Published in final edited form as:
Behav Brain Res. 2012 June 15; 232(1): 159–164. doi:10.1016/j.bbr.2012.04.010.

Middle-aged female rats retain sensitivity to the anorexigenic effect of exogenous estradiol

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Abstract

It is well established that estradiol (E2) decreases food intake and body weight in young female rats. However, it is not clear if female rats retain responsiveness to the anorexigenic effect of E2 during middle age. Because middle-aged females exhibit reduced responsiveness to E2, manifesting as a delayed and attenuated luteinizing hormone surge, it is plausible that middle-aged rats are less responsive to the anorexigenic effect of E2. To test this we monitored food intake in ovariohysterectomized young and middle-aged rats following E2 treatment. E2 decreased food intake and body weight to a similar degree in both young and middle-aged rats. Next, we investigated whether genes that mediate the estrogenic inhibition of food intake are similarly responsive to E2 by measuring gene expression of the anorexigenic genes corticotropin-releasing hormone (CRH), proopiomelanocortin (POMC), the long form of the leptin receptor (Lepr) and serotonin 2C receptors (5HT2CR) and the orexigenic genes agouti-related peptide (AgRP), neuropeptide Y (NPY), prepromelanin-concentrating hormone (pMCH) and orexin in the hypothalamus of young and middle-aged OVX rats treated with E2. As expected, E2 increased expression of all anorexigenic genes while decreasing expression of all orexigenic genes in young rats. Although CRH, 5HT2CR, Lepr, AgRP, NPY and orexin were also sensitive to E2 treatment in middle-aged rats, POMC and pMCH expression were not influenced by E2 in middle-aged rats. These data demonstrate that young and middle-aged rats are similarly sensitive to the anorexigenic effect of E2 and that most, but not all feeding-related genes retain sensitivity to E2.

Keywords

Estradiol; Estrogen Receptor Alpha; Food Intake; Aging

INTRODUCTION

The hypothalamus is a key regulator of a number of homeostatic processes, one of which is the regulation of feeding behavior. In female mammals, estradiol (E2) acts within the hypothalamus to inhibit food intake [1–3]. In cycling rats, food intake is lowest on the day

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of estrus [4–6]. Daily food intake increases following removal of endogenous E2 via ovariectomy [7], which can be prevented by E2 replacement [8, 9]. Moreover, site specific infusion of E2 into the arcuate nucleus (ARC) and medial preoptic area (MPOA) of the hypothalamus decreases food intake [2, 3]. E2 appears to inhibit food intake through nuclear estrogen receptors (ERs) to increase anorexigenic signaling mediated by peptides such as corticotropin releasing hormone (CRH) [10], and by decreasing orexigenic signals, such as neuropeptide Y (NPY) [11], in the hypothalamus. Although there are two nuclear ERs, ERα and ERβ, E2's inhibitory effect on feeding appears to be mediated solely through ERα. ERα knockout mice (αERKO) have increased body weight and food intake compared to wild-type controls [12] and following ovariectomy, E2 treatment does not decrease food intake [13]. In addition, pharmacological studies demonstrated that an ERα but not an ERβ agonist decreases food intake in rats and mice [14–16] and that blockade of ERα but not ERβ with selective antagonists prevents the inhibitory effect of endogenous and exogenous E2 on food intake [17].

E2 also acts within the hypothalamus as an important regulator of the luteinizing hormone (LH) surge. E2 exerts positive feedback actions on gonadotropin releasing hormone (GnRH) neurons, stimulating GnRH release into the hypophysial portal blood system to affect pituitary gonadotropes. The subsequent secretion of LH from the pituitary triggers ovulation [18]. This appears to be mediated by ERα as αERKO mice are infertile due in part to a disruption in the LH surge [19]. Although GnRH neurons do not express ERα [20], E2 regulates these neurons indirectly through afferent inputs from other E2-responsive neurons within the hypothalamus, such as the neurons synthesizing the peptide kisspeptin, along with neurons that release the neurotransmitters GABA, glutamate and norepinephrine [21–24]. Interestingly, as females rodents enter middle age, multiple E2-responsive neurons within the hypothalamus become less sensitive to E2 positive feedback, which results in a delayed and attenuated LH surge [25, 26].

