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Conjugated Equine Estrogen, with Medroxyprogesterone Acetate, Enhances Formation of 5 α -Reduced Progestogens and Reduces Anxiety-like Behavior of Middle-Aged Rats

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Abstract

The mechanisms by which progestogens influence affective behaviors of females are poorly understood despite clear changes in mood/affect that are associated with their decline during menopause. Conjugated equine estrogens (CEE), with or without medroxyprogesterone acetate (MPA), are commonly-prescribed hormone-replacement, but there is heterogeneity in responses to these pharmacotherapies. One way in which these compounds differ is in their capacity to potentiate metabolism of progesterone to its 5 α -reduced products, dihydroprogesterone (DHP) and 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP). The present study investigated whether responses to CEE and MPA may be related to the capacity to metabolize progesterone. Middle-aged female rats that had maintained reproductive status, or those that had a decline, were administered vehicle, CEE and/or MPA. Effects on anxiety-like (open field, elevated plus maze) and social behavior (social interaction test), and plasma and hippocampus steroid levels were determined. We hypothesized that CEE, but not MPA, would decrease anxiety-like behavior coincident with increased hippocampal metabolism of progesterone. CEE, or CEE+MPA, increased central entries in the open field and time spent on the open arms of the plus maze, but did not alter social interaction of rats that had maintained reproductive status. CEE and/or CEE+MPA increased E₂ and 3 α ,5 α -THP in plasma and/or hippocampus of rats, but MPA increased levels of DHP in the hippocampus of rats with declining reproductive status. Simple regressions demonstrated that hippocampus 3 α ,5 α -THP levels accounted for a significant portion of the variance in anxiety-like behavior. Therefore, effects of CEE to reduce anxiety-like behavior of middle-aged rats may be due, in part, to its capacity to enhance levels of 3 α ,5 α -THP in the hippocampus.

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Keywords

estradiol; progesterone; conjugated equine estrogen; medroxyprogesterone acetate; anxiety; menopause; Premarin; Prempro; rat

Introduction

The ovarian steroids 17 β -estradiol (E₂) and progesterone (P₄) can have profound effects on physiological and psychological function throughout the lifespan, including modulating mood and anxiety (Steiner et al., 2003; Estrada-Camarena et al., 2010; Lokuge et al., 2001). The role of steroids in the development and treatment of anxiety disorders, which are widely prevalent (16.6% worldwide by some estimates) with large personal, societal, and therapeutic costs, is of great interest. (Somers et al., 2006) Some women have an increased risk for mood changes, such as anxiety and depression, when ovarian function declines at menopause, and choose hormone replacement therapies (HRT) to manage psychological and/or physical symptoms associated with steroid decline (Soares et al., 2005). However, not all women respond with improved mood to typical HRT, such as conjugated equine estrogen (CEE; Premarin®, Wyeth) or CEE and medroxyprogesterone acetate (MPA; Prempro®, Wyeth), and there also are clear side effects and reproductive cancer and stroke risks associated with HRT (Craig et al., 2005; Sherwin, 2007). It has been proposed that heterogeneity in the response to HRT may be due to individual differences related to aging, health status, timing of treatment, as well as the treatment's mechanism of action (Sherwin and Henry, 2008; Sherwin, 2009). As such, it is important to understand better HRT mechanisms and their functional effects. In this regard, animal models can provide valuable information on the neurobiological underpinnings of anxiety as well as the effects and mechanisms of HRT in mediating anxiety behavior.

One laboratory approach to model menopause of women, which is characterized by changes in menstrual cyclicity, followed by cessation of such cycles, and ovarian production of E₂ and P₄, involves the study of ovariectomized adult rats. For example, there is great variability in the endocrine profile of aging rats (Chakraborty and Gore, 2004). Changes in cyclicity from the typical 4-day estrous cycle of young rats (Long and Evans, 1929; Freeman, 1998; Nelson, 2005) can eventually develop into persistent diestrus or estrus in aged rats. There are also changes in ovarian secretion of E₂ and P₄. Contrary to the decline observed among menopausal women, rats can show increases in ovarian production of steroids with aging (Huang et al., 1978; Lu et al., 1979; Nass et al., 1984). However, factors other than ovarian steroids change with aging of both women and rodents and may influence responses to HRT. As such, the present study aimed to compare the effects of CEE and/or MPA and vehicle on anxiety-like behaviors and levels of E₂, P₄ and P₄ metabolites in intact, middle-aged, female rats.

