that the stimulatory aggressiveness effect of SB is correlated with testosterone levels in the brain, but not in the serum.

Correlation between behavioral data and the studied biochemical markers in group III (50% SB)

As shown in Table 4, there was significant negative correlation of both times spent in aggressive behavior and number of attacks with brain aromatase activity and levels of neuroestrogen, Cyp19 A1, ER-β, RFRP3 and NPFFR ($p < 0.01$). However, significant positive correlation was found between behavioral data and brain levels of dopamine, glutamate, testosterone and ER-α ($p < 0.01$). No significant correlation was found between time spent in aggressive behavior and number of attacks with serum testosterone ($p > 0.05$).

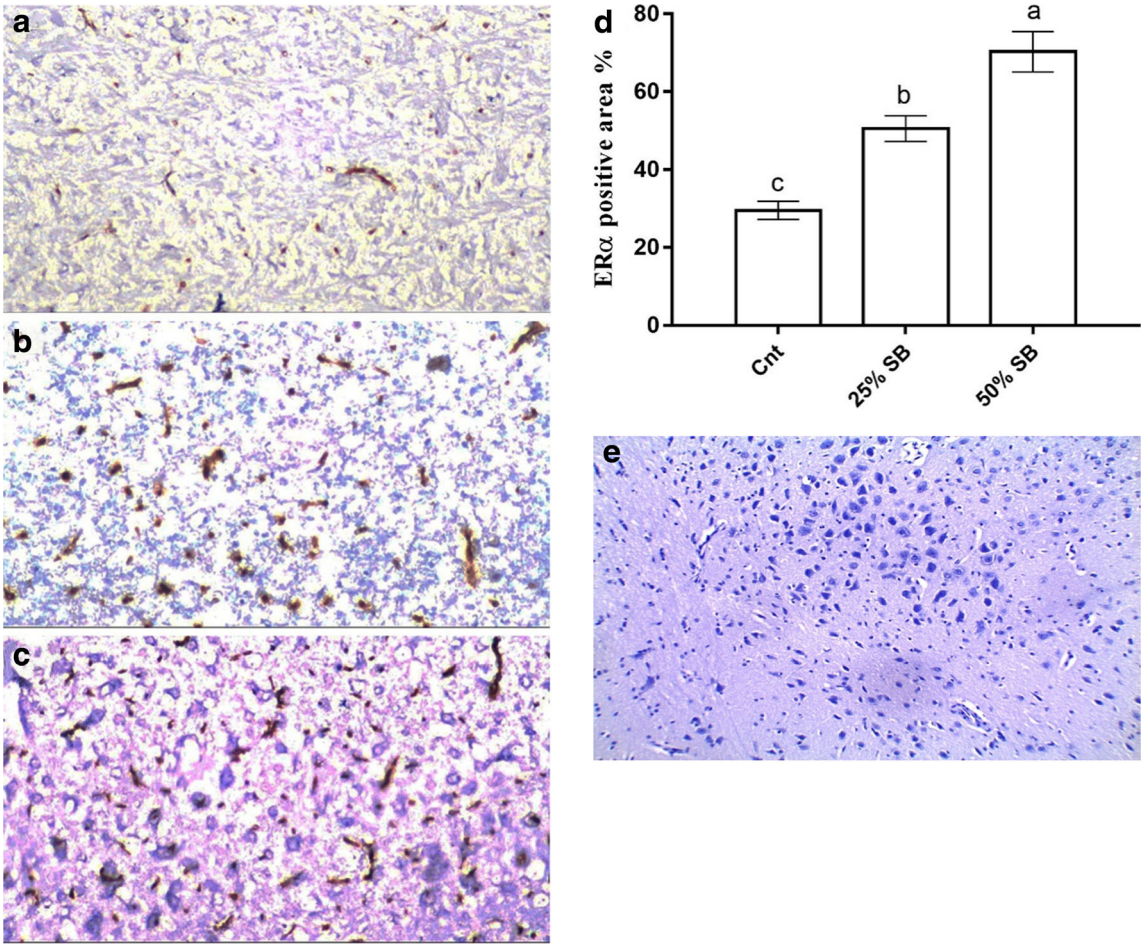

Fig. 2 Photomicrographs of rat brain sections immune-stained with ERα (estrogen receptor alpha) antibody (brown stained nuclei) of group I (**a**), group II (**b**), and group III (**c**), × 400. (**d**) Quantitative evaluation of ERα immuno-staining. Values are expressed as mean of ERα positive area % ± standard error of mean (SEM) and values carrying different lower case letter are significantly different at $p < 0.05$.Cnt, control group (group I) (15 rats); 25% SB, group II (15 rats); 50% SB, group III (15 rats). (E) Represents the negative control rat brain sample

Discussion

The impact of soya bean and its contents of phytoestrogens which are found in high amounts in most food products on brain and social behaviour is still a debating issue. Previous studies on male rat, hypothesized that GnIH may inhibit aggressive behaviors by direct activation of aromatase activity which subsequently induces high neuroestrogen synthesis beyond its optimal level in the brain (Ubuka et al. 2013, 