The menopausal transition is associated with increased morbidity that negatively affects the quality of life [26]. In particular, increased intra-abdominal fat during the perimenopausal years is associated with an elevated risk of metabolic and cardiovascular diseases [27, 28]. Therefore, understanding the interaction of age and hormones on the capacity of the neuroendocrine axis to regulate eating behavior and /or maintain energy balance in middle-aged females may reveal potential interventions that reduce morbidity and improve quality of life. Although it is well known that aging impairs E2's ability to induce the LH surge [25, 26, 29], the effect of age on E2's anorexigenic properties, which are also mediated by the neuroendocrine axis, have not been investigated. Because both the LH surge and E2's anorexigenic effect are mediated by ERα in the hypothalamus, it is possible that the anorexigenic effect of E2 is also affected by reproductive aging. Here we tested the hypothesis that E2's anorexigenic effect is attenuated in middle-aged compared to young female rats. We also determined whether feeding related genes in the hypothalamus are similarly regulated by E2 in young and middle-aged rats.

MATERIALS AND METHODS

Animals and Ovariohysterectomy

Young (2 month) and middle-aged (retired breeders, 8–12 month) Sprague-Dawley female rats (Charles Rivers, Wilmington, MA) were group housed in standard cages with free access to food and water, except where otherwise noted. Other labs have reported no differences in hypothalamic responsiveness to E2 between middle-aged virgin and retired breeders [30–32]; therefore, this study used retired breeders. Room temperature was maintained at 20 ± 2°C with a 14:10 light cycle (lights off at 10 pm). Rats were anesthetized with intraperitoneal injections of a mixture of ketamine (80 mg/kg) and xylazine (4.6 mg/kg)

and then bilaterally ovariohysterectomized (OVX) using an intra-abdominal approach. All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Rats and were approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine.

Experiment 1: Anorexigenic effect of estradiol in young and middle-aged rats

Three days after OVX, six young and six middle-aged rats were transferred to individual test cages designed to continually measure food intake (Med Associates, Georgia, VT). The test cages were equipped with a water spout and a food trough, a 45-mg chow pellet dispenser that used a photobeam to detect the presence of a pellet. Food and water were continuously available except during a daily 1 h period (0900–1000 h) when rats were weighed and food dispensers and water bottles were refilled. Each testing cage was enclosed in an outer box with a 12:12 h light cycle (lights off at 1300 h) and equipped with a white noise generator.

Following a seven day adaptation period, body weight and 24 h food intake were measured daily between 0900 and 1000 h for a four day testing period. All rats received a single subcutaneous injection of 2 μg E2 benzoate (Steraloids, Inc., Newport, RI) dissolved in 0.1 ml peanut oil 4 h before the dark phase on day 3. This schedule of hormone treatment and four-day behavioral assessments were based on a previous report that an acute injection of E2 on day 3 of a 4 day cycle models the changes in E2 secretion observed across the estrous cycle and decreases food intake on day 4 (the day that represents behavioral estrus) in young rats [9].

Experiment 2: Influence of E2 on anorexigenic and orexigenic gene young and middle-aged rats

