Application for Review of Human Research: IRB Protocol Summary **Section II**
Social and Behavioral Sciences **November 28, 2016**

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PROTOCOL TITLE: Interaction of Estrogen and Serotonin in Modulating Brain Activation in Menopause

STUDY SPONSORSHIP

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INTRODUCTION AND PURPOSE

The overarching purpose of this study is to further our understanding of the individual and interactive effects of the hormone estrogen and the neurotransmitter serotonin on certain aspects of cognition and brain activation in menopausal women ages 48 to 60 years. Women will undergo cognitive testing and fMRI sessions both before and after 6 weeks of either estrogen or placebo administration. We will recruit women who are across the first 10 years and 11 months since their last menstrual period so that we can gather information regarding the potential impact of time since menopause on our outcomes of interest. We anticipate that findings from this study will help scientist and clinicians to refine their use of estrogen therapy in menopausal women. In addition, should the role of serotonin be of utmost importance for maintenance of healthy cognition, these data aid future drug development to preserve health cognition and/or to treat dementias in which serotonin is an important factor. This proposal is both novel and timely as results from this study are likely to provide information critical to the on-going discussion regarding the risks and benefits of ET use in menopausal women.

The aim of this study is to examine the interactive effects of estrogen and serotonin (5HT) on cognition, emotional processing, and brain activation. We will study the effects of acute tryptophan (TRP) depletion on cognition and mood in healthy menopausal women before and after estrogen replacement treatment (ERT). Using functional magnetic resonance imaging (fMRI), we will identify differences in brain activation during cognitive tasks with and without TRP depletion and before and after ERT in order to determine which brain regions and cognitive functions are affected by each manipulation.

OBJECTIVES

Specific Aim 1: To replicate and extend our previous behavioral findings of an interaction between ET and TRP-D on verbal memory in a group of early menopausal women randomized to receive ET (n=28). In a previous behavioral study of TRP-D and ET in healthy menopausal women (Amin et al., 2006b), we observed that TRP-D interfered with verbal memory to a lesser extent following ET than before. *In a new sample of women, we again hypothesize* (**H1.1**) *that paragraph recall performance and verbal paired-associates learning will decline as a result of TRP-D, but that ET will attenuate this decline.* In our previous study, we also found that length of hypogonadism (measured by months since last

menstrual period) was significantly associated with waning verbal memory performance. However, we did not have sufficient power with 18 women to detect whether the magnitude of ET effects differed based on length of time since menopause. *In this larger sample of 28 women, we hypothesize* (**H1.2**) *that women who have more recently experienced menopause will benefit more from ET than later menopausal women.*

Specific Aim 2: To evaluate the extent to which effects of ET and TRP-D on verbal working memory are mediated through the dorsolateral prefrontal cortex. Previous neuroimaging studies have associated ET with improved working memory performance and increased dorsolateral prefrontal cortex (DLPFC) activation (Duff & Hampson 2000; Shaywitz et al., 1999). However, it is unclear whether TRP-D has an effect on working memory (Harrison et al., 2004; Riedel et al., 2003). It is hypothesized (**H2.1**) that ET will increase DLPFC activation during working memory more so than PT, but there will be no effect of TRP-D and no interaction between estrogen and serotonin effects in either the ET or PT groups. Very few fMRI studies have examined the impact of practice or rescanning on behavioral measures or the BOLD response during cognitive tasks. We seek to fill that void with the proposed study by including a placebo arm. Since submission of the A2 application, we have obtained test-retest data from our MR colleagues (T. Constable, Ph.D.) demonstrating that with our new sample size of 28 women receiving ET and 14 women receiving placebo we can adequately test our hypothesis regarding ET and TRP-D effects on DLPC activation during the proposed working memory task.

Specific Aim 3: To determine the impact of adverse childhood events on brain activation during performance of the Emotion Identification (EmoID) and N-back Tasks pre and post ET and during sham and ATD. In healthy postmenopausal women, ET reverses the detrimental effects of ATD on verbal memory (Amin et al., 2006) and the impact of ATD on brain activation during cognitive an affective processing (Epperson, et al., 2007). ET will reverse the ATD related increase in amygdala activation to the EmoID Task while ET will reverse the ATD related decrease in dorsolateral prefrontal cortex (DLPFC) activation with the N-back Task in healthy, early postmenopausal women. This effect will be diminished in women with a score of $\geq$ 2 on the Adverse Childhood Event questionnaire (ACE).

Note Regarding Exploratory Aims*:* The following 2 aims have been relegated to an 'exploratory' status as this is now a 2-year grant and the sample size proposed herein may not be sufficient to adequately test this hypothesis. However, these data can be obtained at no additional costs as the main study expenses are related to recruitment and scans. Subjects will complete these tasks while in the scanner as described in the A2 application without increasing the subject burden above that of the previously reviewed application. These data will inform the development of additional RO1s aimed at examining the impact of ET on other aspects of verbal memory and affective processing in menopausal women.

Exploratory Aim 1: To evaluate the extent to which effects of ET and TRP-D on verbal memory are mediated through medial temporal lobe structures. TRP-D interferes with memory consolidation (McAllister-Williams et al., 2002; Park et al., 1994; Schmitt et al., 2000) and several studies have associated estrogen with improved verbal memory, possibly through effects on medial temporal lobe structures (Maki & Resnick 2000; Maki et al., 2001; Resnick et al., 1998; Shaywitz et al., 2003; Wolf et al., 1999). Again, there is little data examining the impact of practice or rescanning on behavioral measures or the BOLD response during a memory consolidation task. Our inclusion of a placebo group will fill the void and allow us to examine the effects that are specific to ET. We hypothesize (**HEA.1**) that hippocampal activation during sham and active depletion will differ during verbal encoding pre-ET, but not post-ET, when compared to the PT group. Within the two-year time frame of this study we will obtain sufficient data to more accurately determine the sample size necessary to adequately power

future investigations focusing on ET effects on the medial temporal lobe memory structures during verbal encoding.

Exploratory Aim 2: To evaluate the effects of ET and TRP-D on performance and brain activation during emotional processing. TRP-D has been associated with negative mood in certain vulnerable subjects and with bias toward negative stimuli (Klaassen et al., 2002). However, in our previous study (Amin et al., 2006a; see Preliminary Studies section, page 30), estrogen levels may be associated with brain activation indicative of bias toward positive stimuli. During an emotional go/no-go task, it is hypothesized (**HEA2.1**) that TRP-D will result in increased prefrontal cortex (PFC) activation to negative distracters, though this bias will be minimized in the ET group to a greater degree than in the PT group. Similarly, in response to an emotional face processing task, it is hypothesized (**HEA2.2**) that TRP-D will result in increased amygdala and orbitofrontal cortex activation to negative emotions, though this bias will be minimized following ET but not significantly following PT.