2014). Similarly, injection of GnIH/RFRP3 decreases aggressive behavior in rats (Johnson et al. 2007). Therefore consumption of SB in large amount may induce aggressive behavior in adult male rats through inhibition of this GnIH/RFRP-aromatase-neuroestrogen (GAN) pathway. If SB targeted the three components, we have at least three hypotheses to speculate about the mode of SB action. Regardless the targeted component of this pathway and the three possible hypotheses, the final outcome was induction of male aggressiveness by consumption of large amount of SB for long time (12 weeks). In the present study, male rat aggressiveness was induced by consumption of large amount of SB for long time (12 weeks). In consistent with our findings, increased aggressive behavior in adult male monkeys was also noticed after long-term consumption of diets rich in soy protein and isoflavones (Simon et al. 2004). Our first hypothesisis that high dose of phytoestrogen (through consumption of large amount SB) can induce male aggressive behavior through a negative feedback mechanism where high level of phytoestrogen may inhibit GnIH/RFRP which further prevents aromatase activity and decrease neuroestrogen levels in brain. As GnIH inhibits the release of gonadotropin hormones and testosterone, hence, increasing aromatase activity which converts testosterone to neuroestrogen thus stabilizes the socio-sexual behavior including aggression and sexual behavior. By phytoestrogen intake, it acts most probably as allosteric inhibitor to aromatase and inhibits the conversion of testosterone to neuroestrogen by aromatase enzyme. The accumulated testosterone worsens the aggressive behavior (Ubuka et al. 2014; Ubuka and Tsutsui 2014). In this hypothesis, SB phytoestrogens could target the upstream component in this