Anxiety behavior of middle-aged rats increases with transition to reproductive-senescence (Walf et al., 2009), and can be reduced with acute CEE administration (Walf and Frye, 2008), but the mechanisms for these effects are unknown. CEE and MPA are prescribed as E₂-like and progestin-like HRTs, respectively, but can have different mechanisms than natural E₂ and P₄. For instance, CEE contains minimal levels of E₂ and ~40 other components, including other estrogens and androgens (Bhavnani, 1998; Stern, 1992). MPA does not function like endogenous progestogens, has androgenic actions and, unlike E₂ and CEE (Genazzani et al., 2004; Frye and Rhodes, 2005), inhibits activity of 3 α -hydroxysteroid dehydrogenase (3 α -HSD; Penning et al., 1985), which can limit metabolism of P₄ to its neuroactive steroids (Frye, 2009). P₄ is converted, by 5 α -reductase, to dihydroprogesterone (DHP) and, by 3 α -HSD, to 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP), which clinical studies suggest may play a role in anxiety disorders (Frye, 2009). Moreover, among young rats, higher 3 α ,5 α -THP levels in circulation and in the hippocampus are associated with less anxiety-like responding (Frye et

al., 2000; Frye, 2009). Given its ability to enhance P_4 metabolism, some of the anti-anxiety effects of E_2 may be subsequent to $3\alpha,5\alpha$ -THP formation (Jensen et al., 2010). Also, blocking formation of $3\alpha,5\alpha$ -THP in the whole brain or hippocampus increases anxiety-like responding of female mice and rats, respectively (Frye et al., 2004; Rhodes and Frye, 2001). These studies suggest that favorable responses to HRT may be related to their capacity to enhance $3\alpha,5\alpha$ -THP formation.

Of interest is whether the response of individuals to HRT is influenced by aging- and reproductive-competence- related changes in metabolism. There is some evidence for changes in P_4 metabolism with aging and hormonal milieu among female rats (Hodges and Karavolas, 1992; Marrone and Karavolas, 1982); however, whether these are due to aging or, specifically, changes in reproductive status are not well understood. Thus, in the present experiments, we determined levels of E_2 , P_4 and P_4 metabolites in circulation and in the hippocampus, and anxiety-like and social behavior of age-matched, middle-aged rats that had maintained reproductive status, or had a decline in reproductive status. The hippocampus was assessed because we have previously demonstrated that this brain region is a likely target of $3\alpha,5\alpha$ -THP to reduce anxiety-like behavior (Frye et al., 2000; 2007; Rhodes and Frye, 2001). We hypothesized that CEE, but not MPA, would decrease anxiety-like behavior coincident with increased hippocampal metabolism of P_4 , which may be particularly apparent among rats that have maintained reproductive status.

Methods

Methods were approved by the IACUC at The University at Albany-SUNY and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications, No 80-23).

Subjects and Housing

Subjects ($N=63$) were middle-aged (12–15 months old) Long-Evans rats, obtained from Taconic Farms (Germantown, NY) at 2 months of age, or bred in our facility from this stock. Rats were group-housed (2–4 per cage) in the Laboratory Animal Care Facility at The University at Albany-SUNY. Six cohorts of rats over the last four years have been included in this study. It should be noted that the first cohort of rats for this study was purchased to set up a colony in the Life Sciences Building when it was first opened. (This first cohort of rats and the subsequent cohorts that are included in this study had similar environmental and reproductive experiences before inclusion in the study. There was no evidence of any cohort effects for behavioral measures.) Subjects were breeders retired at 10–12 months of age that had generated 4–6 litters each. Rats have a decline in reproductive function with aging (Chakraborty and Gore, 2004; Wise, 1999) and categorization of middle-aged rats has been previously described (Walf et al., 2009). Similar criteria, as well as comparison data collected in our laboratory over the last five years, were used in the present study (Frye et al., 2010b; Paris et al., 2010). Briefly, rats were considered to be maintaining reproductive status if they had typical 4–5 days estrous cycles (Long and Evans, 1929; Freeman, 1998; Nelson, 2005), fertility (more than 60% successful pregnancies, or those resulting in a litter following mating with a male), and fecundity (more than 10 pups/litter) similar to that observed among young adult rats. Rats were considered to be declining in reproductive status if they were acyclic and/or in constant diestrus or estrus, became pregnant on fewer than 60% of the occasions that they were mated, and had litters with fewer than 10 pups.