BACKGROUND

The menopause may be marked by changes in cognition and mood in addition to well-characterized vasomotor and urogenital symptoms (Devi et al., 2005; McVeigh 2005; Peeyananjarassri et al., 2006; Schnatz et al., 2006). While the administration of estradiol can relieve depressive symptoms (Schmidt et al., 2000; Soares et al., 2001) and may improve cognitive symptoms, particularly when used early in the menopausal transition (Sherwin 2003), the mechanisms by which estrogen modulates cognition and mood are still unclear. There is evidence of estrogen's interaction with the neurotransmitter serotonin (review in Amin et al., 2005), which is hypothesized to be involved in depression and various cognitive processes. Studies in animals have suggested decreased serotonin transporter (Kakiuchi et al., 2001) and receptor (Nyakas et al., 1997) binding with aging, and that treatment with serotonin precursors reduces age-related cognitive decline (Richter-Levin and Segal, 1996). Studies in humans also show changes in the serotonin system with aging (McEntee and Crook 1991; Palmer and DeKosky, 1993) and associations between cognitive decline in Alzheimer's disease, decreased serotonin levels, and changes in density of certain serotonin receptors (Lai et al 2005; Lai et al 2002). Furthermore, it is possible that there are sex differences in the effects of serotonin on cognition in aging, with women more likely to benefit from increased serotonergic transmission than men (Munro et al., 2004). Research in animals indicates that estrogen enhances serotonin transmission (review in Bethea et al., 2002) and observational evidence in humans has suggested that estrogen treatment may decrease the risk for Alzheimer's disease if initiated early (Henderson et al., 2005), suggesting a possible link between estrogen and age-related changes in the serotonin system. However, there is little information about estrogen-serotonin interactions in humans, largely because there are few methods that allow manipulation of both estrogen and serotonin.

The results of the proposed study are expected to provide insights regarding the clinical use of estrogen in healthy menopausal women. By examining the effects of estrogen treatment in women within 10 years of their last menstrual period, we aim to evaluate whether estrogen administration can improve certain aspects of cognition in women along the peri- and early menopause stages of life. *While the study proposed in this present application will not provide definitive information regarding 'when' and 'how long' women should use ET for cognitive benefits, it will provide data to guide future investigations. In addition, our findings are expected to further our understanding of the individual and interactive effects of estrogen and serotonin on specific aspects of cognition and brain regions thought to underlie those processes.*

Previous studies have found interactions between treatment with estrogen and with selective serotonin reuptake inhibitors (SSRIs). For example, administration of fluoxetine was found to suppress circulating estradiol levels and estradiol treatment-related enhancement of spatial memory in ovariectomized rats (Taylor et al., 2004). Whether the cognitive changes associated with this drug-hormone interaction were due to changes in estradiol level and/or secondary to primary neuronal effects could not be determined. As these rodents were acutely hypogonadal, it is not known whether the same interaction between SSRI and estrogen would occur with the gradual decline in estrogen characterized by a natural menopause. In the human laboratory, women with hot flashes and depressive symptoms demonstrated a better symptom reduction with low-dose conjugated equine estrogen (CEE) treatment plus fluvoxamine than with CEE treatment alone (Nagata et al., 2005). Whether fluvoxamine alone compared to CEE alone was more effective in the treatment of hot flashes and depressive symptoms was not addressed by the investigators. However, these findings suggest that for women experiencing vasomotor and mood symptoms in the menopause, a regimen that combines estrogen with a SSRI may be preferable.

Studies of ET in menopausal women indicate increased serotonin activity, (Halbreich et al 1995; Lippert et al 1996; Sherwin and Suranyi-Cadotte 1990; van Amelsvoort et al., 2001), improved mood (Schmidt et al., 2000; Soares et al., 2001), and, despite some inconsistencies across measures and hormone regimens, improved performance in cognitive domains such as verbal learning and memory (Hogervorst et al., 2000; LeBlanc et al., 2001; Rice and Morse 2003). In addition, estrogen-induced changes in the serotonin system have been found coincident with mood or cognitive changes (Kugaya et al., 2003; Sherwin and Suranyi-Cadotte 1990). Thus, the literature suggests a correlation between estrogen and serotonin activity and a change in mood or cognition in humans, but causation is unclear. It is possible that the regulatory activity of estrogen on the 5-HT system decreases if estrogen levels are low, contributing to the vulnerability of menopausal women to changes in mood and cognitive deficits. By combining ET, tryptophan depletion procedures, and functional magnetic resonance imaging (fMRI), it is possible to safely manipulate both estrogen and serotonin in humans, and non-invasively evaluate their individual and joint effects in the brain.

The acute tryptophan depletion (TRP-D) paradigm, a manipulation which results in rapid reduction of brain TRP and 5-HT levels, has been successfully used in human studies as a probe of central 5-HT function. Sex differences in response to acute TRP-D have been identified, suggesting hormonal influences on the serotonin system. For example, a PET study demonstrated that the rate of serotonin synthesis decreased significantly more in women than in men undergoing TRP-D (Nishizawa et al., 1997). In addition, a study of twenty healthy women found that TRP-D resulted in significant worsening of mood, though data from healthy men had shown no significant change (Ellenbogen et al., 1996). Given the likelihood that sex hormones modulate response to the TRP-D procedure, the combination of ET and TRP-D is expected to reveal interactions between estrogen and serotonin.

Brain Structures and Types of Cognition Modulated by Estrogen and TRP Depletion

The use of TRP-D in conjunction with ET would help to define a pathway by which estrogen-serotonin interactions may influence menopausal symptoms. ET has been associated with improved verbal memory (Maki et al., 2001; Resnick et al., 1998; Shaywitz et al., 2003; Wolf et al., 1999), semantic recall (Henderson et al., 1996), and figural memory (Resnick et al., 1998). Although an advantage to memory is a common finding, there have not been consistent results regarding estrogen's effects on specific fields of cognitive function (such as frontal lobe tasks or hippocampus-dependent memory tasks). Some studies have suggested that estrogen effects on cognition are through medial temporal lobe structures (Maki and Resnick, 2000; Resnick et al., 1998). However, others have found a

lack of advantage in simple recall but a verbal memory advantage in menopausal hormone users compared to non-users, suggesting helpful effects of estrogen on the prefrontal cortex (Duff and Hampson, 2000). Similarly, Keenan et al. (2001) suggest that executive function, managed by the prefrontal cortex, is affected by estrogen and that hippocampal memory deficits are only secondary. While recall ability did not differ between hormone users and non-users, women receiving hormone replacement therapy were better able to inhibit perseverative errors and performed better on an N-Back working memory task (Keenan et al., 2001). Furthermore, a neuroimaging study of figural and verbal working memory showed increased prefrontal cortex activation following estrogen treatment (Shaywitz et al., 1999).