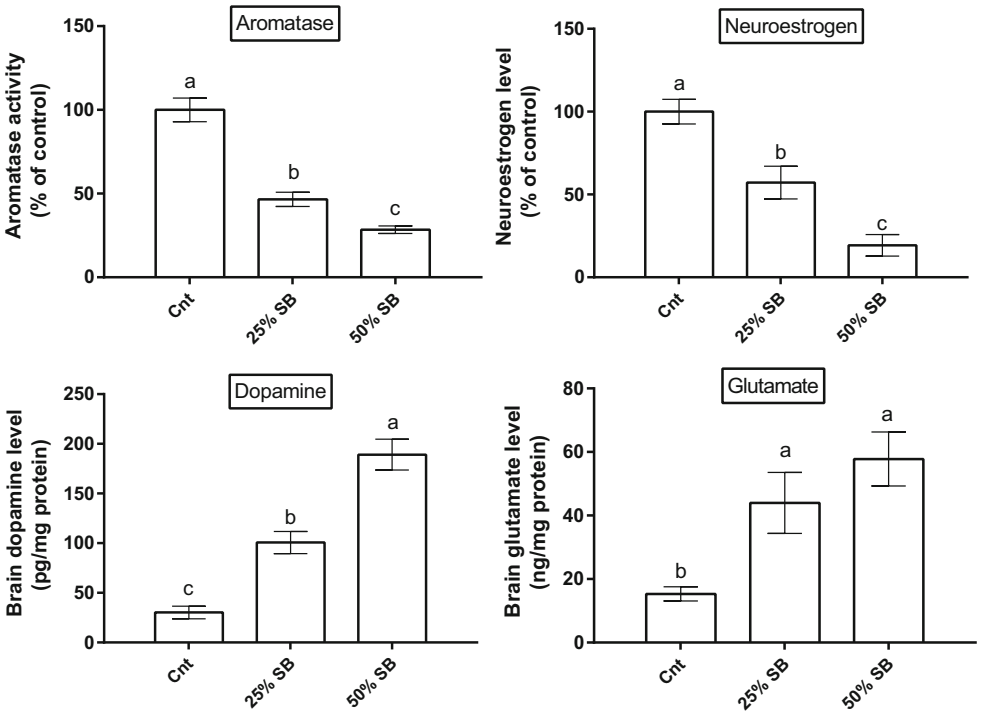

Fig. 3 Effect of feeding on SB-rich diet on aromatase activity, and levels of neuroestrogen, dopamine and glutamate in the brain of adult male rats. Values are expressed as mean ± standard error of mean (SEM) and means in columns with different letters are significantly different at $p < 0.05$. Cnt, control group (group I) (15 rats); 25% SB, group II (15 rats); 50% SB, group III (15 rats)

pathway, GnIH/RFRP, confirming the importance of this molecule in regulation of aggression in adult male mammals (Lephart 1996). This hypothesis was based on our results which exhibited a significant downregulation of *RFRP3*, *NPFFR* and *Cyp19A1* expression and decrease in aromatase activity and neuroestrogen concentration in the brain following consumption of a large amount of SB in a dose dependent manner. In agreement, previous studies also showed that injection of a GnIH receptor (GPR147) antagonist RF9 or an aromatase inhibitor, fadrozole hydrochloride, inhibited the stimulatory action of GnIH on aromatase and neuroestrogen synthesis in the brain in a dose dependant manner (Kriegsfeld et al. 2006; Ubuka et al. 2013, 2014). Moreover, inhibition of GnIH expression by small interfering RNA increased the aggressive behavior in the male quail (as noticed by increasing the number of strutting, pecking, grabbing, and mounting actions), however central administration of GnIH inhibited this aggressive behavior (Kriegsfeld et al. 2006; Ubuka et al. 2013, 2014). Similarly, intra-cerebro-ventricular administration of GnIH/RFRP suppressed male aggressive behavior of rats (Johnson et al. 2007). Co-expression of GPR147 mRNA in brain immunoreactive aromatase cells suggests that GnIH may directly activate aromatase cells in brain (Ubuka et al. 2013). Similarly, for its ability to bind ER (Jefferson et al. 2002), phytoestrogens may also directly inhibit GnIH/RFRP in the brain, but further investigations are required to check whether GnIH/RFRP neurons contain ER.

In support to our first hypothesis, central injection of estrogen significantly downregulated the expression of *GnIH* gene in mice brain, suggesting a mechanism for abolishing of GnIH suppressive effect on social aggressive behavior during times of high estrogen stimulation (Tsutsui et al. 2012). This can also explain downregulation of GnIH expression by high levels of circulating oestradiol levels to allow gonadotrophin secretion in mature animals (Lephart 1996).