Drug regimen

In each of 6 cohorts, rats were randomly assigned to be administered vehicle, CEE, MPA, or CEE+MPA. Vehicle (vegetable oil, 10% EtOH), CEE (Wyeth; 0.625 mg/kg in propylene

glycol, 10% EtOH), and MPA (Sigma; 4 mg/kg in vegetable oil, 10% EtOH) were administered subcutaneously (s.c.) 44–48 hours before testing or tissue collection. The dosing regimen and the vehicle used for suspension were based upon published and pilot data showing that these dosages of CEE and MPA alter P₄ metabolism or anxiety-like behavior of rats (Genazzani et al., 2004; Ciriza et al., 2006; Walf and Frye, 2008). Additionally, historical control data (not shown), as well as recent studies of hippocampus levels of E₂, P₄, DHP, and 3 α ,5 α -THP in intact middle-aged rats, indicate that vegetable oil vehicle, with or without 10% EtOH, does not influence steroid levels or behavior in the open field or elevated plus maze (Paris et al., 2010). Furthermore, while ethanol can have anti-anxiety-like effects in rats when administered i.p. (Taksande et al., 2010), the dose and route of administration (s.c.) in the present investigation are not expected to produce behavioral effects.

Behavioral Testing

Behavioral data were collected with ANY-maze animal tracking software (Stoelting Co., Wood Dale, IL) by staff who were blind to the treatments. Data reported are from testing on one (n=31) or two occasions (n=32). If rats were tested more than once, there was at least 3–4 weeks between testing in different rooms and different testing chambers to obviate test decay effects, which is especially important for elevated plus maze testing (Walf and Frye, 2008). Each experimental condition was represented in groups that were tested once or twice. Analyses of the behavioral data with testing on one or a second trial as a factor did not reveal any significant differences. Data were analyzed collapsed across this variable to maximize the number of observations per experimental condition.

Anxiety-like behavior

Rats were tested in the open field and the elevated plus maze for five minute sessions (Frye et al., 2000; Walf and Frye, 2008; Walf et al., 2009). In the open field (76 × 57 × 35 cm), entries were recorded into each of 48-squares marked as a grid floor (6 × 8 squares, 9.5 cm/side). Entries made by rats into the center of the chamber were used as an index of reduced anxiety-like behavior. For elevated plus maze testing, rats were placed in the junction of the two open and two closed arms. The arms were each 50 cm long and 10 cm wide, elevated 50 cm off the ground, and the closed arms had 30 cm high walls. The time spent on the open arms during this 5-minute task was recorded and used as the primary index of anxiety-like behavior.

Social behavior

The social interaction task was utilized to assess social behavior. Rats were tested in an open field in the social interaction task. During this task, the time spent in interaction with an ovariectomized conspecific was recorded for five minutes (Frye et al., 2000). Interaction was defined as sniffing, grooming, and/or followed with contact.

Steroid measurement

Tissue from some rats in Cohorts 1, 5, and 6 was collected, following vehicle or HRT, and processed for radioimmunoassay analyses of E₂ and progestogens in plasma and hippocampus. Technical and practical issues precluded the collection and/or processing of tissue from all rats. Rats in these three cohorts that were tested in previous behavioral studies were randomly chosen to minimize potential confounding variables related to cohort effects (e.g. time of year), and so that randomly-assigned drug conditions would be represented across cohorts.

