**Estrogen Replacement Therapy Regulation Of Energy Metabolism In Female Mouse Hypothalamus**

(Article begins on next page)

25 April 2024

Fontana R. , Della Torre S., Meda C., Longo A., Eva C., Maggi, A.

ESTROGEN REPLACEMENT THERAPY REGULATION OFENERGY
METABOLISM IN FEMALE MOUSEHYPOTHALAMUS
ENDOCRINOLOGY (2014)
DOI: 10.1210/en.2013-1731

# Estrogen Replacement Therapy Regulation Of Energy Metabolism In Female Mouse Hypothalamus

Roberta Fontana, Sara Della Torre, Clara Meda, Angela Longo, Carola Eva, and Adriana C. Maggi

Center of Excellence on Neurodegenerative Diseases and Department of Pharmacological and Biomolecular Sciences, Via Balzaretti 9, 20133 Milan, Italy,

## Abstract

Estrogens play an important role in the regulation of energy homeostasis in female mammals and a reduced ovarian function, due to natural aging or surgery, is associated with body weight increase and fat redistribution. This disruption of energy homeostasis may constitute a trigger for several pathologies known to be associated with climacterium; however, so far, limited attention has been devoted to the ability of estrogen replacement therapies (ERT) to reinstate the balanced energy metabolism characteristic of cycling female mammals. The purpose of the present study was to compare the efficacy of selected ERTs in reversing the ovariectomy-induced gain in body weight. To this aim female ERE-Luc mice were ovariectomized and, after 3 weeks, treated per os for 21 days with: conjugated estrogens, two selective estrogen receptor modulators (bazedoxifene and raloxifene), and the combination of bazedoxifene plus conjugated estrogens (tissue-selective estrogen complex, TSEC). The study shows that the therapy based on TSEC was the most efficacious in reducing the body weight accrued by ovariectomy (OVX). In addition, by means of in vivo imaging, the TSEC treatment was shown to increase estrogen receptor (ER) transcriptional activity selectively in the arcuate nucleus, which is a key area for the control of energy homeostasis. Finally, quantitative analysis of the mRNAs encoding orexigenic and anorexigenic peptides indicated that following ERT with TSEC there was a significant change in Agrp, NPY, and Kiss-1 mRNA accumulation in the whole hypothalamus. Considering that prior studies showed that ERT with TSEC was able to mimic the rhythm of ER oscillatory activity during the reproductive cycle and that such fluctuations were relevant for energy metabolism, the present observations further point to the ER tetradian oscillation as an important component of the ER signaling necessary for the full hormone action and therefore for an efficacious ERT.

Obesity and overweight have nearly doubled since 1980 (1) and have recently been demonstrated as an important risk factor for a number of metabolic, cardiovascular, and skeletal disorders (2). Epidemiological evidence shows that the occurrence of obesity is higher in women (in 2009–2010 the percentage of obese US women was 11% higher than men) and aging is a predisposing factor as the frequency of overweight in women is increased more than 30% after 60 years of age (3).

It is now well accepted and demonstrated that in women with reproductive dysfunctions, such as Turner syndrome and polycystic ovary syndrome, or after menopause, there is a gain in body weight and a redistribution of fat to the visceral area (4–8); these changes are associated with an increased risk of chronic health problems such as insulin-resistance, hyperlipidemia, hypertension, and low-grade inflammation (9–11). The mechanisms potentially involved in these dysmetabolic diseases may be multiple, but sex hormones play a significant role (12). Indeed, we now know that estrogens are important modulators of lipid and glucose metabolism both peripherally (13–15) and centrally, where these sex hormones control several mechanisms that preside food intake and fat distribution in the hypothalamus (16–18). Experimentally, it is well established that estrogen replacement therapy (ERT) reverts the rapid increase in body weight induced by ovariectomy (OVX) (19). In women the effects of hormone replacement

therapy on energy metabolism have not been a primary object of study, however several investigations have shown that ERT counteracts body weight gain, preserves lean mass, and prevents the shift of fat deposition from the gluteo-femoral area to the abdomen (20–22). These effects appear to differ depending on the nature (23) and on the route of hormone administration (23–25). However, a clear understanding of the ERT regimen necessary to restore energy homeostasis and the mechanism underlying such an effect is still lacking.

In previous studies based on in vivo imaging we investigated the dynamics of estrogen receptor (ER) transcriptional activity in selected body areas of mice prior or after OVX (26) and the receptor transcriptional response to long term administration of the following ERTs: conjugated estrogens (CE), bazedoxifene (BZA), conjugated estrogens plus bazedoxifene (tissue-selective estrogen complex, TSEC), and raloxifene (RAL) (26, 27). These studies showed that ER activity is differentially regulated by the chemical nature and the concentration of the estrogenic compound administered (26, 27), and demonstrated the ability of the TSEC treatment to reproduce the natural oscillation of ER in several organs (26, 28). Most relevant, TSEC was also shown to be the most efficacious in preventing the formation of liver lipid deposits after OVX (14). This initial observation leads us to hypothesize that this compound could be effective on the regulation of energy metabolism not only in the peripheral organs, but also centrally.

The purpose of our investigation was to compare the efficiency of selected ERTs in reverting the body weight gain induced by OVX in female mice. The ERT treatments, carried out for 21 days, showed that TSEC was the most efficacious in reducing the body weight accrued by OVX. Furthermore, we tested the effectiveness of the ERTs on the regulation of the hypothalamic neuropeptides involved in the control of energy metabolism. We report that in the group of animals treated with TSEC we observed a significant increase of estrogen receptor transcriptional activity in the arcuate nucleus and changes in the content of the mRNA encoding Agrp, NPY, and Kiss-1. Therefore, this study further indicates that a treatment able to reinstate the oscillatory activity of ER is the most efficacious in overcoming the metabolic alterations induced by the lack of ovarian functions possibly because it involves an activity at hypothalamic level. Furthermore, the study underlines the ability of ERT to normalize metabolic functions that may represent a primary event for the onset of most of the pathologies associated with the climacterium and the importance of evaluating metabolic parameters as an end point for a successful ERT.