Seven days after OVX, six young and six middle-aged rats were injected with either vehicle or 2 μg E2 in 0.1 ml peanut oil for two consecutive days at 0900 h. The two injection E2 paradigm was used to be consistent with previous studies examining E2 regulation of orexigenic and anorexigenic gene expression [33] and because this type of E2 treatment more closely models the short-term fluctuations of a natural estrous cycle. Additionally, similar changes are reported when acute and chronic E2 treatment is used. For example, both acute and chronic E2 treatment decreases MCH gene expression in the hypothalamus [34, 35]. In the afternoon on the third day (1300 h) rats were anesthetized with an overdose of ketamine and xylazine and then decapitated. The anterior hypothalamus, which included the MPOA, and the posterior hypothalamus, which included the ARC, paraventricular nucleus (PVN) and the lateral hypothalamus (LH), were dissected as previously described [36], frozen on dry ice and stored at −80°C. CRH mRNA was analyzed in the anterior hypothalamus, and proopiomelanocortin (POMC), the long form of the leptin receptor (Lepr) and serotonin 2C receptors (5HT2CR) and the orexigenic peptides agouti-related peptide (AgRP), NPY, prepromelanin-concentrating hormone (pMCH) and orexin mRNAs were analyzed in the posterior hypothalamus.

cDNA Synthesis and RT-PCR

Real-time PCR was used to quantify mRNA levels in hypothalamic samples. DNA-free total RNA was purified using the RNeasy lipid minikit (QIAGEN, Valencia, CA), including a deoxyribonuclease step. Reverse transcription (RT) was performed with 500 ng of RNA using the high-capacity cDNA RT kit with ribonucleaseinhibitor (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using SYBR GREEN gene master mix (AppliedBiosystems) according to the manufacturer's instructions. The primer sequences are listed in Table 1.

Data Analysis

Data are expressed as means ± SEM throughout. Due to the significant difference in body weight between the young and middle-aged rats (middle-aged>young, $F = 33.7$, $P < 0.05$), food intake was normalized to 100 g body weight. A mixed design ANOVA (within factor: hormone; between factor: age) was used to compare the anorexigenic effect of E2 in young and middle-aged rats. The anorexigenic effect of E2 was assessed by comparing food intake and body weight on day 2 (the day that represents diestrus 2) and day 4 (the day that represents estrus) [15]. Additionally, the effect of E2 on the percent change in body weight from day 2 to day 4 in young and middle-aged rats was analyzed via an independent measures t-test. RT-PCR values were calculated using the ΔΔCT quantification method using β-actin as the normalizing housekeeping gene. Two factor ANOVAs (hormone by age) were used to determine differences in mRNA expression. Bonferroni post hocs were used throughout to determine individual group differences following significant main or interaction ANOVA effects ($P< 0.05$).

RESULTS

Experiment 1

Both age and E2 had significant effects on food intake (Fig. 1A). Following E2 injection on day 3, food intake on day 4 (the day that models estrus) decreased compared to food intake on day 2 (the day that models diestrus 2; $F = 17.5$, $P< 0.01$). Regardless of hormone treatment, middle-aged rats consumed less food than young rats ($F = 17.8$, $P< 0.01$). However, the magnitude of decrease in food intake following hormone treatment was similar in young and middle-aged rats (hormone × age interaction, $F = 0.14$, n.s.). The change in body weight following hormone treatment was different between young and middle-aged rats ($t = 2.97$, $P< 0.05$; Fig. 1B). Middle-aged rats lost ~1.4% of their body weight compared to a gain of 0.8% in young rats. This difference is likely the result of the different growth curves in young and middle-aged rats [37]. An additional analysis revealed an interaction between E2 and age on body weight ($F = 12.5$, $P < 0.05$). E2 treatment decreased body weight in middle-aged rats ($P < 0.05$) but not in young rats. However, prior to hormone treatment young rats gained ~6 g per day, while middle-aged rats gained ~ 2 g per day. Therefore, in two days one would expect a 12 g increase in body weight in young rats and a 4 g increase in body weight in middle-aged rats. Following E2 treatment, young rats only gained ~ 2 g while middle-aged rats lost ~ 6 g, which translates into a loss of ~10 g body weight for both age groups.