In contrast, TRP-D has repeatedly been shown to impair learning, particularly verbal memory (McAllister-Williams et al., 2002; Park et al., 1994; Schmitt et al., 2000). Other studies have found response inhibition, decision-making, and processing of reward cues to be disrupted by TRP-D as well (Murphy et al., 2002; Park et al., 1994; Rogers et al., 1999; Rogers et al., 2003). Thus, it is hypothesized that temporal lobe memory structures and the orbitofrontal cortex are most affected by TRP-D. Consistent with hypotheses, fMRI studies have found that TRP-D reduces inferior prefrontal cortex activation during response inhibition (Rubia et al., 2005) and reduces hippocampal activation during encoding of verbal stimuli (van der Veen et al., 2006). In contrast to the deficits observed in memory consolidation and response inhibition, focused attention (Gallagher et al., 2003; Schmitt et al., 2000) and verbal fluency (Schmitt et al., 2000) measures have been improved by TRP-D and it is unclear whether the procedure has an effect on working memory (Harrison et al., 2004; Riedel et al., 2003). Also consistent with behavioral data suggesting that TRP-D improves attention, TRP-D has been found to improve performance and increase activation in the anterior cingulate during incongruent color word trials of the Stroop task, which require increased cognitive control (Evers et al., 2006).

Estrogen's and Serotonin's Effects on Affective Processing

Estrogen may also be an effective treatment for certain mood disorders (reviews in Epperson et al., 1999; Halbreich and Kahn, 2001) and has been associated with increased activation to positive stimuli (Amin et al., 2006a). Several studies have demonstrated the efficacy of estrogen administration for depression during the perimenopause (Schmidt et al., 2000; Soares et al., 2001) and suggest a possible role for estrogen in the treatment of postpartum psychiatric disorders (Ahokas et al., 2001; Gregoire et al., 1996). In studies from our lab (see Preliminary Studies section), we found that healthy young women undergoing an increase in estrogen during the course of the menstrual cycle exhibited brain activation indicative of bias toward positive stimuli (Amin et al., 2006a; Amin et al., submitted). Furthermore, activation to positive stimuli positively correlated with plasma estradiol level and activation to negative stimuli negatively correlated with estradiol level, suggesting that estrogen greatly influences processing of affective stimuli (Amin et al., 2006a).

In contrast, TRP-D has been associated with depressive relapse (Delgado et al., 1990). In healthy populations, only those who are vulnerable to affective disorders, for example, as indicated by family history or serotonin transporter genotype, are likely to experience mood-lowering effects of TRP-D (Benkelfat et al., 1994; Klaassen et al., 1999; Neumeister et al., 2002). However, there is evidence of healthy participants showing bias toward negative stimuli while undergoing TRP-D (Klaassen et al., 2002). In addition, a double-blind, placebo controlled, cross-over design study of TRP-D in healthy participants found that although there were no mood effects or general frontal lobe deficits, there was increased reaction time to happy words but not sad words in a go/no-go response inhibition task following TRP-D (Murphy et al., 2002). An fMRI study involving the same task (Elliott et al., 2002) suggests brain regions that may be affected by TRP depletion. Depressed participants showed

decreased activation in the anterior cingulate for emotional compared to neutral targets and there was increased medial prefrontal cortex activation to happy stimuli in healthy participants, but to sad stimuli for depressed participants (Elliott et al., 2002).

Use of ET Early and Late in Menopause

Differences in estrogen response are possible depending on how recently the menopause took place. While reviews and meta-analyses suggest a positive, albeit modest, effect of menopausal hormone therapy (HT) on verbal memory, attention, and reasoning, and associate it with a decreased risk of dementia (e.g., Hogervorst et al., 2000; Rice and Morse, 2003), most reports also cite methodological differences across studies. For example, there may be adverse effects depending on the type of HT or timing of HT use (Espeland et al., 2004). Several studies in rodents and non-human primates have also shown that treatment with estradiol can improve learning and memory. In aged non-human primates, as well, cyclic estradiol treatment improves spatial working memory and object recognition (Rapp et al., 2003). Working memory in aged rats was improved when chronic estradiol treatment occurred shortly after ovariectomy, but not if it took place after a delay of five months (Daniel et al., 2006). Initial data in humans parallel these preclinical findings. Some meta-analyses suggest that mainly symptomatic perimenopausal women (who are within 12 months of their last menstrual period) experience cognitive improvement as a result of HT (LeBlanc et al., 2001; Yaffe et al., 1998). This may relate to coincident improvement in menopausal symptoms such as insomnia, but recent evidence suggests that estrogen use early during menopause can exert positive effects on cognition (Bagger et al., 2005; Sherwin, 2003). Similarly, while estrogen administration does not appear effective in the treatment of major depression in *post*menopausal women (Morrison et al., 2004), two double-blind placebo controlled studies (Schmidt et al., 2000; Soares et al., 2001) have shown that estradiol administration was effective in treating *peri*menopausal women experiencing major or minor depression. Moreover, recent findings from the Nurses Health Study, a large-scale observational study of HT in menopausal women, found that onset of HT within the first 4 years of the LMP was associated with a reduction in coronary heart disease (CHD) while there was no association between CHD and onset of HT 10 years or more since LMP (Grodstein, 2006). Reassessment of data from the Women's Health Initiative (WHI) also suggested cardiovascular benefit of ET over placebo for women who were 50-59 years old at the time enrollment (Hsia et al., 2006). Findings from a WHI ancillary study indicates that in these same women aged 50-59 years old, ET use was associated with a lower coronary artery calcified-plaque burden at the end of the study (Mason et al., 2007). Thus, for multiple organ systems, it appears that timing of HT may be critical to the risk benefit profile of ET and HT.

Prepubertal Adversity, Depression & Cognition: Importance of Individual Factors

Findings from this investigation will advance knowledge regarding individual factors, such as early life adversity, that may contribute to risk for depression and poor cognitive aging during periods of waning estrogen in human subjects. This investigation will add to the growing literature that adverse childhood experiences have powerful effects on adult disease, as well as help public policy, development of new interventions, and consideration of life experience when making clinical recommendations to an individual. By examining history of early life adversity, a known detriment to intact serotonin function (Bethea et al., 2011; Murrough et al., 2011; Shively, et al., 2003) we will significantly enhance our understanding of the estrogen serotonin interaction in affective processing and working memory in the 'real world'. Although adverse childhood experiences are unfortunately quite common, occurring in up to 65% of women (http://www.cdc.gov/ace/prevalence.htm), this will be the first study, to our knowledge, to take into consideration the potential impact of prepubertal adversity on serotonergic function as it relates to emotion processing, working memory, and neural response to ET.

STUDIES OF TRYP-D IN MENOPAUSAL WOMEN

Impact of TRP-D on Verbal Memory: Menopausal Women in Remission from Depression

Pilot data from our group at Yale provided additional evidence that TRP-D has adverse effects on performance on verbal memory. In a study of 11 peri- and post-menopausal women who were treated for major depression, there was a significant effect of TRP-D compared to sham depletion on cognition, but not mood (Epperson et al., 2006). Subjects recalled fewer items in a delayed as compared to immediate paragraph recall task during both sham and active depletion, $F(1,10) = 25.43$, $p<0.01$, but active depletion resulted in significantly lower recall scores, $F(1,10)= 7.34$, $p<0.05$, particularly during delayed recall (Figure 1).