## Materials and Methods

### Experimental animals

We used female C57BL/6 heterozygous repTOPTMERE-Luc mice of 2–3 months of age (weight range: 20.4 ± .4 g). In this animal model the reporter gene, firefly luciferase, is expressed under the transcriptional control of the ERE sequence: the construct presents two palindromic EREs spaced 8 bp located at 55 bp from the constitutive thymidine kinase promoter; and the presence of two insulator sequences guarantees the ubiquitous expression of the transgene (29). Animals were housed 3 to 4 per cage under 12 hours light-dark cycle and maintained at a temperature of 22–25 C. Mice were fed ad libitum with standard diet (4RF21, Mucedola, Italy) and had free access to water. Three weeks before the beginning of the treatments mice were ovariectomized under ketamine/xylazine anesthesia and then shifted to an estrogen-free diet (AIN93M, Mucedola, Italy). At day 0 the average body weight of the OVX animals was 23.8 ± .2 g; at this time point mice were divided in the different group to ensure that the body weight was comparable among groups.

All animal experimentations were carried out in accordance with the European guidelines for animal care and use of experimental animals, approved by the Italian Ministry of Research and University, and controlled by the panel of experts of the Department of Pharmacological and Biomolecular Sciences, University of Milan.

## Pharmacological treatments

Conjugated estrogens and bazedoxifene were provided by Pfizer (USA); raloxifene was from Sigma-Aldrich (Italy). The compounds were dissolved in dimethyl sulfoxide and diluted in vehicle solution (2% Tween 80 and .5% carboxymethylcellulose water solution). Treatments were administered daily (between 0930 hours and 1030 hours) by gavage at the dose of 10 mg/kg for BZA and RAL, 3 mg/kg for CE, 10 mg/kg BZA and 3 mg/kg CE for TSEC treatment in a volume of about 0.1 ml. The control groups received 0.1 ml of vehicle solution. Compounds were administered at doses equivalent to those used in humans as calculated by the allometric approach, and further harmonized with the companies that developed the drugs (26).

## Bioluminescence-based imaging

Bioluminescence in body areas and in brain slices were measured in female heterozygous repTOPTMERE-Luc mice as described in the Supplemental Materials and Methods (30, 31). Cycling (CYC) animals were analyzed at metestrus. All bioluminescence-based studies were carried out in the early afternoon after 6 hours fasting to avoid confounding effects due to liver estrogen receptor activation associated with protein intake (28).

## Body weight, food intake, and spontaneous locomotor activity

Animals were weighed every 3 days during the experiment while food intake was measured daily. Spontaneous locomotor activity was evaluated in mice after 7, 14, and 21 days from the beginning of the treatment. Animals were single-housed in an isolated dark room with infrared lights and their locomotor activity was recorded for 3 hours during the dark phase (from 2100 hours to 2400 hours). Data were analyzed using Ethovision XT video track system (Noldus Information Technology, The Netherlands).

## Gene expression

Animals were euthanized after 21 days of treatment between 1500 hours and 1700 hours after 6 hours of fasting. We decided to kill mice in a very short period of time (just 2 hours) to avoid a possible confounding element dependent upon circadian rhythm; indeed it was demonstrated that food intake and many other metabolic parameters are highly dependent upon circadian regulation (32). After euthanasia, the hypothalami were rapidly collected, snap frozen on dry ice and stored at −80 C. Total hypothalamic RNA was extracted with RNeasy Mini Kit (Qiagen, Germany) and reversed transcribed to cDNA. Real-time PCR experiments were performed with TaqMan technology and TaqMan gene expression assay: Agrp (Mm00475829_g1), Npy (Mm03048253_m1), Pomc (Mm00435874_m1), Cart (Mm04210469_m1), Kiss-1 (Mm03058560_m1), and the reference gene assay 18S rRNA VIC-MGB-PDAR (Applied Biosystems). The reactions were carried out according to the manufacturer's protocol using 7900HT fast real-time PCR system (Applied Biosystems). The data were analyzed using the Sequence Detection System Software version 2.3 (Applied Biosystems) and the 2-ΔΔCt method (33).

## Statistical analysis

Statistics were carried out with the GraphPad Prism version 5.02 for Windows by 1-way or 2-way ANOVA followed by Bonferroni post hoc test or unpaired t test.

The coefficient of variation % (CV%), also known as relative variability, equals the standard deviation divided by the mean and expressed as a percentage, has been calculated by using the GraphPad Prism software.

## Results

In 3-month old C57BL/6 ERE-Luc mice, 3-week OVX induced a significant increase in body weight with respect to the average weight of intact, CYC animals (from 20.83 to 23.86 g, +12%) (Figure 1A). Figure 1B shows that all ERTs induced a trend of progressive diminution of body weight; however, only TSEC was able to significantly decrease mice body weight by day 14 and, at day 21, the average body weight of TSEC-treated animals was indistinguishable from the CYC controls. The other ERTs were not so efficacious possibly due to the high variability in mice response (Supplemental Figure 2). It was of interest to note that the oral administration of the vehicle induced a trend toward a decrease in body weight in OVX animals, but not in CYC animals. This phenomenon was ascribed to the stress of manipulation; clearly CYC mice were less sensitive to such a stress as also indicated by the fact that in all experimental groups, but not in CYC, we noticed a trend to a decrease in body weight at the beginning of the treatment. As mentioned, the experimental setting did not cause any significant change in the body weight of CYC mice which, as expected, was observed to slightly oscillate due to the fact that food intake is regulated by the different phases of the estrous cycle (34).