Rats were euthanized via rapid decapitation, and whole brains were collected and flash frozen. Trunk blood was collected into chilled test tubes, spun in a refrigerated centrifuge, plasma was decanted into clean 1.5 ml tubes and stored frozen until radioimmunoassay. Immediately before radioimmunoassay, brains were briefly thawed on ice, the cortex was gently peeled back and

then the entire hippocampus was dissected out from the whole brain (Frye et al., 2007). Each hemisphere of the hippocampus was homogenized. Steroids from hippocampus and plasma samples were extracted and radioimmunoassays were conducted for E₂, P₄, DHP, and 3 α ,5 α -THP using standard protocols developed in our laboratory (Frye et al., 2000; 2007).

Statistical Analyses

Given the uneven sample sizes per group, one-way analyses of variance (ANOVAs) were used to analyze the contribution, if any, of the 'between subjects' factor, experimental condition (e.g., vehicle or HRT to middle-aged rats with maintaining or declining reproductive status) to behavioral and endocrine data. Fisher's PLSD tests, with corrections for multiple comparisons, were utilized to determine group differences when a main effect was detected. To account for multiple *post hoc* comparisons against the control group (rats maintaining reproductive status and vehicle-administered), a *p*-value of <0.05 was considered significant for main effects, and a *p* value of <0.007 was considered significant for *post hoc* tests, when seven comparisons were taken into account. Simple regressions were utilized to determine how much of the variance in performance in the open field and elevated plus maze could be accounted for by plasma and hippocampus steroid levels.

Results

Anxiety-like behavior

One-way ANOVAs demonstrate that there were significant effects of condition to alter behavior in the open field [$F(7,92)=3.10$, $p<0.01$; Figure 1, upper panel] and elevated plus maze [$F(7,92)=3.71$, $p<0.01$; Figure 1, lower panel]. *Post hoc* tests of open field data revealed that rats maintaining reproductive status made significantly more central square entries after CEE or CEE + MPA than did the control group (rats maintaining reproductive status administered vehicle). Similarly, in the elevated plus maze, *post hoc* tests revealed that the administration of CEE or CEE + MPA to rats maintaining reproductive status significantly increased time spent on the open arms, compared to vehicle controls. Compared to vehicle controls, however, no differences in the open field or elevated plus maze were apparent among rats with maintaining reproductive status that that received MPA, or among rats with declining reproductive status that received CEE and/or MPA.

Social behavior

One-way ANOVAs revealed no significant effects of condition on behavior in the social interaction task [$F(7,92)=1.36$, *NS*]. However, there appeared to be effects of CEE in rats with declining reproductive status. More specifically, time spent interacting with a conspecific was reduced compared to all other groups; however, this effect did not reach significance. Within the control group, rats maintaining reproductive status and receiving vehicle spent a similar duration (mean secs $\pm$ SEM) engaging in social interaction as did rats maintaining reproductive status that received CEE (49.6 $\pm$ 9.0), MPA (48.1 $\pm$ 6.0), or both (42.1 $\pm$ 7.3), or rats with declining reproductive status that received vehicle (40.0 $\pm$ 4.0), CEE (26.3 $\pm$ 5.0), MPA (39.3 $\pm$ 4.0), or both (56.4 $\pm$ 8.5).

Plasma Steroid levels

There were significant main effects of condition for plasma levels of E₂ [$F(7,34)=5.30$, $p<0.01$] and 3 α ,5 α -THP [$F(7,34)=4.29$, $p<0.01$], but not P₄ [$F(7,34)=0.38$, $p=0.91$] or DHP [$F(7,34)=1.88$, $p=0.10$] (Figure 2). *Post hoc* tests revealed that, compared to rats maintaining reproductive status and receiving vehicle, rats with declining reproductive status that were treated with CEE had significantly increased plasma E₂ levels. There were no significant differences between other groups for plasma E₂ levels. As revealed by *post hoc* tests, compared

to vehicle controls, rats maintaining reproductive status and receiving CEE had significantly higher plasma 3 α ,5 α -THP, and rats with declining reproductive status that were treated with CEE + MPA had significantly lower plasma 3 α ,5 α -THP. There were no significant differences between other groups for plasma E₂ levels.