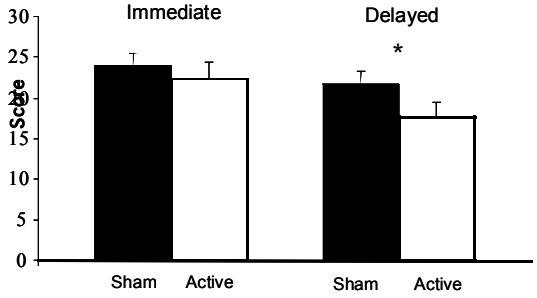
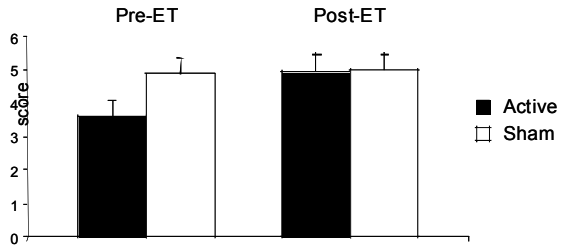


Figure 1: Paragraph recall scores during sham and active TRP-D in recently depressed menopausal women (Mean ± SEM; asterisk marks condition significantly different from all others; Immediate Sham and Delayed Sham were also significantly different from each other)

Impact of TRP-D and ET on Verbal Memory: Healthy Menopausal Women

In another study of 19 healthy peri- and post-menopausal women, we found that TRP-D significantly worsened delayed recall on the logical memory (paragraph recall) and paired associates subtests of the Wechsler Memory Scale (Amin et al., 2006b). Interestingly, 8-10 weeks of estradiol (Vivelle patch 0.075-0.150 mg/d) administration was associated with an improvement in performance when undergoing TRP-D, $F(1, 43.4)= 4.23$, $p= 0.046$, such that there was little difference between TRP-D and sham depletion with respect to performance on these cognitive measures (Figure 2). Thus, estradiol appears to "protect" subjects from the adverse effects of TRP-D and thus rapid lowering of brain serotonin.

Figure 2: Interaction between ET and TRP-D on mean paired-associates score. (Mean ± SEM; asterisk marks condition significantly different from all others)



fMRI STUDIES OF OVARIAN HORMONE EFFECTS ON EMOTIONAL PROCESSING

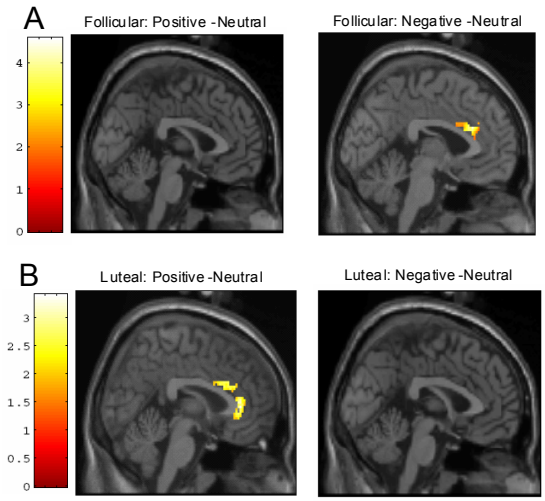
We have conducted fMRI experiments similar to the proposed studies in healthy cycling women in order to examine whether brain activation during cognitive-affective tasks is modulated by ovarian hormones. Using the emotional Stroop task, ovarian steroid fluctuation was found to influence attention toward emotional information in a valence-specific manner (Amin et al., submitted). While the low-hormone follicular phase was characterized by increased anterior cingulate cortex (ACC) activation to negative words, the mid-luteal phase (high estrogen and progesterone) was characterized by increased activation to positive words, suggesting that high levels of ovarian hormones may be associated with attention to positive emotional stimuli (Figure 3). Similarly, the subgenual ACC, whose dysfunction has been associated with mood disorders (Mayberg et al., 1997), showed significantly decreased activation to negative words during the mid-luteal phase compared to the follicular phase. Using an emotional response inhibition task (identical to the proposed emotional go/no-go task), we found increased anterior cingulate and dorsolateral prefrontal cortex activation during response inhibition to positive stimuli in the luteal phase of the menstrual cycle, when there are high levels of estradiol and progesterone (Amin et al., 2006a; Please see Appendix for complete manuscript).

Figure 3: Significant (p < 0.05) increased ACC activation to emotional, relative to neutral, stimuli.

A. **Follicular phase activation to negative, relative to neutral, words.**

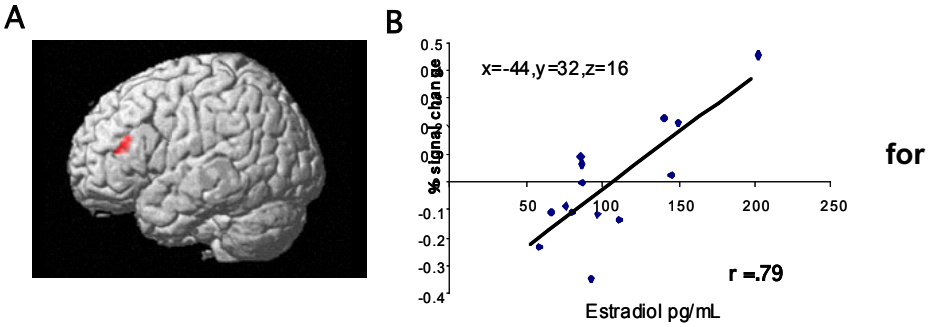
B. **Luteal phase activation to positive, relative to neutral, words.**

Scale represents *t*-values.



Furthermore, across participants, luteal phase levels of estradiol but not progesterone were significantly positively correlated with activation in several structures (including in the DLPFC; Figure 4) during response inhibition to positive stimuli, and negatively correlated with activation during response inhibition to negative stimuli. Thus, estrogen levels were not only significantly associated with brain activation, but these associations were also valence-specific, suggesting a direct relationship between estrogen and brain activation during emotional processing.

Figure 4: Activation (p < 0.005) within the DLPFC associated with luteal estradiol level (controlling progesterone) in response to positive distracters, relative to targets. *A*. Location of clusters of significant activation. *B*. Correlation between luteal estradiol level and DLPFC activation.



for

ABILITY TO MEASURE BASELINE CHANGES IN BLOOD FLOW

Because estrogen is known to have vascular effects and may influence blood oxygen level dependent (BOLD) signal via mechanisms unrelated to cognition, we will use perfusion scans to measure cerebral blood flow (CBF) at rest and enter these data in our statistical models when analyzing fMRI data in order to control for changes in baseline blood flow. If there are effects on CBF due to the TRP-D procedure, these may be controlled for as well.