Next, we measured food intake: the average amount of chow eaten daily by the animals of the different experimental groups during the entire length of the experiment showed that OVX mice ate 13% more than CYC. All ERTs tested significantly reduced food intake, yet each hormonal treatment had a different
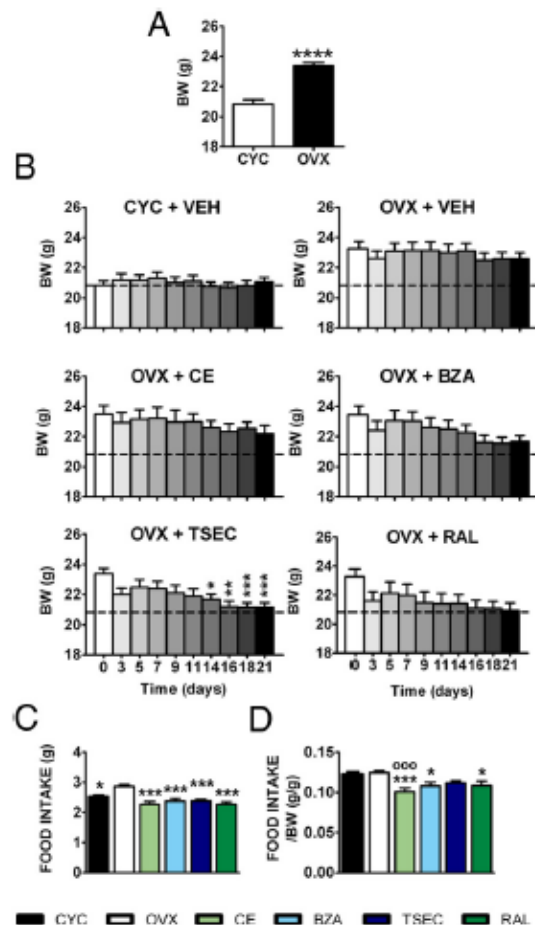


**Figure 1.** Effect of ovariectomy and ERT on body weight. A, Body weight of cycling (CYC, n = 30) and 3 weeks ovariectomized female mice (OVX, n = 120). ****, $P < .0001$ compared with CYC. P values were calculated with unpaired t test. B, After the beginning of the therapy, the body weight of cycling vehicle-treated mice (CYC+VEH) and ovariectomized mice treated with vehicle (OVX+VEH) or with conjugated estrogens (OVX+CE) or with bazedoxifene (OVX+BZA) or with tissue-selective estrogen complex (OVX+TSEC) or with raloxifene (OVX+RAL) was measured between 0900 hours and 1000 hours every three days. Data represent mean ± SEM of 20–30 animals/group. *, $P < .05$; **, $P < .01$; ***, $P < .001$ compared with time 0. P values were calculated with 1-way ANOVA followed by Bonferroni post hoc test. C, Cumulative food consumption of each group of animals in the 21 days of treatment. Data represent mean ± SEM of 20–30 animals/group. *, $P < .05$ and ***, $P < .001$ compared with OVX. P values were calculated with 1-way ANOVA followed by Bonferroni post hoc test. D, Food intake was normalized on mice body weight and plotted as food intake/BW (g/g). Data represent mean ± SEM of 20–30 animals/group. *, $P < .05$ and ***, $P < .001$ compared with OVX; ooo, $P < .001$ compared with CYC. P values were calculated with 1-way ANOVA followed by Bonferroni post hoc test.

4

effect (CE- and RAL-treated mice ate 21% less than vehicle-treated; BZA and TSEC 17% less than vehicle-treated) (Figure 1C). When we calculated the daily food intake relative to the body weight of each animal there was a further indication of a differential effect of the ERTs on the control of food intake (Figure 1D). In particular, only for TSEC we observed a strict correlation between body weight and food intake, but this was not the case for the CE, BZA, and RAL treated groups.
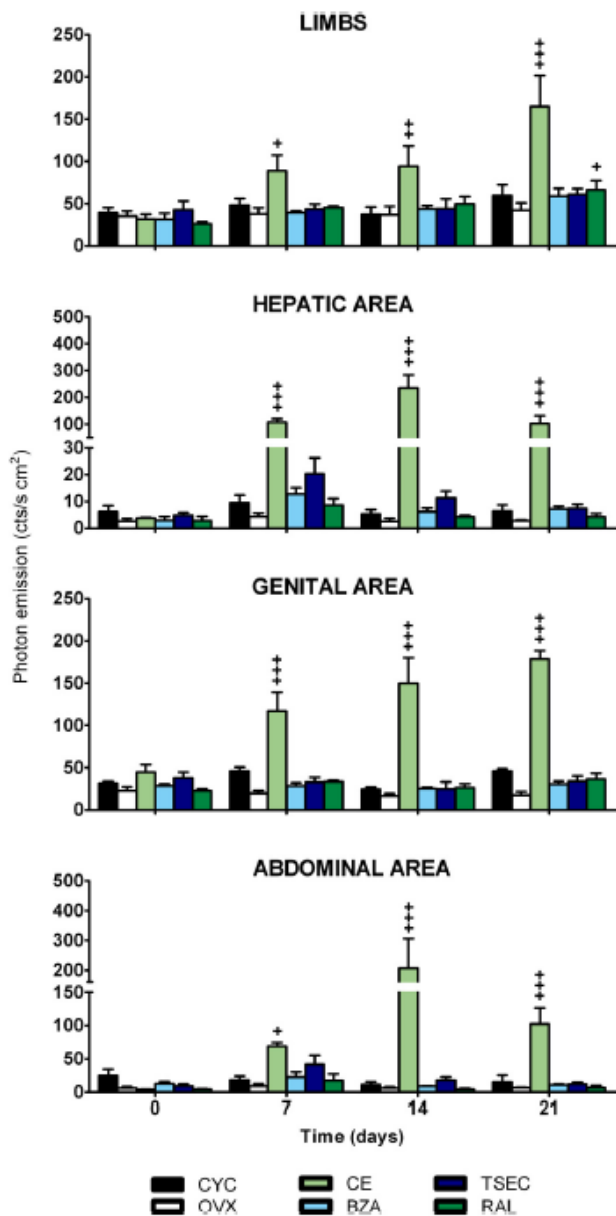
When we analyzed the feeding behavior of the 6 groups in each experimental day (Figure 2A), we found that food intake fluctuated in time in CYC animals; this was expected because of the known effects of the changes of circulating hormones during the estrous cycle (34), additionally the food intake curve was flatter in OVX mice, further underlying the effects of circulating estrogens. Interestingly, ERT with CE, BZA, RAL, but not TSEC, induced a strong variability in food intake with time establishing an oscillatory pattern more robust than in CYC mice as also indicated by the analysis of the CV% of the daily food intake in the 21-day period (Figure 2B): CE and RAL showed the highest variability of food intake while the CV% relative to TSEC was comparable to CYC mice, indicating once again the differential effect of TSEC from the other compounds administered.
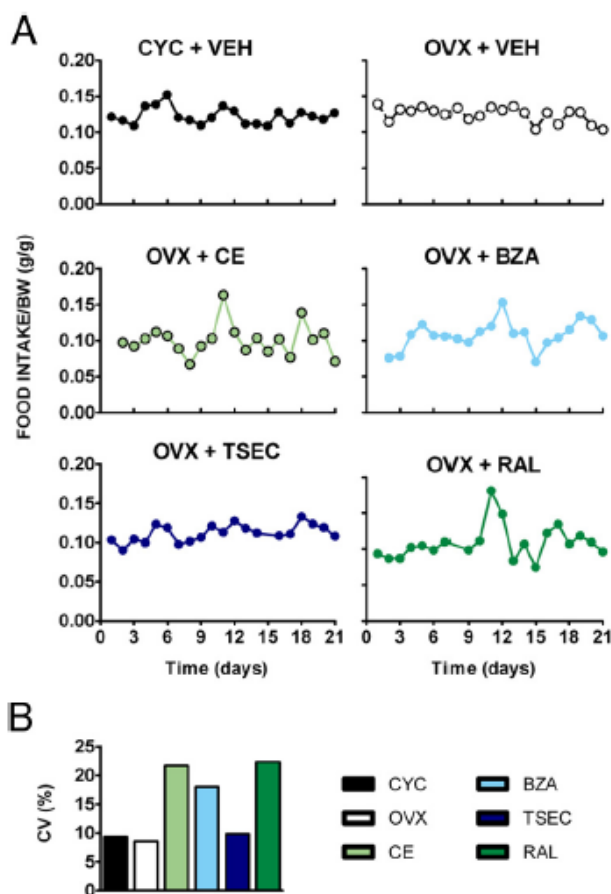