Experiment 2

As expected, E2 increased expression of anorexigenic genes in the hypothalamus (Fig. 2). Regardless of age, E2 increased expression of CRH mRNA ($F = 10.3$, $P< 0.01$) in the anterior hypothalamus and of Lepr ($F = 25.2$, $P< 0.01$) and 5HT2CR ($F = 32.8$, $P < 0.01$) mRNA levels in the posterior hypothalamus. Regardless of hormone treatment, expression of Lepr ($F = 6.4$, $P< 0.05$) and 5HT2CR ($F = 12.3$, $P< 0.01$) mRNA was lower in middle-aged compared to young rats. There was no interaction between hormone and age on CRH ($F = 0.25$, n.s), Lepr ($F = 0.06$, n.s.), nor 5HT2CR ($F = 2.3$, n.s.) expression. However, there was an interaction between E2 and age on expression of POMC in the posterior hypothalamus ($F = 8.27$, $P< 0.05$). Although POMC expression increased in young rats following E2 treatment, this effect was absent in middle-aged rats.

As expected, E2 decreased expression of orexigenic genes in the posterior hypothalamus (Fig. 3). Regardless of age, expression of AgRP ($F = 29.4$, $P< 0.01$), NPY ($F = 6.8$, $P< 0.05$), pMCH ($F = 6.1$, $P< 0.05$), and orexin ($F = 10.5$, $P< 0.01$) decreased following E2 treatment. Although there was a significant main effect of hormone on pMCH expression (F

= 6.1, P < 0.05), this is due primarily to the very large decrease in young rats as a priori post hoc tests demonstrated a reduction in pMCH expression in young ($P< 0.05$) but not middle-aged rats ($P> 0.05$). Additionally, regardless of hormone treatment, young rats had lower NPY mRNA levels than middle-aged rats (F = 13.3, $P< 0.01$).

DISCUSSION

This study tested the hypothesis that middle-aged female rats, which show delayed and attenuated E2- dependent LH surges, are less sensitive to the anorexigenic effect of exogenous E2 and determined whether E2 regulates feeding-related genes in the hypothalamus of middle-aged females. Our results demonstrate that the anorexigenic effect of E2 is similar in middle-aged and young females; both age groups showed a comparable suppression in food intake and body weight gain following E2 treatment. We also found that most genes that mediate E2's anorexigenic effect retain sensitivity to E2 regulation in the hypothalamus of middle-aged rats. These effects of E2 are likely mediated by ERα as it is the nuclear receptor that is both necessary and sufficient for E2's inhibitory effect on food intake [14–17]. It is interesting to note that this is in contrast with another ERα mediated response in the hypothalamus, most notably positive feedback regulation of the LH surge, which is impaired by aging [25, 26].

Because the anorexigenic effect of E2 was unaffected by age, we tested the hypothesis that E2-sensitive, feeding related genes in the hypothalamus are regulated similarly by E2 in young and middle-aged rats. We tested 4 anorexigenic genes whose mRNA levels in the hypothalamus increase in response to E2 and may be involved in mediating E2's inhibitory effect on food intake. CRH in the MPOA is an important mediator of E2's inhibitory effect on food intake. CRH and ERα are co-expressed in MPOA neurons [10], E2 treatment increases CRH expression in young OVX rats [38], and central treatment with a CRH antagonist blocks E2's anorexigenic effect in young OVX rats [10]. In addition, site specific infusion of E2 into the MPOA decreases 24 h food intake in young OVX rats [2, 3]. The ARC is another hypothalamic site where E2 infusion decreases 24 h food intake [3]. POMC neurons are abundant within the ARC, and E2 treatment increases POMC mRNA expression in young OVX mice [39]. Leprs are also expressed throughout the hypothalamus, with particularly high expression in the ARC where they are co-expressed with ERs [40]. In young rats long-term OVX (22 weeks) decreases hypothalamic Lepr expression, and E2 treatment throughout this period prevents the decrease [41]. A shorter period of E2 replacement (8 days) also increases Lepr expression in young OVX rats [42]. Additionally, central and peripheral E2 treatment increases leptin's anorexigenic effect in young OVX rats [43]. Finally, in young OVX rats, a 5HT2C receptor agonist increases E2's inhibitory effect on food intake [44]. As expected, E2 treatment increased expression of CRH, POMC, Lepr and 5HT2CR mRNAs in young rats. E2 also increased expression of CRH, Lepr and 5HT2CR mRNA in middle-aged rats, but POMC expression did not increase in middle-aged females. This could suggest that CRH, Lepr and 5HT2CR are of particular importance in mediating E2's anorexignic effect in both young and middle-aged rats whereas POMC neurons serve a lesser role. This is supported by Geary and colleagues who reported that in young OVX rats E2 does not influence the inhibitory effect of MTII, a synthetic melanocortin 3/4 agonist [45]. These observations suggest that E2's inhibitory effect on food intake is not mediated by α-MSH, a functional derivative of POMC neurons.