Hippocampus Steroid levels

Although one-way ANOVAs did not reveal significant differences in levels of P₄ in the hippocampus [$F(7,34)=1.21$, *NS*], there were differences in E₂ [$F(7,34)=2.98$, $p<0.01$] and P₄ metabolites, DHP [$F(7,34)=8.28$, $p<0.01$] and 3 α ,5 α -THP [$F(7,34)=2.79$, $p<0.02$] (Figure 3). *Post hoc* tests revealed that, compared to vehicle controls, rats with maintenance of their reproductive status had significantly higher hippocampal E₂ levels after treatment with CEE + MPA. There were no significant differences between other groups for hippocampal E₂ levels. *Post hoc* tests for DHP analyses revealed that rats that maintained reproductive status and received MPA had significantly higher DHP levels than did vehicle controls. Additionally, rats with declining reproductive status that received CEE + MPA had higher hippocampus DHP levels than did controls. Although *post hoc* tests did not reveal a significant difference between groups compared to vehicle controls, rats with maintenance of reproductive status that were treated with CEE or CEE + MPA had the highest levels of 3 α ,5 α -THP in the hippocampus.

Simple regression results

It was of interest to determine how much variance in behavior in the open field and elevated plus maze could be attributed to steroid levels. Simple regressions demonstrated that hippocampal, but not plasma, 3 α ,5 α -THP levels accounted for a significant proportion of variance in the open field [$R^2_{\text{hippocampus}} = 0.11$, $F(1,37) = 4.58$, $p < 0.05$] and elevated plus maze tests [$R^2_{\text{hippocampus}} = 0.21$, $F(1,37) = 9.27$, $p < 0.01$] (Figure 4). Plasma and hippocampus levels of E₂ accounted for significant variance in the open field [$R^2_{\text{plasma}} = 0.22$, $F(1,37) = 9.95$, $p < 0.01$; $R^2_{\text{hippocampus}} = 0.18$, $F(1,37) = 7.82$, $p < 0.01$], but did not reach significance in the elevated plus maze test [$R^2_{\text{plasma}} = 0.08$, $F(1,37) = 3.04$, $p < 0.09$; $R^2_{\text{hippocampus}} = 0.09$, $F(1,37) = 3.58$, $p < 0.06$]. Neither plasma nor hippocampal levels of P₄ or DHP contributed significantly to variance in either open field or elevated plus maze behavior.

Discussion

Our hypothesis that HRT that increase P₄ metabolism to 3 α ,5 α -THP in the hippocampus would decrease anxiety-like behavior of middle-aged female rats was partially supported. CEE and CEE+MPA, but not MPA alone, increased central entries in the open field and open arm time in the elevated plus maze in rats maintaining reproductive status, compared to vehicle controls. This same pattern of effects was not observed among rats that had declined in reproductive status. There were no differences in social behavior among groups, suggesting that there may be some specificity for effects of HRT on anxiety-like behaviors. This pattern of effects in the open field and elevated plus maze was similar to what was observed when plasma and hippocampal levels of E₂ and 3 α ,5 α -THP were analyzed. Compared to vehicle controls, CEE or CEE + MPA significantly increased plasma 3 α ,5 α -THP levels, and apparently increased hippocampal 3 α ,5 α -THP levels, when administered to rats that had maintained their reproductive status. Also, compared to controls, CEE + MPA increased hippocampal E₂ levels and MPA increased hippocampal DHP levels in rats that were maintaining reproductive status. On the other hand, rats with declining reproductive status had significantly lower plasma 3 α ,5 α -THP and higher hippocampal DHP levels after CEE + MPA, and CEE significantly increased plasma E₂ levels. It was of interest to determine whether these changes in steroid levels contributed to some of the variance in the open field and elevated plus maze behavior. Simple regressions showed that hippocampus 3 α ,5 α -THP levels accounted for a significant proportion of variance in the results of open field and elevated plus maze experiments.

Although the effect was less robust than observed for hippocampus $3\alpha,5\alpha$ -THP levels, plasma and hippocampus levels of E_2 also accounted for significant variance in the open field, but not elevated plus maze data. In contrast, neither plasma nor hippocampal, levels of P_4 or DHP contributed significantly to variance in either open field or elevated plus maze behavior. Thus, the present study shows that middle-aged rats can respond favorably to some HRT, but responses may be influenced in part by their capacity to form P_4 metabolites, including $3\alpha,5\alpha$ -THP, in the hippocampus.