**Figure 2.** Daily food intake in animals subjected to ERT. A, Animals were caged in groups of 3/4 mice/cage. The amount of food used up was measured daily at 0930 hours, normalized on mice body weight and plotted as a profile of daily food intake/BW (g/g). B, The coefficient of variation % (CV%), also known as relative variability, equals the standard deviation divided by the mean and expressed as percentage. The CV% was calculated by using the GraphPad Prism software.

To investigate the overall estrogenic effect of the treatments carried out, we measured the accumulation of the estrogen surrogate reporter luciferase in the limbs and in the genital, hepatic, and abdominal areas in the ERE-Luc mice. In all the areas taken into consideration, conjugated estrogens (CE) was able to induce the accumulation of the reporter enzyme; such an accumulation increased quite significantly with time in the genital area (mainly vagina) and in bones (limbs), and appeared to be maximal at 14 days in the hepatic and abdominal areas. In contrast, none of the other compounds used for the ERT had effects in the genital area. RAL increased ER activity in limbs where a trend to an increase was observed also with BZA and TSEC. RAL and BZA did not show effects in the hepatic and abdominal areas in which TSEC showed a trend toward an increase at the seventh day, an effect that disappeared at day 14 and 21. The amount of bioluminescence elicited by the treatments was in line with our previous observations (27); in
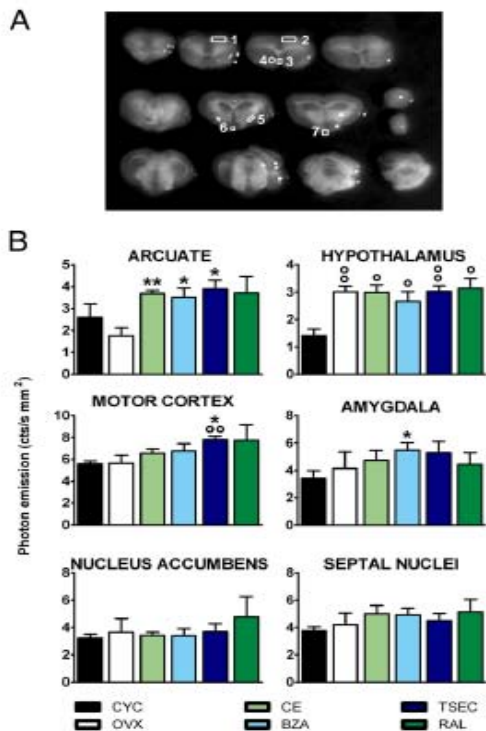
particular, the low luciferase activity in the genital area confirmed the lack of any ER agonist effect of these selective estrogen receptor modulators (SERMs) on reproductive tissues demonstrated also by the uterus weight (data not shown, 27). Additionally, the observation that CE was more effective on luciferase induction than TSEC demonstrated that the final effects of the combination of the two compounds (CE and BZA) do not simply derive from the mere sum of the activity of the two, but it produces novel responses due to the blend of their respective actions, which might include the BZA antagonist action (Figure 3).

Further analysis of the estrogenic activity of the ERTs in brain areas (Figure 4) provides evidence of their ability to penetrate the blood brain barrier. In the arcuate nucleus we observed a trend to a decrease of luciferase activity in OVX mice: this effect was reversed by all treatments with the exception of RAL possibly, once more, due to high variability in this experimental group. Interestingly, when we considered the entire hypothalamus, luciferase activity was increased by OVX and none of the treatments reversed such an effect. TSEC was the only treatment able to change significantly ER activity after 21 days of treatment in motor cortex (+ 39%). No significant changes in bioluminescence were observed in the other brain nuclei with the exception of the group treated with BZA in which increased luciferase activity was measured in the amygdala (+ 60%).