We also examined expression of 4 orexigenic genes in the hypothalamus that are decreased by E2 treatment or likely involved in mediating E2's inhibitory effect on food intake. In young rats, AgRP and NPY gene expression in the ARC increases after OVX [46]; both decrease *in vitro* and *in vivo* following E2 treatment [11, 39, 47] and E2 decreases NPY-induced feeding [48]. pMCH gene expression decreases in young OVX female and male rats

following E2 treatment [34, 35], and in young rats endogenous and exogenous E2 decrease MCH-induced feeding [49]. Finally, in young OVX rats, E2 treatment decreases orexin mRNA expression in the hypothalamus [42]. As expected, E2 decreased expression of AgRP, NPY, pMCH and orexin mRNA in young rats. In middle-aged rats AgRP, NPY and orexin neurons were still sensitive to E2 as indicated by a decrease in mRNA expression following E2 treatment. However, E2 did not alter pMCH mRNA in middle-aged rats. These results suggest that AgRP, NPY and orexin, but not MCH, may be of particular importance in mediating E2's inhibitory effect on food intake, especially in middle-aged rats. Interestingly, MCH neurons, similar to GnRH neurons, do not express ERα, suggesting that any influence of E2 on MCH-induced feeding is through afferent inputs, which could also be influenced by aging [50].

It is well known that ERα expressed within hypothalamic neurons is the crucial mediator of E2's inhibition of feeding and its positive feedback effects on the LH surge. However, E2 regulation of the LH surge is impaired with aging, as middle-aged female rats have a delayed and attenuated LH surge [25, 26]. Accumulating evidence suggests that LH surge dysfunction reflects reduced sensitivity of specific hypothalamic neurons to E2 in middle-aged females. For example, kisspeptin neurons in the anteroventral periventricular region of the hypothalamus (AVPV) provide potent excitatory input to GnRH neurons [24]. In young rats E2 treatment increases the number of kisspeptin immunoreactive neurons in the AVPV. In middle-aged rats, the E2-induced increase in kisspeptin-positive neurons decreases markedly compared to young rats [51], suggesting that neurons involved in mediating the LH surge lose sensitivity to E2 with aging. Again, this is in contrast with these results, which demonstrate that most feeding related genes are regulated similarly by E2 in both young and middle-aged rats. Interestingly, we found that POMC and MCH mRNAs were not responsive to E2 treatment in middle-aged rats. Clegg and colleagues recently demonstrated that selective deletion of ERα in POMC neurons produced both hyperphagia and impaired fertility in female mice through inhibition of E2 negative feedback [52]. Therefore, although POMC is important for both feeding and reproduction, our results may suggest that POMC is more important for E2 regulation of gonadotropin release than of feeding.