Using pulsed arterial spin labeling (ASL), we have measured baseline resting state absolute CBF in a series of volunteers and measured changes in CBF during auditory stimulation (Figure 5). Clear primary auditory cortex activation is seen in this example.

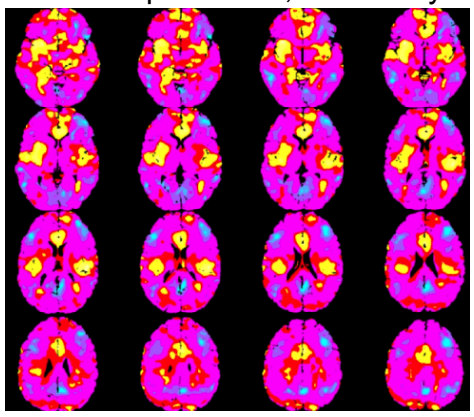
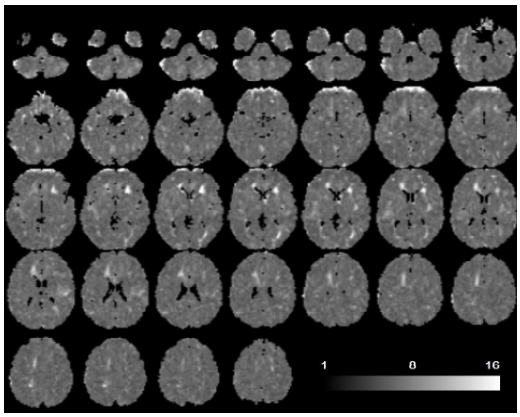


Figure 5: Multisubject data set showing (right) whole brain coverage moving from the bottom of the brain to the top in 16 slices.

The ASL approach has also been applied in a test/retest manner in order to get a sense of the reliability of this approach both between and across subjects. The data show that the variance is regionally specific and that good within subject repeatability and excellent across subject repeatability are obtained (Figure 6). The data indicate that 16 subjects are required



to generate a $p < 0.05$ with a 40% increment in blood flow with activation, suggesting that we will have sufficient power to detect changes in blood flow as a result of ET or TRP-D.

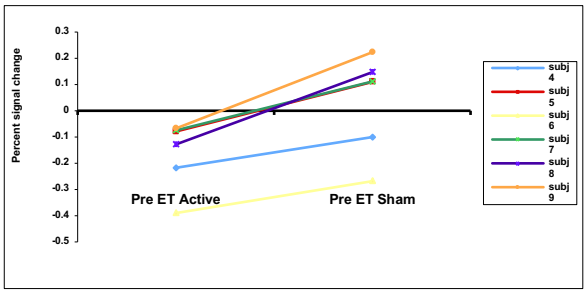
Figure 6: Resting state CBF maps in 16 subjects scanned twice to generate inter- and intra-subject variance maps for CBF. The colorbar represents the number of subjects required to obtain a p<0.05 level of significance corrected for multiple comparisons in the awake state.

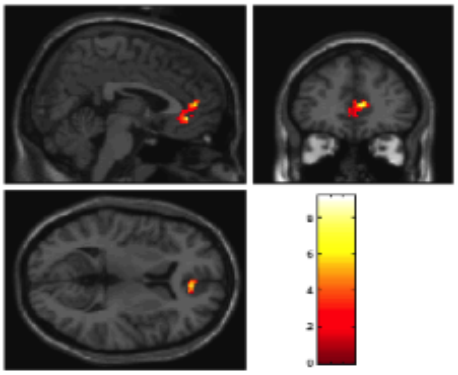
fMRI Studies of Menopausal Women Undergoing TRP-D and ET

To date we have pilot data collected on 9 subjects; this data is from the PIs study at the Yale University School of Medicine. This data was used for our R01 grant submission. The figures below include data from 6 of the 9 subjects. One subject withdrew before her first test day could be scheduled and another was excluded when we decided to limit our recruitment to women 10 years within LMP. A third subject discontinued estrogen during travel between Test Day 3 and Test Day 4. Thus, we have full data from 6 subjects who completed all 4 test days. It is very important to reiterate that this sample size is too small to conduct meaningful statistical analyses. These data are presented solely to demonstrate 1) our ability to conduct the proposed study, 2) that the experiments are appropriately designed to investigate BOLD signal changes within the regions of interest, and 3) to show similar changes in activation across subjects across test days, supporting that we will find group effects.

To demonstrate these effects, we have conducted a preliminary group analysis to identify similarities in activation in regions of interest (p<0.05 uncorrected). We include the data below as they demonstrate differences ***between test days*** *and the* ***individual effects of TRP-D*** *on brain activation during the 2-back verbal working memory task. (Rt ACC shown in figure)*

Figure 5: Prior to ET, Active TRP-D resulted in decrease in ACC activation in the 2-back verbal working memory task.





In order to examine the ***individual effects of ET*** *on verbal working memory, we compared brain activation on sham depletion test days pre-ET and post-ET. As reported in our previous application, we continue to see ET-enhancement in DLPFC activation during the 2-back test.*

Figure 6: ET enhanced activation in the DLPFC on the 2-back test.

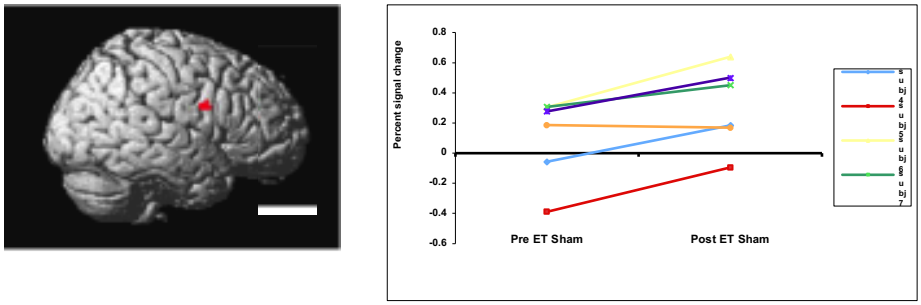


Figure 7: Supporting our hypothesis, there was a decrease in right amygdala activation during the faces task (angry relative to neutral) on post-ET sham test days compared to pre-ET sham test days.
p < 0.05, FDR-corrected, masked for amygdala