Figure 3. Whole body bioluminescence-based measurement of luciferase activity in ERE-Luc reporter mice. Photon emission measured as counts/sec · cm² (cts/s · cm²) from specific body areas at 0, 7, 14 and 21 days of chronic oral treatment with VEH, CE, BZA, TSEC and RAL. Body areas evaluated were: hepatic area, abdominal area, reproductive area (mainly vagina) and limbs. Data represent mean ± SEM of 10–14 animals/group. +, $P < .05$; ++, $P < .01$; +++, $P < .001$ compared with time 0. P values were calculated with 2-way ANOVA followed by Bonferroni post hoc test.

6

**Figure 4.** Photon emission of selected brain areas of cycling and ovariectomized ERT-treated ERE-Luc mice. A, Brains were sliced for semiquantitative analysis of photon emission in specific brain areas. B, Photon emission measured as counts/sec · mm² (cts/s · mm²) after 21 days of chronic oral treatment with VEH, CE, BZA, TSEC and RAL from specific brain areas: 1,2 motor cortex; 3 septal nuclei; 4 nucleus accumbens; 5 amygdala; 6 arcuate; and 7 hypothalamus. Data represent mean ± SEM of 10–14 animals/group. *, $P < .05$ and **, $P < .01$ compared with OVX; $^\circ$, $P < .05$ and $^{\circ\circ}$, $P < .01$ compared with CYC. P values were calculated with unpaired t test.
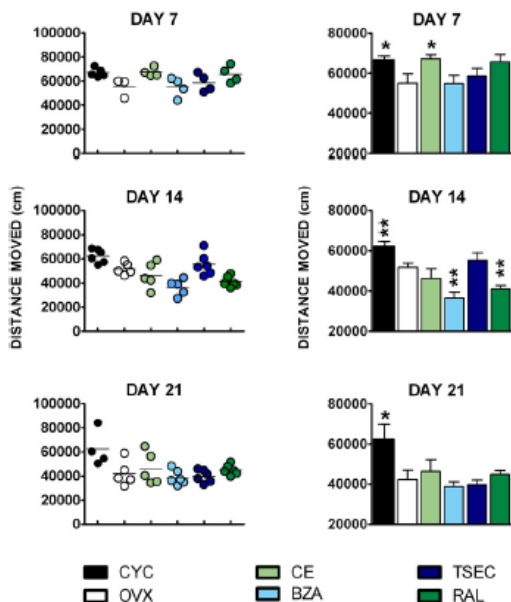


**Figure 5.** Effect of OVX and ERT on locomotor activity. Spontaneous locomotor activity was evaluated in mice after 7, 14, and 21 days from the beginning of the treatment as described in the methodology section. Data represent mean ± SEM of 4–6 animals. *, $P < .05$ and **, $P < .01$ compared with OVX. P values were calculated with unpaired t test.

TSEC effect in the motor cortex led us to hypothesize that the efficacy of this treatment in inducing weight loss could be owed to higher motility. Therefore, we compared the spontaneous locomotor activity in all the experimental groups. The tests, carried out at 7, 14, and 21 days of treatment, showed that most of the treatments appear to be unable to overcome the decrease in locomotor activity consequent to OVX suggesting that the effect of TSEC on ER activity present in the motor cortex had no significant consequence for mice spontaneous motility (Figure 5).

It is now well established that the different nuclei of the hypothalamus estrogenic compounds regulate the expression of genes encoding orexigenic and anorexigenic peptides mainly through ERα (35); in the whole hypothalamus no significant changes were observed in the levels of mRNA encoding for this gene (Supplemental Figure 3) despite the increased luciferase activity (Figure 4). Quantitative analysis of the respective mRNAs after 21 days of treatment showed that, in the hypothalamus, the orexigenic Agrp mRNA was significantly higher in the TSEC group compared with controls (+151%); the amount of hypothalamic Agrp mRNA found in the TSEC-treated mice was comparable to the amount of mRNA found in the physiological range of CYC mice. A similar effect was observed also when we measured Npy mRNA: a slight, significant increase was observed in the TSEC group. Npy mRNA was also found to be elevated in the animals treated with BZA, while no effects were observed with the other treatments (Figure 6). These results were unanticipated because of the efficacy of this ERT in reducing the OVX-induced body weight gain, and led us to also measure the mRNAs encoding anorexigenic peptides such as proopiomelanocortin (Pomc), cocaine- and amphetamine-regulated transcript (Cart), and kisspeptin (Kiss-1), in spite of the fact that their respective mRNA did not appear to be

7

particularly affected by the estrous cycle when measured in the whole hypothalamus. Our study supported previous observations showing that mRNAs encoding anorexigenic peptides do not fluctuate significantly during the reproductive cycle (35), however the content of Kiss-1 mRNA was significantly increased with respect to all phases of the cycle in the BZA (+ 263% compared with proestrus) and even more in the TSEC (+ 306% compared with proestrus) group (Figure 6).
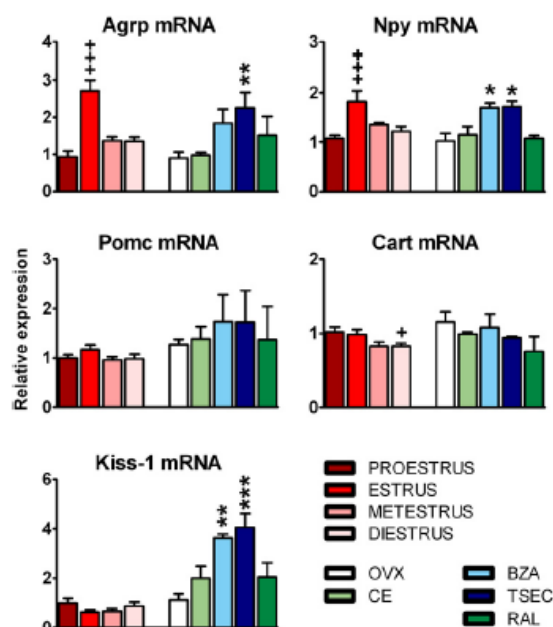