Our second hypothesis suggests that consumption of large amount of SB could induce aggressive behavior in adult male rats in a dose dependant manner through inhibition of aromatase (independent on GnIH) which subsequently decreases neuroestrogen contents in the brain. This hypothesis was based on our results which showed that consumption of large amount of SB led to significant decreases in aromatase activity, *Cyp19A1* mRNA levels, and neuroestrogen concentration in the brain. In support, previous studies also reported a similar negative association between aromatase activity or neuroestrogen concentration and the male social behavior as revealed by enhanced aggressive behavior of mice by targeted disruption of *Cyp19A1*gene (Harada et al. 2009; Toda et al. 2001). Other studies also showed ability of phytoestrogens to inhibit aromatase enzyme activity in peripheral endocrine tissue sites (in vitro) (Ibrahim and Abul-Hajj 1990; Wang et al. 1994). In the present study, feeding rats on high SB diet downregulated GnIH/RFRP and reduced aromatase activity and *Cyp19A1* mRNA levels in the hypothalamus and thalamus regions, which are known for their higher aromatase activities (Kriegsfeld et al. 2006; Lephat, 1996). Higher aromatase activity was also detected in the hippocampus and cerebral cortex suggesting a potential role aromatase in modulation of mood, pain, and social behaviors (Stanić et al. 2014). In contrast to our results, a previous study concluded that

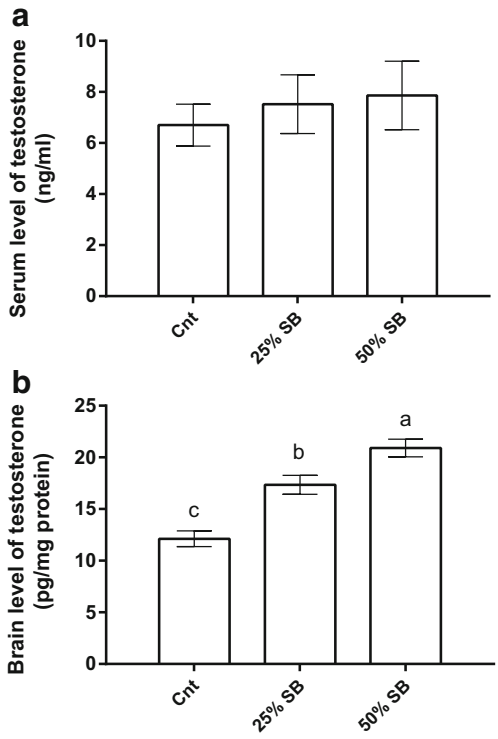

Fig. 4 Effect of feeding on SB-rich diet on serum (**a**) and brain (**b**) levels of testosterone. Values are expressed as mean ± standard error of mean (SEM) and means in columns with different letters are significantly different at $p < 0.05$.Cnt, control group (group I) (15 rats); 25% SB, group II (15 rats); 50% SB, group III (15 rats)

administration of phytoestrogen diet to adult rats did not significantly change aromatase levels in brain, suggesting that the phytoestrogens target brain areas with no aromatase activity (Lephart et al. 2000). However, these contradictory results may be attributed to variation in amount of SB or phytoestrogens and duration of the study. In our study we used a very larger amount of SB (25% and 50% SB) for longer time (12 weeks), than those used in other studies. Thus, the very high doses of phytoestrogens used in the present study may be enough to inhibit aromatase activity and *Cyp19A1* expression via negative feedback mechanism or via direct inhibition because the *Cyp19A1* gene was shown to be co-expressed with ER in neurons of thalamus and hypothalamus with abundant expression in male than in female mice (Stanić et al. 2014). Although, testosterone plays an important role in male aggressive behavior we did not find a significant change in serum testosterone concentration after administration of SB-rich diet. Additionally, long term consumption of soy led to increased aggressiveness but no effects on serum testosterone or estradiol concentrations (Simon et al. 2004). This suggests that the aggressive behavior may also be induced by factor(s) other than testosterone produced by testis. Indeed, our results revealed that feeding SB-rich diets accompanied by a significant increase in testosterone level in the brain which may be attributed to the inhibition of aromatase by high levels of phytoestrogens. This highlights the significant effect of brain testosterone in modulation of aggressive behaviors and so aromatization is not necessary for induction of aggressiveness in rats. In the present study, feeding rats on SB rich diets led to a significant increase in brain level of dopamine and glutamate, which are known for their stimulatory effect on male aggressive behavior (Balthazart et al. 2003; Guadarrama-Bazante et al. 2014). In agreement with these results, Balthazart et al. (2002), who found a similar dopamine and glutamate inhibitory effect on aromatase activity in animal brain. Aromatase activity is regulated at long term