Independent of hormone treatment, we observed age related decreases in Lepr and 5HT2CR mRNA within the hypothalamus. This extends reports that show less Lepr expression in aged male rats [53]. This likely contributes to decreased leptin signaling in aged animals. We are unaware of studies examining the expression of 5HT2CR in the hypothalamus in aged male or female rats. Because 5HT is involved in many behaviors, the reduced receptor expression could have a wide range of implications. We also observed that hypothalamic NPY mRNA expression increases in middle-aged compared to young rats. This finding is consistent with previous studies demonstrating that hypothalamic NPY gene expression increases in postmenopausal compared to premenopausal women [54] and that on the morning of proestrus NPY mRNA in the median eminence is higher in middle-aged compared to young rats [55]. This effect appears to sexually dimorphic in that male rats express less NPY with aging [56]. Additional studies will be necessary to understand the effect of sex and age on NPY mRNA expression and the functional consequence of age-related changes in NPY mRNA expression.

In conclusion, the hypothalamus is the major site of E2 regulation of food intake and reproduction. Here we demonstrate that E2's inhibitory effect on feeding behavior and on most genes that mediate E2's anorexigenic effect remain sensitive to E2 in middle-aged rats. Future studies will be necessary to determine if the observed changes in hypothalamic gene expression following hormone treatment translate into comparable changes in hypothalamic protein expression in young and middle-aged rats. Our results here contrast with the loss of

sensitivity to E2 positive feedback regulation of the LH surge in middle-aged rats. Although the underlying mechanisms are unclear, because there is little evidence that levels of ERα in hypothalamic nuclei differ between young and middle-aged female rats [57], it is interesting to speculate that ERα function is influenced by aging in some but not all hypothalamic neuronal populations. Recently, Brann and colleagues demonstrated that in hippocampal but not uterine tissue of middle-aged rats, ERα protein levels decrease through proteasomal degradation of ERs through enhanced interaction with E3 ubiquitin ligase C terminus of heat shock cognate protein 70 interacting protein [58]. It is possible that in middle-aged rats ERα protein decreases in certain hypothalamic neuronal populations involved in mediating the LH surge but not in those mediating feeding behavior. Future studies are needed to test this hypothesis.

Acknowledgments

This work was supported by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development/National Institutes of Health through cooperative agreement U54 HD058155 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research and by NIH grants P60 DK020541 and T32 AG023475 and the Department of Obstetrics and Gynecology and Women's Health, Albert Einstein College of Medicine.

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Research Highlights

- E2 decreases food intake to a similar degree in young and middle-aged female rats.
- E2 increased expression of CRH, Lepr and 5HT2CR in young and middle-aged females.
- However, E2 increased expression of POMC in young, but not in middle-aged females.
- E2 decreased expression of NPY, AgRP and orexin in young and middle-aged females.
- However, E2 decreased expression of pMCH in young, but not in middle-aged females.

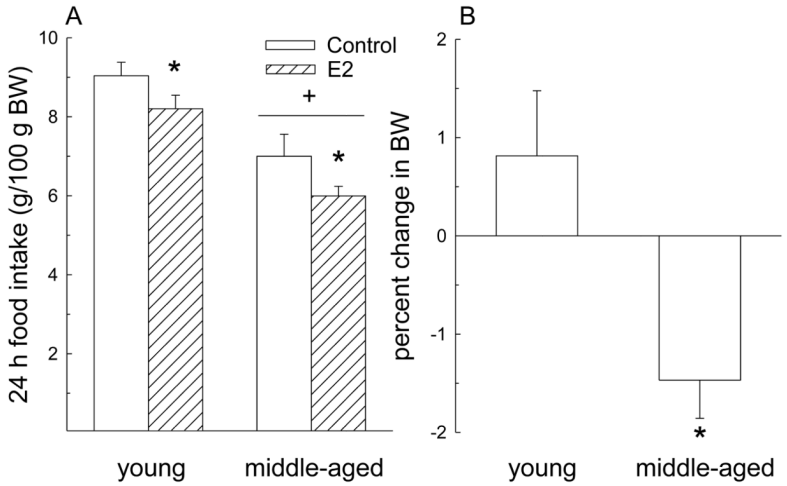

Figure 1.