**Figure 6.** Effect of the estrous cycle, OVX and ERT on the accumulation of mRNAs encoding orexigenic and anorexigenic peptides in the hypothalamus of female mice. All animals were euthanized between 1500 hours and 1700 hours. For cycling animals the phase of the estrous cycle was analyzed on the basis of vaginal smears carried out between 0900 hours and 1000 hours. Real-time PCR was carried out as described in the methodology section. Data represent mean ± SEM of 10−14 animals/group. $^{+}$, $P < .05$ and $^{+++}$, $P < .001$ compared with proestrus; $^{*}$, $P < .05$; $^{**}$, $P < .01$, and $^{***}$, $P < .001$ compared with OVX. $P$ values were calculated with 1-way ANOVA followed by Bonferroni post hoc test.

## Discussion

In mammals, a large body of evidence showed that the cessation of ovarian functions is associated with a change in body weight and a fat distribution characterized by an increased waist to hip ratio indicative of visceral adiposity (4). This has been observed also in humans where this phenomenon has relevant repercussions for health because increased visceral adiposity, independent of body mass index, is associated with hypertension, glucose intolerance, and increased risk for cardiovascular disease in women (9–11, 36).

The present study was undertaken to assess the efficacy of selected hormone replacement therapies in overcoming the weight gain induced by surgical menopause in female mice. The results presented here provide evidence that with all compounds there was a trend toward a decrease in weight; however, the combination of CE and BZA was the most efficacious as the weight reduction reached statistical significance

by day 14 and at the end of the treatment the average body weight of TSEC treated mice was comparable with the intact controls.

It is well established that in the central nervous system (CNS) estrogenic compounds act at the hypothalamic level to control food intake, energy expenditure, body fat distribution, and the reproductive axis. These effects are mainly mediated by a modulation of ERα activity (37, 38). More recently, genetic studies based on the Cre-Lox approach have established that estrogens regulate food intake mainly by directly targeting the POMC neuronal population located in the arcuate nucleus while their effect on body fat distribution is mainly associated with their action on the steroidogenic factor 1 (SF-1) neurons in the ventromedial hypothalamus (VMH) (16). Both CART/POMC and SF-1 neurons signal to second order neurons in different hypothalamic nuclei to integrate the estrogenic effect with other signaling from peripheral organs and to communicate the energetic status to the gonadotropin-releasing hormone neurons responsible for the release of gonadotropins from the pituitary, therefore controlling reproduction (39–41). More recently, the ablation of neurons expressing kisspeptin was found to be associated with changes in body weight and visceral fat distribution indicating that also these neurons may participate to the overall, central, control of energy metabolism (42). It is important to underline that estrogens may act also in the periphery to modulate the metabolism of organs involved in energy metabolism like the liver (14), pancreas (13, 15), and muscle (43).

By using the ERE-Luc reporter mouse, the present study shows that all the treatments tested were able to modulate ERs in the CNS; indeed, for the first time, we show that synthetic estrogens or combination of estrogenic compounds may exert a dissimilar effect on the ER transcriptional activity in the brain. None of the treatments affected ER activation consequent to OVX in the hypothalamus, whereas, in the arcuate nucleus, only CE, BZA, and TSEC increase ER activity. Furthermore, BZA and TSEC were the only treatments able to modulate ER transcriptional activity in the amygdala and motor cortex, respectively. These results were unanticipated and more detailed studies need to be carried out to understand the extent to which the differential responses observed are the consequence of the pharmacokinetic characteristics of each compound or, more likely, are due to a differential brain distribution of the coregulators able to interact with ERs in the spatial conformation induced by each ligand. Our findings are therefore of potential therapeutic relevance because they suggest that each ERT may result in the activation of ERs in a diversified neuronal population thus producing a variety of effects. The ERE-Luc model presents the limit of focusing only on the transcriptional effects of the ER activation, but could not report the rapid, nongenomic effects of estrogen and SERMs (44). In particular, in recent years, it has been described that membrane ER (mER) could modulate energy metabolism possibly by participating in the regulation of the central mechanism important for the control of food intake (45). This was demonstrated pharmacologically in OVX rats and guinea pigs by the use of selective ligands for membrane ER (46, 47) and in mouse models of mER knock in (48); thus, we could not rule out the contribution of mER in the effects observed.

We found of interest the lack of a strict correlation between food intake and weight loss: the anorexigenic response was the highest in the CE group where the change in body weight was the least significant; and on the other hand in the TSEC group we observed a substantial reduction of body weight despite minimal changes in eating habits. The biochemical analysis of the mRNAs encoding hypothalamic orexigenic and anorexigenic peptides may provide an explanation for our observations showing that prolonged administration of TSEC had a significant effect on the expression of several genes involved in the control of energy metabolism. The increased accumulation of mRNAs encoding orexigenic peptides remains to be explained, while the effect on the kisspeptin gene expression is very remarkable for a number of reasons: 1) the hypothalamic content of Kiss-1 mRNA does not appear to be influenced during the estrous cycle; 2) the

effect of the treatment was very remarkable (+ 265% increase vs. OVX); and 3) kisspeptin neurons were reported to play a significant role in the control of visceral fat accumulation (42). Thus the major effect of TSEC on the expression of the Kiss-1 gene may explain the efficacy of the therapy in decreasing the body weight after OVX. The identification of Kiss-1 as a primary target of an estrogenic treatment able to normalize energy balance after menopause is very intriguing considering that the peptide encoded by this gene was reported to be modulated whenever metabolic shifts are induced by a change in the reproductive stage. In fact the regulation of Kiss-1 gene expression was reported to be critical for the preovulatory surge of gonadotropins at puberty (49–51), in late pregnancy, and during lactation (52), all reproductive stages associated with major changes in energy metabolism. Thus, this peptide may represent the central switch for changes in energy metabolism associated with changes in women reproductive stages and, therefore, a very relevant target to avoid the undesired effects associated with alterations of ovarian functions (12).