Table 4 Correlation between behavioral data and the studied biochemical markers in group III (50% SB)

Group III (50% SB) N = 15	Time spent in aggressive behavior (seconds)		Number of attacks	
	r	p value	r	p value
Brain aromatase (μU/mg protein)	- 0.632	0.001*	- 0.713	0.001*
Brain dopamine (pg/mg protein)	0.548	0.002*	0.587	0.001*
Brain glutamate (ng/mg protein)	0.563	0.001*	0.685	0.001*
Brain neuroestrogen (pg/mg protein)	- 0.574	0.001*	- 0.632	0.001*
Serum testosterone (ng/ml)	0.148	0.532	0.217	0.254
Brain testosterone (pg/mg protein)	0.423	0.021*	0.475	0.016*
Brain Cyp19A1 mRNA relative expression	-0.532	0.003*	-0.593	0.001*
Brain ER-α mRNA relative expression	0.498	0.012*	0.521	0.001*
Brain ER-β mRNA relative expression	- 0.398	0.031*	- 0.427	0.020*
Brain RFRP3 mRNA relative expression	- 0.435	0.019*	−0.497	0.012*
Brain NPFFR mRNA relative expression	- 0.472	0.017*	−0.512	0.006*

*Significant at $p < 0.01$. N: number. SB: soya bean, Cyp19A1: cytochrome P450, family 19, subfamily A, polypeptide 1, ER-α: estrogen receptor alpha, ER-β: estrogen receptor beta, RFRP3: RF amide-related peptide 3, NPFFR: neuropeptide FF receptor

(hourstodays) by *Cyp19A1* gene transcription and decreased at short term (minutes) by enhancing intracellular Ca^{2+} concentration and phosphorylation by glutamate (Balthazart et al. 2003). Exposure to stress or corticosterone injection can induce GnIH/RFRP release and aromatase activity in brain (Tsutsui et al. 2012; Son et al. 2014) suggesting a mediator role for GnIH/RFRP and aromatase against stressors. Although aggressive behavior is strongly stimulated by stress, we found a decline in GnIH/RFRP and aromatase in highly aggressive animals (groups II and III). This may reflect the powerful inhibitory effect of SB on GnIH/RFRP and aromatase which may predominate on the stimulatory effects of stressors.