The present results confirm previous reports in the literature on the effects of CEE and/or MPA in animal and *in vitro* models for anxiety or other behaviors mediated by the hippocampus, such as cognition. Acute CEE + MPA or CEE alone in this study, and acute CEE in a previous study, similarly reduced anxiety-like behavior of middle-aged rats in the open field and elevated plus maze (Walf and Frye, 2008). In that study, CEE, when administered post-training during memory consolidation, also enhanced object recognition (Walf and Frye, 2008). In addition, chronic administration of CEE to middle-aged ovariectomized has been shown to improve spatial reference and working memory, as well as attenuated memory deficits produced by scopolamine (Acosta et al., 2009). As in the present study, few beneficial effects of MPA alone have been observed for protection against cellular insult *in vitro* (Nilsen and Brinton, 2002; Zhao and Brinton, 2006;), ictal activity or traumatic brain injury *in vivo* (Ciriza et al., 2006; Craig et al., 2005; Wright et al., 2008), or cognitive performance (Braden et al., 2010; Frye et al., 2010a; Toufexis et al., 2004). Also, MPA may attenuate the beneficial effects of E_2 on water maze performance in ovariectomized middle-aged rats (Lowry et al., 2010), or those of P_4 administration on cognitive performance across several tasks in ovariectomized young mice as well as their effects on hippocampal $3\alpha,5\alpha$ -THP levels (a et al., 2010). Interestingly, in the present study, we did not find that MPA abrogated the effects of CEE on behavior or steroid levels, which could be due to the timing or dosing of MPA utilized. Another question raised by the data in these studies is the specificity of the effects of HRT. Although direct performance measures were not systematically obtained in the present study, it is noteworthy that a clinical study did not find CEE, with or without MPA, to alter elderly women's physical activity or performance (Greenspan et al., 2005). In the present study in rats, there were no differences among groups for social interaction behavior; albeit we have previously demonstrated that acute CEE can increase the time spent in social interaction in middle-aged rats, but did not alter another socially-relevant behavior, lordosis (Walf and Frye, 2008). Although the present data suggest that there may have been some specificity in the effects of CEE, with or without MPA, on anxiety-like behavior, such other findings that are more focused on cognitive and social behavior clearly indicate that further investigation of the functional effects of HRT are warranted.

Endocrine status may influence individual differences in responses to HRT. Lack of effect, or detrimental effects, of HRT among women in clinical trials may be due to reduced responsiveness to HRT with aging and ovarian cessation (Sherwin, 2007; Sherwin and Henry, 2008). A similar pattern for reduced responsiveness to E_2 -replacement following ovarian cessation has been demonstrated in animal models. Middle-aged rats respond more to E_2 immediately after OVX than after a long delay (Daniel et al., 2006; Bohacek et al., 2008; Walf et al., 2009). Because it is difficult to determine the contribution of aging versus ovarian decline in the effects of steroids, we utilized a model in the present study in which age-matched rats with similar reproductive experiences had differing reproductive status at the time of HRT treatment, testing and measurement of circulating and hippocampal steroids. We found that rats that were maintaining reproductive status seemed to respond more favorably with less anxiety-like behavior to acute CEE, alone or co-administered with MPA, than did rats in declining reproductive status. Moreover, the general pattern of steroid levels in plasma and the hippocampus supported our predictions. The highest levels of plasma and hippocampal steroids with CEE or CEE + MPA were observed for E_2 and $3\alpha,5\alpha$ -THP. Moreover, notwithstanding

some contribution of E₂ levels, regression analyses supported the notion that the greatest part of the variance in anxiety-related behaviors was due to levels of hippocampal 3 α ,5 α -THP, but not P₄ or DHP. Of note, E₂ can increase activity of metabolism enzymes required for 3 α ,5 α -THP production (Cheng and Karavolas, 1973; Vongher and Frye, 1999), and, therefore, these effects of E₂ may be mediated by increased levels of 3 α ,5 α -THP. Other studies in rats have also suggested that increased 3 α ,5 α -THP levels in the hippocampus occur concomitant with reduced anxiety- or depression-like behavior (Frye et al., 2000; Walf and Frye, 2002), and can predict variance in other measures of hippocampal function such as object recognition (Walf et al., 2006).