The observation of a fluctuation of Agrp and Npy, but not Pomc mRNAs during the estrous cycle here reported is in agreement with Olofsson et al (35). To clarify, it is the lack of a significant effect of the ERTs with CE and RAL on the contents of mRNAs encoding peptides centrally involved in the regulation of appetite and energy availability. Indeed the mRNA quantitative analysis carried out in the entire hypothalamus did not allow identifying changes, which might have occurred in discrete neuronal populations. Further investigation based on immunohistochemistry will provide a more detailed knowledge.

Previous reports from our group showed that ERT carried out by continuous administration of estrogenic compounds may determine a fluctuation of ER activity mimicking what occurs during the estrous cycle (26, 27). This oscillatory activity of ERs appears to be as functionally relevant in the control of energy homeostasis in the liver as TSEC treatment, that showed to faithfully reproduce the tetradian oscillation of liver ERα transcriptional activity, was the most efficacious in the protection from steatosis induced by OVX (14). The finding here described on the efficacy of TSEC in reducing OVX-induced weight gain, provide a further indication that the reestablishment of ER physiological, oscillatory activity is associated with health benefits.

Technical limitations prevent the measurement of brain ER activity in living mice, yet it was of interest to observe that animals treated with TSEC showed a fluctuation of food intake in time that more closely reproduced what was observed in the intact, CYC mice. This was not the case with the other therapies that resulted in abnormal oscillation in the daily food intake (Figure 2).

In conclusion, this study shows that the combination therapy based on the continuous administration of CE and the SERM BZA is able to rapidly reduce the weight gain induced by surgical menopause possibly through the modulation of the activity of kisspeptin hypothalamic neurons, thus underlying the relevance of kisspeptin in the control of body weight. Because kisspeptin has a significant effect on the synthesis of hypothalamic hormones, it is conceivable that the effect of this peptide is due to a combination of responses at the central as well as peripheral level, which remains to be further elucidated.

*Abbreviations:*

BZA     bazedoxifene
CNS     central nervous system
CV%     coefficient of variation percentage
CYC     cycling
ERT     estrogen replacement therapy

ER      estrogen receptor
mER     membrane estrogen receptor
OVX     ovariectomy
RAL     raloxifene
SERMs   selective estrogen receptor modulators
SF-1    steroidogenic factor 1
TSEC    tissue-selective estrogen complex.

## Acknowledgments

## References

1.  WHO: obesity (online). Available at: http://www.who.int/mediacentre/factsheets/fs311/en/. 2013.
2.  Haslam DW, James WP. Obesity. Lancet. 2005;366(9492):1197–1209.
3.  Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity in the United States, 2009–2010. NCHS Data Brief. 2012(82):1–8.
4.  Rogers NH, Perfield JW 2nd, Strissel KJ, Obin MS, Greenberg AS. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology. 2009;150(5):2161–2168.
5.  Ainslie DA, Morris MJ, Wittert G, Turnbull H, Proietto J, Thorburn AW. Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. Int J Obes Relat Metab Disord. 2001;25(11):1680–1688.
6.  Lovejoy JC, Champagne CM, de Jonge L, Xie H, Smith SR. Increased visceral fat and decreased energy expenditure during the menopausal transition. Int J Obes (Lond). 2008;32(6):949–958.
7.  Essah PA, Nestler JE. The metabolic syndrome in polycystic ovary syndrome. J Endocrinol Invest. 2006;29(3):270–280.
8.  Gravholt CH. Epidemiological, endocrine and metabolic features in Turner syndrome. Eur J Endocrinol. 2004;151(6):657–687.
9.  Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev. 2000;21(6):697–738.
10. Phillips GB, Jing T, Heymsfield SB. Does insulin resistance, visceral adiposity, or a sex hormone alteration underlie the metabolic syndrome? Studies in women. Metabolism. 2008;57(6):838–844.
11. Krotkiewski M, Bjorntorp P, Sjostrom L, Smith U. Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J Clin Invest. 1983;72(3):1150–1162.
12. Della Torre S, Benedusi V, Fontana R, Maggi A. Energy metabolism and fertility–a balance preserved for female health. Nat Rev Endocrinol. 2014;10(1):13–23.
13. Alonso-Magdalena P, Ropero AB, Carrera MP, et al. Pancreatic insulin content regulation by the estrogen receptor ER alpha. PLoS One. 2008;3(4):e2069.

14. Villa A, Della Torre S, Stell A, Cook J, Brown M, Maggi A. Tetradian oscillation of estrogen receptor alpha is necessary to prevent liver lipid deposition. Proc Natl Acad Sci USA. 2012;109(29):11806–11811.

15. Tiano JP, Delghingaro-Augusto V, Le May C, et al. Estrogen receptor activation reduces lipid synthesis in pancreatic islets and prevents beta cell failure in rodent models of type 2 diabetes. J Clin Invest. 2011;121(8):3331–3342.

16. Xu Y, Nedungadi TP, Zhu L, et al. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. Cell Metab. 2011;14(4):453–465. CrossRef, Medline

17. Santollo J, Torregrossa AM, Eckel LA. Estradiol acts in the medial preoptic area, arcuate nucleus, and dorsal raphe nucleus to reduce food intake in ovariectomized rats. Horm Behav. 2011;60(1):86–93.

18. Roepke TA. Oestrogen modulates hypothalamic control of energy homeostasis through multiple mechanisms. J Neuroendocrinol. 2009;21(2):141–150.