Our third hypothesis suggests that large phytoestrogens levels (due to consumption of high-SB diet) may induce aggressive behavior through completive antagonism with neuroestrogen on binding ERβ, diminishing the inhibitory influence of ERβ on ERα aggressive stimulatory effect. This hypothesis was based on our findings that consumption of large amount of SB induced aggressive behavior and this is accompanied by decreased neuroestrogen concentration, *ER β* expression, and increased *ER α* expression and immunostaining in the brain. In agreement, previous studies showed that phytoestrogens can reach the brain (Bakker and Baum 2008) and can induce many of the biological responses evoked by endogenous steroidal estrogens when given in higher doses such as stimulation of aggressive behavior in male animals (Michael et al. 1995). Moreover, previous studies revealed that male aggressive behavior was significantly reduced by the lack of ER-alpha gene (Nomura et al. 2002). In contrast, mice lacking ER-beta gene tended to be more aggressive than wild type control mice. These findings can lead to hypothesize that aggression may be facilitated by estrogen via ER-alpha; however, it may inhibit aggression via ER-beta (Liu et al. 2002). The phytoestrogens present in SB can preferentially bind ER β, than ER α although at a lower affinity relative to neuroestrogen, and produce dose- and tissue-dependent estrogenic effects (Toda et al. 2001), such as promotion of aggressive behavior in rats (Patisaul and Jefferson 2010). Our study provides a new explanation that decreasing the neuroestrogen below the optimal levels may also induce higher aggressive behavior and so the effect of neuroestrogen mainly depends on its concentration in brain. Optimal concentration of neuroestrogen maintains aggressive behavior; however sub- or supra-optimal concentrations may induce or inhibit this behavior, respectively. Although, consumption of SB and its phytoestrogens may have some health benefits (Patisaul and Jefferson 2010), our data suggest that consumption of large amount of SB can induce aggression and so careful attention is needed when consume SB to balance benefits and potentially side effects. The ability of phytoestrogens to combine with ER (Liu et al. 2002) and co-expression of ER and Cyp19A1 in the same neurons of thalamus and hypothalamus (Stanić et al. 2014) suggests that phytoestrogens may directly inhibit at least two components of the GAN pathway, which are aromatase and neuroestrogen. However, further in situ hybridization and immunofluorescent staining investigations are required to check whether ER can be detected in GnIH neurons. It is also important to characterize the different cellular mechanisms that mediate the effects of phytoestrogens in the brain, and to check the effect on other estrogen dependent behaviors. Interestingly, the significant negative correlation which was found between both time spent in aggressive behavior and number of attacks and brain aromatase activity, and levels of Cyp19 A1, neuroestrogen, ER-β, RFRP3 and NPFFR ($p < 0.01$) together with the significant positive correlation which was found between behavioral data and brain levels of dopamine, glutamate, testosterone and ER-α confirm the results of the present work.

Conclusion

Our results showed that feeding adult male rats with large amount of soy beans has the ability to induce aggressive behavior in a dose dependant manner possibly through inhibition of GnIH/RFRP-aromatase-neuroestrogen pathway. These effects may be through decreasing aromatase activity, neuroestrogen concentration, Cyp19A1 and ERβ mRNA levels and increasing ERα mRNA levels and immunostaining as well as testosterone level in the brain. These findings also provide further support for the inhibitory role of RFRP3on the aggressive behavior of adult male rats. The data reported herein may open new avenues for the potential harmful effects of consumption large amounts of soya bean rich food on humans.

Compliance with ethical standards

Conflict of interests The authors declare no conflict of interests.

References

Adgent MA, Daniels JL, Edwards LJ, Siega-Riz AM, Rogan WJ (2011) Early-life soy exposure and gender-role play behavior in children. Environ Health Perspect 119:1811–1816

Albert DJ, Dyson EM, Walsh ML, Petrovic DM (1988) Cohabitation with a female activates testosterone-dependent social aggression in male rats independently of changes in serum testosterone concentration. Physiol Behav 44:735–740

Angoa-Perez M, Kuhn DM (2015) Neuroanatomical dichotomy of sexual behaviors in rodents: a special emphasis on brain serotonin. Behav Pharmacol 26: 595–606

Bakker J, Baum MJ (2008) Role for estradiol in female-typical brain and behavioral sexual differentiation. Front Neuroendocrinol 29:1–16

Balthazart JM, Baillien M, Ball GF (2002) Interactions between aromatase (estrogen synthase) and dopamine in the control of male sexual behavior in quail. Comp Biochem Physiol B Biochem Mol Biol 132:37–55

Balthazart J, Baillien M, Charlier TD, Cornil CA, Ball GF (2003) Multiple mechanisms control brain aromatase activity at the genomic and non-genomic level. J Steroid Biochem Mol Biol 86:367–379

Balthazart J, Baillien M, Ball GF (2006) Rapid control of brain aromatase activity by glutamatergic inputs. Endocrinology 147:359–366

Barnes S (2010) The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. Lymphat Res Biol 8:89–98

Chavarro JE, Toth TL, Sadio SM, Hauser R (2008) Soy food and isoflavone intake in relation to semen quality parameters among men from an infertility clinic. Hum Reprod 23:2584–2590