There was some indication of differences in metabolism between rats that had maintained reproductive status and those that were in decline. It is interesting that MPA alone increased levels of DHP in the hippocampus of rats in reproductive decline. First, this demonstrates that MPA was able to cross the blood-brain barrier to have a functional effect. It also suggests that shunting one enzymatic pathway (i.e. 3 α -HSD) may have consequently altered the activity of 5 α -reductase and back-conversion to DHP. Previous work has suggested that some reduced responses to steroids with aging may be due to differences in their metabolism. For example, reproductively senescent rats in constant estrus have an approximately 2-fold increase in pituitary activity of 5 α -reductase and 3 α -HSD, and levels of metabolites such as DHP and 3 α ,5 α -THP are about 50% lower than those observed in reproductively senescent rats in anestrus or in young cycling female rats (Marrone and Karavolas, 1982; Hodges and Karavolas, 1992). Additionally, ovariectomy alters this pituitary response depending upon the age and/or endocrine status of the individual. Ovariectomy of reproductively senescent rats in constant estrus decreased 3 α -HSD activity, but did not alter 5 α -reductase activity. Conversely, ovariectomy increased 5 α -reductase activity in anestrus rats, and this was reversed with replacement back with E₂ benzoate for 3 days (Hodges and Karavolas, 1992). In the present study, subjects were intact middle-aged rats, and given the above considerations, it may be of interest in future investigations to compare behavioral and endocrine responses to HRT between middle-aged rats that are ovariectomized or intact. Given the role of E₂ to enhance progesterone metabolism, previous findings, and the present results showing that HRT most consistently increased E₂ and 3 α ,5 α -THP levels, support the idea that beneficial effects of HRT such as CEE may be related to the capacity for 3 α ,5 α -THP formation.

The role of reproductive/endocrine status in the effects of HRT on hippocampally-mediated processes is of interest and deserves thorough investigation. For example, previous exposure to endogenous steroids (i.e. pregnancy) can influence subsequent responses to endogenous or exogenous steroids in anxiety-like and cognitive behavior of young and middle-aged rats (Kinsley et al., 1999; Byrnes and Bridges, 2006; Pawluski et al., 2006ab; Macbeth et al., 2008; Paris and Frye, 2008; Walf and Frye, 2008). Indeed, multiparous, compared to nulliparous, rats exhibit less memory decline with aging in a dry-land version of the water maze and lesser reductions in amyloid precursor protein in the CA1 and dentate gyrus regions of the hippocampus (Gatewood et al., 2005). Also, middle-aged multiparous, but not age-matched nulliparous, rats showed increased hippocampus cell proliferation following administration of E₂ or other estrogens, such as 17 α -E₂ and estrone (Barha and Galea, 2010a). Together, these data suggest that individual differences in responses to hormone replacement with aging may be related to present and prior endocrine milieu as well as individual capacity for metabolism. Thus, a limitation of the present study is that all rats were retired breeders from our colony, and may have responded differently than would have aged-matched nulliparous rats with a different life-time experience.

It is also of obvious importance to understand the role of timing in the effects of HRT. Indeed, the general heuristic is for women to use HRT for the briefest time possible so as to optimize symptom reduction while minimizing risk of reproductive cancers and stroke, which increases

with longer treatments. We do not know how long rats in the present study may have been in a state of altered ovarian production of steroids but, based upon the data in the vehicle-administered rats in the cohorts that could be assessed, there was low production of ovarian E₂ and P₄ among these middle-aged rats. Possibly, chronic dosing with HRT, which is more clinically-relevant, would have produced more robust effects on behavioral and steroid level endpoints. Other studies have demonstrated that there are clear effects of the dosing and timing of E₂, P₄, and HRT on central nervous system functions, such as cognition and affective behavior, that are coincident with trophic effects in peripheral, cancer-prone tissues (i.e. uterine growth or carcinogen-induced mammary tumors (Bohacek et al., 2008; Bohacek and Daniel, 2010; Daniel and Bohacek, 2010; Gibbs, 2000; Walf et al., 2009; Walf and Frye, 2009a^b; 2010a).