E2 effects on food intake and body weight in young and middle-aged rats. (A) E2 treatment decreased 24 h food intake in both young and middle-aged rats. Regardless of hormone treatment, 24 h food intake was lower in middle-aged than in young rats. *< control, P< 0.05. +< young, P< 0.05. (B) The percent change in body weight following E2 treatment was different in young and middle-aged rats. Young rats gained ~0.8% whereas middle-aged rats lost ~1.4% of their body weight. Data are expressed as mean ± SEM. *< young, P< 0.05.

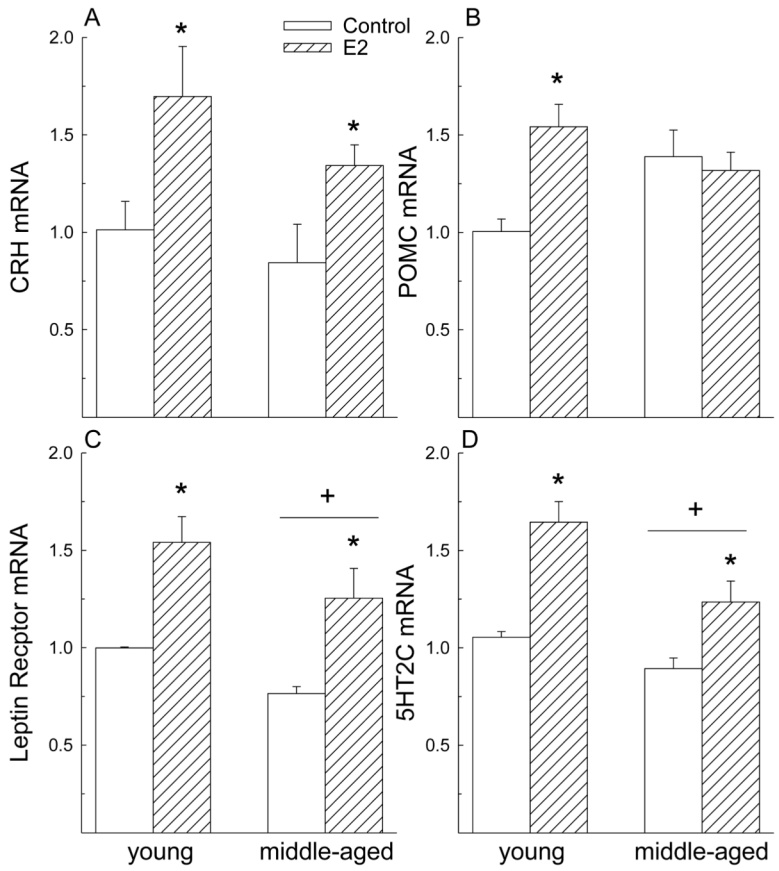

Figure 2.

E2 increased expression of most anorexigenic genes in the hypothalamus. (A) E2 increased expression of CRH in the anterior hypothalamus of young and middle-aged rats. (B) E2 increased expression of POMC in the posterior hypothalamus of young but not middle-aged rats. (C, D) E2 increased expression of the leptin and 5HT2C receptors in the posterior hypothalamus of young and middle-aged rats. Regardless of hormone, leptin and 5HT2C receptor expression was lower in middle-aged than in young rats. Data are expressed as mean ± SEM. RT-PRC values are expressed as fold change relative to young, oil-treated control. *>control, $P< 0.05$.+< young, $P< 0.05$.