Dominguez JM, Gil M, Hull EM (2006) Preoptic glutamate facilitates male sexual behavior. J Neurosci 26:1699–1703

El-Magd MA, Khamis A, Nasr Eldeen SK, Ibrahim WM, Salama AF (2017) Trehalose enhances the antitumor potential of methotrexate against mice bearing ehrlich ascites carcinoma. Biomed Pharmacother 92:870–878

Guadarrama-Bazante IL, Canseco-Alba A, Rodríguez-Manzo G (2014) Dopamine receptors play distinct roles in sexual behavior expression of rats with a different sexual motivational tone. Behav Pharmacol 25:684–694

Harada NT, Wakatsuki T, Aste N, Yoshimura N, Honda SI (2009) Functional analysis of neurosteroidal oestrogen using gene-disrupted and transgenic mice. J Neuroendocrinol 21:365–369

Hess WR, Akert K (1955) Experimental data on role of hypothalamus in mechanism of emotional behavior. AMA Arch Neurol Psychiatry 3: 127–129

Ibrahim AR, Abul-Hajj YJ (1990) Aromatase inhibition by flavonoids. J Steroid Biochem Mol Biol 37:257–260

Jefferson WN, Padilla-Banks E, Clark G, Newbold RR (2002) Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses. J Chromatogr B Analyt Technol Biomed Life Sci 777:179–189

Johnson MA, Tsutsui K, Fraley GS (2007) Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm behave 51:171–180

Kleitz-Nelson HK, Dominguez JM, Ball GF (2010) Dopamine release in the medial preoptic area is related to hormonal action and sexual motivation. Behav Neurosci 124:773–779

Koolhaas JM, Coppens CM, de Boer SF, Buwalda B, Meerlo P, Timmermans PJ (2013) The resident-intruder paradigm: a standardized test for aggression, violence and social stress. J Vis Exp (77): e4367

Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R (2006) Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. Proc Natl Acad Sci U S A 103:2410–2415

Kriegsfeld LJ, Gibson EM, Williams WP 3rd, Zhao S, Mason AO, Bentley GE, Tsutsui K (2010) The roles of RFamide-related Peptide-3 (RFRP-3) in mammalian reproductive function and behavior. J Neuroendocrinol 22:692–700

Lee H, Kim DW, Remedios R, Anthony TE, Chang A, Madisen L, Zeng H, Anderson DJ (2014) Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. Nature 509:627–632

Lephart ED (1996) A review of brain aromatase cytochrome P450. Brain Res Rev 22:1–26

Lephart ED, Thompson JM, Setchell KD, Adlercreutz H, Weber KS (2000) Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. Brain Res 859:123–131

Lin D, Boyle MP, Dollar P, Lee H, Lein ES, Perona P, Anderson DJ (2011) Functional identification of an aggression locus in the mouse hypothalamus. Nature 470:221–226

Liu Q, Guan XM, Martin WJ, McDonald TP, Clements MK, Jiang Q, Zeng Z, Jacobson M, Williams DL Jr, Yu H, Bomford D, Figueroa D, Mallee J, Wang R, Evans J, Gould R, Austin CP (2001) Identification and charecterization of novel mammalian neuropeptide FF like peptides that attenuate morphine-induced antinociception. J Biol Chem 276:36961–36969

Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH Jr, Pestell RG, Kushner PJ (2002) Opposing action of estrogen receptors a and h on cyclin d1 gene expression. Biol Chem 277: 2453–24360

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1952) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Michael RP, Clancy AN, Zumpe D (1995) Distribution of androgen receptor-like immuno-reactivity in the brains of cynomolgus monkeys. J Neuroendocrinol 7:713–719

Nomura M, Durbak L, Chan J, Smithies O, Gustafsson JA, Korach KS, Pfaff DW, Ogawa S (2002) Genotype/age interactions on aggressive behavior in gonadally intact estrogen receptor beta knockout (betaERKO) male mice. Horm Behav 41:288–296