Further investigation of how trophic effects may limit the therapeutic options of HRT is of great interest. Natural E₂ and progestogens, as well as HRT, have clear trophic effects in the body, including mammary glands and reproductive tract (Jensen et al., 2010). Indeed, MPA is prescribed primarily to counter the growth-enhancing effects of natural estrogens or E₂-mimetic HRT in the uterus. Steroids also produce trophic effects in the brain (Barha and Galea, 2010ab; Frye and Walf, 2008; 2010). Ovarian steroids enhance neural plasticity in the cortex, hippocampus, and hypothalamus (Galea, 2008). *In vitro* studies find that CEE promotes neuronal growth in these regions (Diaz Brinton et al., 2000), which may be related to their functional roles in reproduction, brain protection/cognition, and stress/affect (Genazzani et al., 2004; Littleton-Kearney et al., 2005; Walf and Frye, 2008). In the present study, effects in the hippocampus were the focus, and we found reduced anxiety-like responding and increased hippocampus levels of E₂ and 3 α ,5 α -THP of middle-aged rats. While not determined at present, the factors that may lie downstream of these effects are of clear interest for further study. For instance, CEE increases neurotrophin levels in the hippocampus of middle-aged rats following chronic treatment (Engler-Chiurazzi et al., 2010), and can increase hippocampus synaptic density of young ovariectomized rats (Littleton-Kearney et al., 2005). Together, these findings support a clear role of steroids for growth-enhancing processes in the body as well as in the central nervous system.

Conclusion

The typically-prescribed HRT, CEE, when administered alone or in combination with MPA, reduces anxiety-like behavior of middle-aged female rats. There were apparent differences depending upon whether rats were maintaining reproductive status or in declining reproductive status. Also, these effects on anxiety-like behavior seem to be partly related to the capacity to form 3 α ,5 α -THP in the hippocampus. Investigations are ongoing to determine whether the beneficial effects of progestogens are due to trophic actions in the brain, independent of potentially unwanted trophic actions in the body.

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Figure 1.

Effects of vehicle (VEH), conjugated equine estrogens (CEE) and/or medroxyprogesterone acetate (MPA) on open field central entries (upper panel; mean $\pm$ sem) and elevated plus maze open arm time (lower panel; mean $\pm$ sem; sec) of middle-aged rats that were maintaining or had a decline in reproductive status. * $p < 0.007$ in *post hoc* tests, compared to the control group (maintaining reproductive status rats administered VEH).

Figure 2.

Effects of vehicle (VEH), conjugated equine estrogens (CEE) and/or medroxyprogesterone acetate (MPA) on plasma levels (mean + sem) of 17β-estradiol (E₂; pg/ml), progesterone (P₄; in ng/ml), dihydroprogesterone (DHP; ng/ml) and 5α-pregnan-3α-ol-20-one (3α,5α-THP; ng/ml), in middle-aged rats that were maintaining or had a decline in reproductive status. * p < 0.007 in *post hoc* tests, compared to the control group (maintaining reproductive status rats administered VEH).

Figure 3.

Effects of vehicle (VEH), conjugated equine estrogens (CEE) and/or medroxyprogesterone acetate (MPA) on hippocampus levels (mean + sem) of 17 β -estradiol (E₂; pg/g), progesterone (P₄; ng/g), dihydroprogesterone (DHP; in ng/g) and 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP; ng/g) of middle-aged rats that were maintaining or had a decline in reproductive status. * $p < 0.007$ in *post hoc* tests compared to the control group (maintaining reproductive status rats administered VEH).

Figure 4. Regression analyses showing that hippocampus levels of 5α -pregnan- 3α -ol-20-one ($3\alpha,5\alpha$ -THP) accounted for a significant proportion of variance in the open field (top) and elevated plus maze (bottom) of middle aged rats whose reproductive status was maintained or in decline.