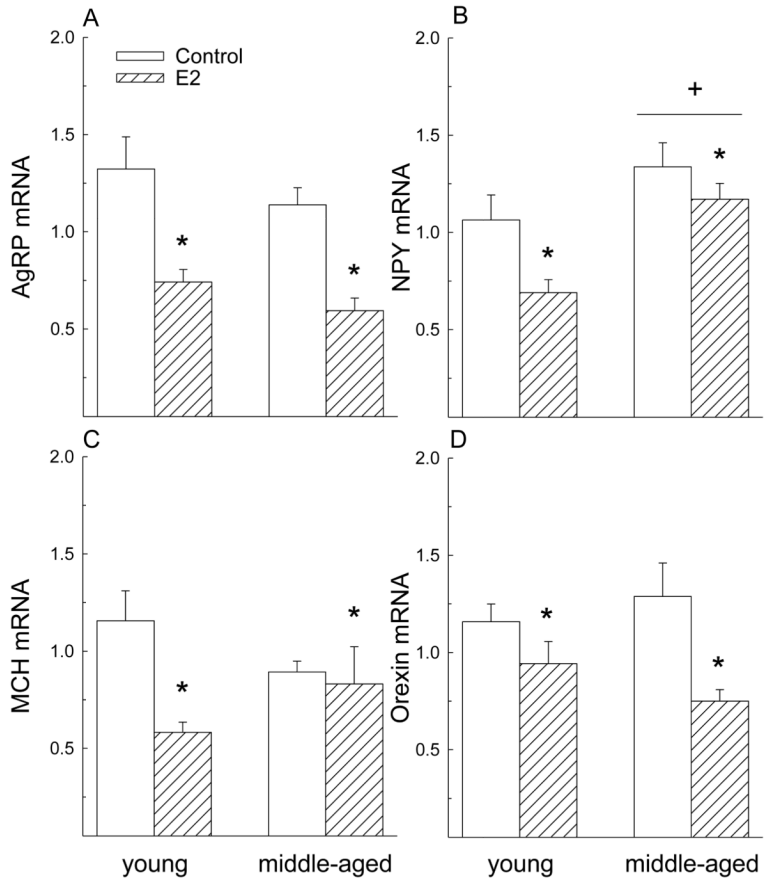

Figure 3.

E2 decreased expression of orexigenic genes in the posterior hypothalamus. (A) E2 decreased expression of AgRP in young and middle-aged rats. (B) E2 decreased expression of NPY in young and middle-aged rats. Regardless of hormone, NPY expression was higher in middle-aged than in young rats. (C, D) There was a main effect of hormone on MCH and orexin expression. E2 decreased expression of MCH and orexin in young and middle-aged rats. Data are expressed as mean ± SEM. RT-PRC values are expressed as fold change relative to young, oil-treated control *<control, $P < 0.05$. +> young, $P < 0.05$.

Table 1

Primer Sequences Used for RT-PCR

Gene	Sense	Antisense
CRH	5'GCT GTC CCC CAA CTC CAC	5' CAG CTC CGT GCT GCT GTC
POMC	5' CTC CAT AGA CGT GTG GAG CTG	5' TCA GTC AAG GGC TGT TCA TCT
Lepr	5'AGG GAA CCT GTG AGG ATG AGT	5' TGT CTC AGT GGG GAA TGT TTC
5HT2CR	5' AGA AAG AAA AGC GTC CCA GAG	5'CCA CAA AGA ACC GAC AGG ATA
AgRP	5'TGA AGA AGA CAG CAGCAG ACC	5'AAG GTA CCT GTT GTC CCA AGC
NPY	5'GTA ACA AAC GAA TGG GGC TGT	5' CGC AGA GCG GAG TAG TAT CTG
pMCH	5' ATG CTG GCC TTT TCT TTG TTT	5' AAG GAG CAA CAA CCG ATC TTT
Orexin	5'GCA TCC TCA CTC TGG GAA AG	5' GCA GGG ATA TGG CTC TAG CTC
β-actin	5'AGA TTA CTG CCC TGG CTC CTA	5'CTC ATC GTA CTC CTG CTT GCT

Abbreviations: CRH = corticotropin releasing hormone; POMC = proopiomelanocortin; Lepr = long form of the leptin receptor; 5HT2CR = serotonin 2C receptor; AgRP = agouti-related peptide; NPY = neuropeptide Y; pMCH = prepromelanin-concentrating hormone.