19. Bryzgalova G, Lundholm L, Portwood N, et al. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. Am J Physiol Endocrinol Metab. 2008;295(4):E904–E912.

20. Gambacciani M, Ciaponi M, Cappagli B, et al. Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women. J Clin Endocrinol Metab. 1997;82(2):414–417.

21. Genazzani AR, Gambacciani M. Effect of climacteric transition and hormone replacement therapy on body weight and body fat distribution. Gynecol Endocrinol. 2006;22(3):145–150.

22. Sites CK, Brochu M, Tchernof A, Poehlman ET. Relationship between hormone replacement therapy use with body fat distribution and insulin sensitivity in obese postmenopausal women. Metabolism. 2001;50(7):835–840.

23. Godsland IF. Effects of postmenopausal hormone replacement therapy on lipid, lipoprotein, and apolipoprotein (a) concentrations: analysis of studies published from 1974–2000. Fertil Steril. 2001;75(5):898–915.

24. O'Sullivan AJ, Crampton LJ, Freund J, Ho KK. The route of estrogen replacement therapy confers divergent effects on substrate oxidation and body composition in postmenopausal women. J Clin Invest. 1998;102(5):1035–1040.

25. dos Reis CM, de Melo NR, Meirelles ES, Vezozzo DP, Halpern A. Body composition, visceral fat distribution and fat oxidation in postmenopausal women using oral or transdermal oestrogen. Maturitas. 2003;46(1):59–68.

26. Rando G, Horner D, Biserni A, Ramachandran B, Caruso D, Ciana P, Komm B, Maggi A. An innovative method to classify SERMs based on the dynamics of estrogen receptor transcriptional activity in living animals. Mol Endocrinol. 2010;24(4):735–744.

27. Della Torre S, Biserni A, Rando G, Monteleone G, Ciana P, Komm B, Maggi A. The conundrum of estrogen receptor oscillatory activity in the search for an appropriate hormone replacement therapy. Endocrinology. 2011;152(6):2256–2265.

28. Della Torre S, Rando G, Meda C, Stell A, et al. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. Cell Metab. 2011;13(2):205–214.

29. Ciana P, Di Luccio G, Belcredito S, et al. Engineering of a mouse for the in vivo profiling of estrogen receptor activity. Mol Endocrinol. 2001;15(7):1104–1113.

30. Stell A, Belcredito S, Ciana P, Maggi A. Molecular imaging provides novel insights on estrogen receptor activity in mouse brain. Mol Imaging. 2008;7(6):283–292.

31. Maggi A, Rando G. Reporter mice for the study of intracellular receptor activity. Methods Mol Biol. 2009;590:307–316.

32. Tahara Y, Shibata S. Chronobiology and nutrition. Neuroscience. 2013;253:78–88.

33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–408.

34. Asarian L, Geary N. Modulation of appetite by gonadal steroid hormones. Philos Trans R Soc Lond B Biol Sci. 2006;361(1471):1251–1263.

35. Olofsson LE, Pierce AA, Xu AW. Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. Proc Natl Acad Sci USA. 2009;106(37):15932–15937.

36. Lapidus L, Bengtsson C, Larsson B, Pennert K, Rybo E, Sjostrom L. Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden. Br Med J (Clin Res Ed). 1984;289(6454):1257–1261.

37. Ribas V, Nguyen MT, Henstridge DC, et al. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice. Am J Physiol Endocrinol Metab. 2010;298(2):E304–E319.

38. Musatov S, Chen W, Pfaff DW, Mobbs CV, et al. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proc Natl Acad Sci USA. 2007;104(7):2501–2506.

39. True C, Verma S, Grove KL, Smith MS. Cocaine- and amphetamine-regulated transcript is a potent stimulator of GnRH and kisspeptin cells and may contribute to negative energy balance-induced reproductive inhibition in females. Endocrinology. 2013;154(8):2821–2832.

40. Roa J, Herbison AE. Direct regulation of GnRH neuron excitability by arcuate nucleus POMC and NPY neuron neuropeptides in female mice. Endocrinology. 2012;153(11):5587–5599.

41. Kim KW, Li S, Zhao H, et al. CNS-specific ablation of steroidogenic factor 1 results in impaired female reproductive function. Mol Endocrinol. 2010;24(6):1240–1250.

42. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, et al. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology. 2012;153(6):2800–2812.

43. Campbell SE, Febbraio MA. Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. Am J Physiol Endocrinol Metab. 2002;282(5):E1139–E1146.

44. Vasudevan N, Pfaff DW. Non-genomic actions of estrogens and their interaction with genomic actions in the brain. Front Neuroendocrinol. 2008;29(2):238–257.

45. Smith AW, Ronnekleiv OK, Kelly MJ. Gq-mER signaling has opposite effects on hypothalamic orexigenic and anorexigenic neurons. Steroids. 2013;81:31–35.

46. Santollo J, Eckel LA. Oestradiol decreases melanin-concentrating hormone (MCH) and MCH receptor expression in the hypothalamus of female rats. J Neuroendocrinol. 2013;25(6):570–579.

47. Roepke TA, Xue C, Bosch MA, Scanlan TS, Kelly MJ, Ronnekleiv OK. Genes associated with membrane-initiated signaling of estrogen and energy homeostasis. Endocrinology. 2008;149(12):6113–6124.

48. Park CJ, Zhao Z, Glidewell-Kenney C, et al. Genetic rescue of nonclassical ERalpha signaling normalizes energy balance in obese Eralpha-null mutant mice. J Clin Invest. 2011;121(2):604–612.

49. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. Endocr Rev. 2009;30(6):713–743.

50. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology. 2005;146(9):3686–3692.

51. Pineda R, Garcia-Galiano D, Roseweir A, et al. Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. Endocrinology. 2010;151(2):722–730.

52. Scott V, Brown CH. Beyond the GnRH axis: kisspeptin regulation of the oxytocin system in pregnancy and lactation. Adv Exp Med Biol. 2013;784:201–218.