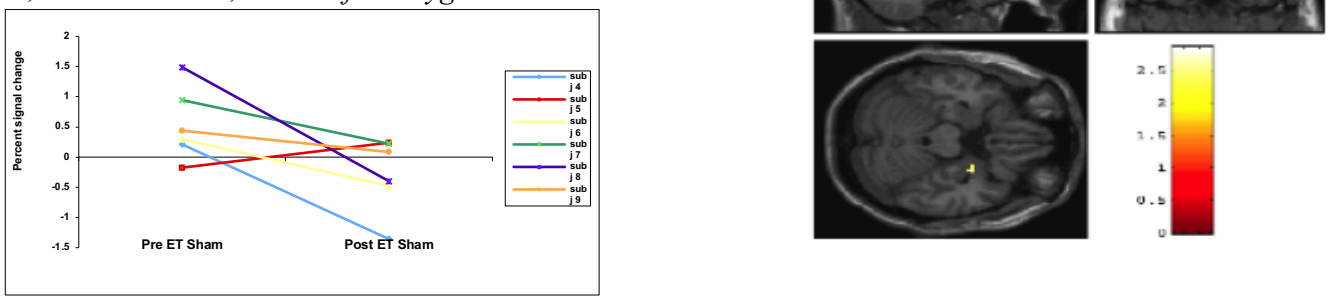
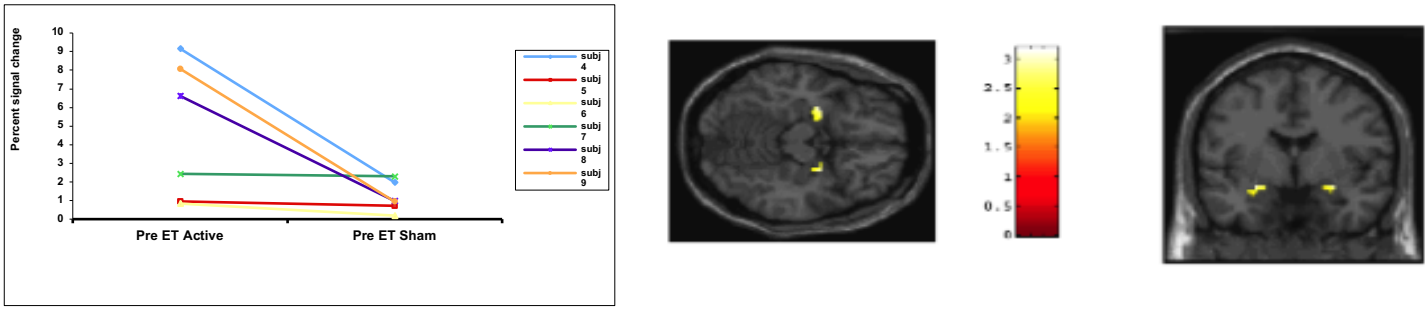


Figure 8: Data depicted here suggests that a future study examining the frequency of genetic polymorphisms of the serotonin transporter may be informative. Three of 6 women experienced an accentuation in amygdala activation during the faces task (all faces relative to fixation) on active TRP-D test days compared to sham test days prior to ET. These findings suggest that there may be genetic factors or other individual variables that lead to more dramatic CNS effects of reduction in serotonin. *(Rt Amygdala 25 voxels, x=28, y=2, z=14, p<0.05)*



Summary of Preliminary Studies

Preliminary studies verify our ability to conduct studies of TRP-D and ET in menopausal women and support that we will find significant interactive effects of TRP-D and ET on verbal memory. Furthermore, using identical imaging parameters as in this proposed study, we have previously found significant ovarian steroid modulation of brain activation during cognitive-affective tasks. Considering estrogen's vascular effects, we are also capable of addressing a possible confound of baseline CBF change following ET. Data presented in Figures 5 and 6 indicate that under certain conditions we can expect to find whole group effects, while data presented in Figure 8 suggests that there may be divergent within group effects. Finally, pilot fMRI data from 6 menopausal women who have completed the proposed procedures confirm that the experiments are appropriately designed to investigate changes in BOLD signal across test days within regions of interest.

CHARACTERISTICS OF THE STUDY POPULATION

1. Target Population and Accrual

Women from the greater Philadelphia and surrounding areas who are ages 48 – 60 and determined to be peri- or post-menopausal per the following guidelines will be considered for enrollment into this study:

Women will be included in the study if they have a FSH ≥ 30 IU/ml and either report that their LMP was at least 12 months prior to presentation (postmenopausal women) OR if they report menstrual cycle irregularity with menstrual cycle length of <24 or > 36 days duration (perimenopausal women). Length of menopause will be used as a continuous variable of number of months since LMP when analyzing performance on cognitive tasks and activation within regions of interest. For women who have previously used HT, months since LMP will be determined as the total length of time since their last menstrual period during which they have not used HT.

We anticipate the composition of the proposed study population to reflect the greater Philadelphia referral base. The projected composition from Philadelphia is African American 42.58%, Hispanic 8.5%, Asian 4.42%, Other 2.04% and White, not of Hispanic Origin, 42.46%. The size of our expected samples will limit our ability to detect effects due to ethnicity or minority status, but we may have sufficient data to begin to examine the impact of ethnicity on estrogen's and serotonin's effects on brain activation. We will make attempts to enhance recruitment of minorities by making sure that fliers and brochures are available at a variety of locations throughout the Philadelphia area as well as promoting a marketing campaign that includes direct home mailings throughout the greater Philadelphia metropolitan area.

2. Key Inclusion Criteria:

Women ages *48* to 60 (at the time of enrollment) will be eligible for this study if they:

1. Have no history of major depressive disorder, generalized anxiety disorder, and or panic disorder within the last three years according to the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-NP) (First et al., 1995), or a history of major depressive disorder, generalized anxiety disorder, and or panic disorder greater than 3 years ago, but now resolved according to the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-NP) (First et al., 1995);
2. Have no substance abuse disorders (this includes alcohol, prescription, and illicit substances) within the last three years according to the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-NP) (First et al., 1995);
3. Subject has history of substance abuse disorders (this includes alcohol, prescription, and illicit substances) ≥3 years ago but the period of abuse did not last more than 5 years according to the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-NP) (First et al., 1995);
4. No first-degree relative (excluding children) with a known psychotic disorder or bi-polar disorder per patient report. Psychotic disorders include schizophrenia, schizoaffective disorder, psychotic disorder;
5. Have not taken hormonal contraceptives, ET or HT for at least 3 months as per self-report;
6. Are within 10 years and 11 months of LMP as per self-report;
7. Have a follicular stimulating hormone level (FSH) of ≥30 IU/ml as per hormone testing results; women with an FSH below 30 will have the option to undergo an additional blood draw between 3-9 months following the initial blood draw (see note 2 below);

8. Are able to give written informed consent;
9. Provide written documentation of having had a normal mammogram and a PAP within the recommended timeframe as defined by the American College of Obstetricians and Gynecologists (ACOG) – please visit their website for current recommendations;
10. Must have clear urine toxicology screen upon recruitment;
11. Are fluent in written and spoken English;
12. Are right-handed.