Patisaul HB, Jefferson W (2010) The pros and cons of phytoestrogens. Front Neuroendocrinol 31:400–419

Rietjens IMCM, Louisse J, Beekmann K (2017) The potential health effects of dietary phytoestrogens. Br J Pharmacol 174:1263–1280

Roselli CE, Ellinwood WE, Resko JA (1984) Regulation of brain aromatase activity in rats. Endocrinology 114:192–200

Simon NG, Kaplan JR, Hu S, Register TC, Adams MR (2004) Increased aggressive behavior and decreased affiliative behavior in adult male monkeys after long-term consumption of diets rich in soy protein and isoflavones. Horm Behav 45:278–284

Smith JT, Clarke IJ (2010) Gonadotropin inhibitory hormone function in mammals. Trends in Endocrinol Metab 21:255–260

Son YL, Ubuka T, Narihiro M, Fukuda Y, Hasunuma I, Yamamoto K, Belsham DD, Tsutsui K (2014) Molecular basis for the activation of gonadotropin-inhibitory hormone gene transcription by corticosterone. Endocrinology 155:1817–1826

Soucy NV, Parkinson HD, Sochaski MA, Borghoff SJ (2006) Kinetics of genistein and its conjugated metabolites in pregnant Sprague Dawley rats following single and repeated genistein administration. Toxicol Sci 90:230–240

Stanić D, Dubois S, Chua HK, Tonge B, Rinehart N, Horne MK, Boon WC (2014) Characterization of aromatase expression in the adult male and female mouse brain. I. Coexistence with oestrogen receptors alpha and beta, and androgen receptors. PLoS One 9:e90451

Thelen P, Wuttke W, Seidlova-Wuttke D (2014) Phytoestrogens selective for the estrogen receptor beta exerts anti-androgenic effects in castration resistant prostate cancer. J Steroid Biochem Mol Biol 139: 290–293

Toda K, Okada T, Takeda K, Akira S, Saibara T, Shiraishi M, Onishi S, Shizuta Y (2001) Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17beta-oestradiol to aromatase gene (cyp19) knockout mice. J Endocrinol 168:455–463

Tsutsui K, Ubuka T, Bentley GE, Kriegsfeld LJ (2012) Gonadotropininhibitory hormone (GnIH): discovery, progress and prospect. Gen Comp Endocrinol 177:305–314

Ubuka T, Tsutsui K (2014) Review: neuroestrogen regulation of socio-sexual behavior of males. Front Neurosci 28:323

Ubuka T, Mizuno T, Fukuda Y, Bentley GE, Wingfield JC, Tsutsui K (2013) RNA interference of gonadotropin-inhibitory hormone gene induces aggressive and sexual behaviors in birds. Gen Comp Endocrinol 181:179–186

Ubuka T, Haraguchi S, Tobari Y, Narihiro M, Ishikawa K, Hayashi T, Harada N, Tsutsui K (2014) Hypothalamic inhibition of socio-sexual behaviour by increasing neuroestrogen synthesis. Nat Commun 5:3061

Ubuka T, Parhar I, Kriegsfeld LJ, Tsutsui K (2018) Editorial: the roles of GnIH in reproductive function and behavior. Front Endocrinol (Lausanne) 9:19

Wang C, Mäkelä T, Hase T, Adlercreutz H, Kurzer MS (1994) Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. J Steroid Biochem Mol Biol 50:205–212

Yang CF, Shah NM (2014) Representing sex in the brain, one module at a time. Neuron 82:261–278

Yang T, Yang CF, Chizari MD, Maheswaranathan N, Burke KJ Jr, Borius M, Inoue S, Chiang MC, Bender KJ, Ganguli S, Shah NM (2017) Social control of hypothalamus-mediated male aggression. Neuron 95:955–970.e4

Angoa-Perez M, Kuhn DM (2015) Neuroanatomical dichotomy of sexual behaviors in rodents: a special emphasis on brain serotonin. Behav Pharmacol 26: 595-606