3. Key Exclusion Criteria

1. Currently smoking more than 10 cigarettes/day by self report;
2. History of clinical CVD including myocardial infarction, angina, or congestive heart failure;
3. History of thromboembolic disease (deep vein thrombosis or pulmonary embolus);
4. History of untreated (no cholecystectomy) gallbladder disease as per self-report during PE;
5. History of triglyceridemia by subject report;
6. Undiagnosed vaginal bleeding as per self-report;
7. History of estrogen responsive cancers as per self-report;
8. Known hypercoagulable state (thrombophilias) as per self-report;
9. Severe lactose intolerance (sham depletion requires lactose/microcellulose administration; mild to moderate lactose intolerance is acceptable); Dr. Epperson will make the final decision whether an individual's lactose intolerance is severe enough to require exclusion;
10. Use of estrogen- or progestin-containing medication or phytoestrogen containing supplements (e.g. soy concentrates or extracts) within 3 months of participation as per self-report; foods containing soy (e.g. tofu, soy milk) will be permissible; estrogen-based localized treatments such as creams and vaginal inserts will be permissible, so long as said treatments do not effect systemic estrogen levels (women using localized treatments must have estrogen levels similar to other women in the study of their age and menopause status). PI will have final decision about enrollment (see note 3 below);
11. Have a Mini Mental Status Score of $\leq$ 25;
12. Hamilton Depression Score > 14;
13. As per self-report, have taken a psychotropic medication within the previous month, with the exception of sleeping aids if the participant is willing to forgo use during study participation;
14. Have a metallic implant as per self-report;
15. Are claustrophobic as per self-report;
16. Are pregnant (pertains to peri-menopausal women only).

Note 1: In the case of participants with full or partial hysterectomy, timing of final menstrual period will be determined by Dr. Epperson (the study PI) or one of the study MDs. In cases in which final menstrual period cannot be established, subjects will be excluded from the study.
Note 2: Women who undergo the repeat FSH blood test will be enrolled if their levels are $\geq$ 30. Women will not be required to repeat all admission procedures unless they report experiencing a life event which would impact their mental or physical health and well-being. The PI will make the final determination regarding what, if any, screening procedures need to be repeated.
Note 3: Women on localized estrogen treatments who show elevated systemic estrogen levels will not be enrolled. Instead, they will need to discontinue use for 1 month and then have their estrogen levels retested with an additional blood draw. PI will have the final decision regarding eligibility.

4. Subject Recruitment and Screening

This research study will be conducted at the Penn Center for Women's Behavioral Wellness (PCWBW), which is a clinical research endeavor in the Departments of Psychiatry and Obstetrics and Gynecology. Women will be recruited for this study by paid advertising, home mailings, and flyers and brochures in doctor's offices, libraries, and local stores. We will also be utilizing the Penn Data Store analysis in which we will request names, phone numbers, home addresses and email addresses for menopausal women between the ages of 48-60. Women who provide email addresses acquired via the Penn Data Store may be contacted via email, including an e-letter and a link to a survey assessing their qualification for study participation. Women who do not provide an email address may be contacted via a letter mailed to the provided address and/or by a phone call to the number provided to us. All women who contact PCWBW regarding study participation will have the study explained to them using the IRB approved phone script. Following the phone script all women who express interest in the study will have the option to complete a phone interview to determine eligibility. We have requested a waiver of documentation of informed consent to complete this interview. Women will also have the option to complete the ACE interview at the time of their phone interview. Women who appear to meet the entrance criteria after a brief phone screen will be scheduled for an in-office appointment in the Program's private office setting at 3535 Market Street. During recruitment, potential participants will be explained that to offset their time commitment, they will be compensated $200 for each test day. In addition, they will receive an additional $250 if all four tests are completed, which encourages participants to complete the study. ***Recruitment materials have been previously submitted to and approved by the IRB.***

5. Early Withdrawal of Subjects

Early withdrawal of subjects may occur for the following reasons:

1. If the subject fails to return for visits.
2. Failure to locate the subject.
3. The subject withdraws their consent for participation.
4. The PI determines that the subject is no longer eligible for participation in the study.

A subject can withdraw their consent for participation in this study by contacting the study PI and or the research staff personnel who is following them in the study via telephone and informing them of their decision. In this case it would be preferable if the subject is willing to come in to the office for a debriefing regarding whether or not to continue estrogen treatment under the care of their personnel physician. If this is not possible, then the PI will speak with the subject by telephone to conduct this debriefing and find out whether or not the subject plans to stay on estrogen treatment. The subject's decision to withdraw and their disposition upon withdrawing from the study will be recorded in their research chart.

6. Vulnerable Populations:

Children, pregnant women, fetuses, neonates, or prisoners are not included in this research study.

7. Populations vulnerable to undue influence or coercion

Penn students and employees (both of appropriate age and study criteria), and economically disadvantaged persons, are welcome to participate in this study. The participants will be appropriately consented and their information will be kept in a locked cabinet, filed by a code, in a locked room that only the research study personnel has access to.

STUDY DESIGN

7:30 AM	Subject met at CHPS/PCWBW and initial assessments conducted; this includes the blood draw conducted by research nurse to determine estrogen, progesterone, serum hormone binding globulin, DHEA, and tryptophan levels
8:00 AM	Subject begins ingestion of 70 capsules with nurse present
8:30 AM	Subject completes ingesting capsules
2:00 PM	Assessments and memory testing
2:30 PM	Blood draw conducted by research nurse to determine change in free and total tryptophan
3:00 PM	Begin fMRI scan
3:45 PM	Post-scan recognition test
4:00 PM	Subject given meal and discharged after seeing a physician

METHODS

1. Study Instruments

We will collect the following information and administer the following instruments during the screening phase after obtaining written, informed consent from each participant. Those assessments in **bold font** will also be administered on test days. Those in *italics* will only be collected during admission and on test days 2 and 4. **Please note the ACE questionnaire will typically be administered verbally over the phone prior to presenting for the first in-office visit, however, women who do not want to complete the assessment over the phone will be given the option to complete the assessment in-office. The ACE will be administered a second time at test day three to assess the reliability of participant report. All discrepancies between initial ACE report and follow-up ACE will be documented and clarified with the participant.

1. Demographic information including age, education, marital status, race, income and insurance status.
2. Full gynecologic history including age at menarche, menstrual cycle length/regularity and menstrual cycle-related changes in mood and behavior, obstetrical history, mood related to pregnancies, and onset of menopausal symptoms and menstrual irregularity.
3. Information about family history of psychiatric disorders, alcoholism, and nicotine dependence in parents and siblings (obtained from subject report).
4. Structured Clinical Interview for Diagnosis (SCID) based upon the DSM-IV: The SCID interview is a widely used clinical interview to assess the presence of lifetime and present history of psychiatric and substance dependence disorders (First et al., 1997).
5. Mini-Mental State Examination (MMSE): MMSE is a brief quantitative screening measure of cognitive status. With a total 30 possible points, the instrument measures orientation, auditory registration, working memory, recall, language, and constructional skills.
6. Clinician Administered PTSD Scale (CAPS): The CAPS is a 30-item structured interview that corresponds to the DSM-IV criteria for PTSD. The CAPS can be used to make a current (past month) or lifetime diagnosis of PTSD or to assesses symptoms over the past week. (Blake et al., 1995)
7. Traumatic Life Events Questionnaire (TLEQ)*:* The TLEQ is a 23-item self-report measure of 22 types of potentially traumatic events including natural disasters, exposure to warfare, robbery involving a weapon, physical abuse and being stalked. The last question asks respondents to identify the one event that “causes you the most distress” among those endorsed. Respondents

are also asked about their age upon first occurrence, date of last occurrence, and amount of distress the event causes. This measure is commonly used for research purposes (Kubany et al, 2000).

8. **Adverse Childhood Experiences (ACE)*:* The ACE is used to determine a person's number of experienced adverse events prior to age 18. Exposure to one category (not incident) of ACE, qualifies as one point. When the points are added up, the ACE Score is achieved. An ACE Score of 0 (zero) would mean that the person reported no exposure to any of the categories of trauma listed as ACEs above. An ACE Score of 9 would mean that the person reported exposure to all of the categories of trauma listed above (Felitti et al., 1998).
9. Brief COPE: This short scale assesses how and how successful an individual's coping ability is when faced with adversity. (Carver, 1997).
10. Social Support Scale (SSS): The SSS assesses the amount of perceived social support individual's believe they have. (Zimet et al., 1998)
11. Utian Quality of Life Questionnaire (UQoL): The Utian QoL questionnaire is a brief instrument, which measures QoL during the perimenopausal years with 23 questions, demonstrating four separate intercorrelated domains (Utian et al., 2002).
12. **The Speilberger State-Trait Anxiety Scale (STAI)**: is a commonly used measure of trait (current) and state anxiety (general) (Spielberger et al.,1983). All items are rated on a 4-point scale (e.g., from "Almost Never" to "Almost Always"). Higher scores indicate greater anxiety.
13. **Hamilton Depression Scale (HAM-D)**: The HAM-D is a clinician-rated instrument widely used to assess severity of depressive symptoms (Hamilton, 1960).
14. **The Profile of Mood States** (**POMS)**: The POMS asks subjects to rate their mood over the last week (or other defined period of time), according to 65 adjectives, such as "friendly," "listless," and "on edge," using a 5-point Likert scale. The psychometric properties of this commonly-used test are well characterized (McNair et al., 1992).
15. **Perceived Stress Scale (PSS):** The PSS is the most widely used psychological instrument for measuring the perception of stress. It is a measure of the degree to which situations in one's life are appraised as stressful. Items were designed to tap how unpredictable, uncontrollable, and overloaded respondents find their lives. The 10-item scale also includes a number of direct queries about current levels of experienced stress. The PSS was designed for use in community samples with at least a junior high school education. The items are easy to understand, and the response alternatives are simple to grasp. Moreover, the questions are of a general nature and hence are relatively free of content specific to any subpopulation group. The questions in the PSS ask about feelings and thoughts during the last month. In each case, respondents are asked how often they felt a certain way (Cohen at al., 1983).
16. **Visual Analogue Scale (VAS)**: A VAS instrument listing 17 adjectives, including "talkative," "drowsy," and "nervous," and allowing subjects to mark how much of each they feel by choosing a point on a horizontal line will be used as another measure of mood.
13. **Daily Hot Flash (HF) Diary**: Women will be given a form to use in reporting the number of vasomotor events and their severity. Subjects will be instructed to complete this form daily throughout the course of their study participation. Women will rate each HF on a scale of 1 to 4 with 1=mild to 4=very severe. The score is calculated by multiplying the total number HFs by their severity score and summing the results for each day. The mean for the week prior to testing and the 6th week of ET or PT will be compared. This method for assessing HF severity has been validated in a series of pharmacologic trials. *This rating is only given to participants who presently report experiencing hot flashes.
14. **Beck Depression Inventory (BDI):** The BDI was originally developed to detect, assess, and monitor changes in depressive symptoms among individuals. The sum of scores on the BDI

indicates the severity of depressive symptoms. In the general population, a score of ≥ 21 represents depression.

15. *Pittsburgh Sleep Quality Index (PSQI):* The PSQI questionnaire is used to assess recent quality of sleep and sleep disturbance (Buysse et al., 1989).

Additionally, all subjects will undergo a blood draw that includes a cholesterol screen and urine pregnancy test (if perimenopausal) during the screening process. The PI will also administer an ECG at this time to rule out for cardiovascular disease. Subjects must also provide documentation of having had a normal mammogram within the previous year, and a normal PAP and pelvic exam within the previous three years.

2. Group Modifications

Not applicable.

3. Method for Assigning Subjects to Groups

After completing the first two TRP depletion test sequences, participants will be randomized to receive either treatment with 17β-estradiol (Vivelle Dot® 0.10 - 0.15 mg/day) or a look-alike placebo patch for a total of 8 weeks. The research pharmacist who is responsible for dispensing the estrogen or placebo patches will also be responsible for the randomization of all subjects together with the statistician on the study. Randomization will be blocked and stratified. We will use a block size of 3 to be able to maintain a 2:1 ratio of estrogen to placebo within each stratum. To stratify randomization based upon time since last menstrual period by tertiles (1^{st}=0-40 mos; 2^{nd}=40-80 mos; 3^{rd}=80-120 mos), we will prepare separate randomization tables for each stratum. The research pharmacist, PI, and research project manager will know to which group the subject has been randomized. The research pharmacist, PI or research project manager can be reached at any time should it become necessary to unblind a study participant immediately. The subject and all other study-related personnel (clinicians, raters, fMRI technicians, etc.) will be blind to subject assignment.

4. Administration of Surveys and/or Process

Assessments to be administered during the intake interviews:

- Demographic information including age, education, income, health insurance status, marital status, and race;
- Full gynecologic history including age at menarche, menstrual cycle length/regularity and menstrual cycle-related changes in mood and behavior, obstetrical history, mood related to pregnancies, and onset of menopausal symptoms and menstrual irregularity;
- Information about family history of psychiatric disorders, alcoholism, and nicotine dependence in parents and siblings (obtained from subject report);
- Structured Clinical Interview for Diagnosis (SCID; First et al., 1997);
- Mini-Mental State Examination (MMSE);
- The Profile of Mood States (POMS; McNair et al., 1992);
- Utian Quality of Life Questionnaire (Utian QoL; Utian et al., 2002);
- Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989);

- Clinician Administered PTSD Scale (CAPS; Blake et al., 1995);
- Social Support Scale (SSS; Zimet et al., 1998);
- Hamilton Depression Scale (HAM-D; Hamilton, 1960);
- Traumatic Life Events Questionnaire (TLEQ; Kubany et al, 2000);
- Adverse Childhood Experiences (ACE; Felitti et al., 1998)—to be assessed over the phone OR during the in-office admission-screening process;
- Brief COPE (Carver, 1997);
- Perceived Stress Scale (PSS; Cohen at al., 1983).
- Beck Depression Inventory (BDI)
- The Speilberger State-Trait Anxiety Scale (STAI; Spielberger et al.,1983)
- Visual Analogue Scale (VAS)

Assessments to be administered on test days:

- The Profile of Mood States (POMS; McNair et al., 1992);
- Visual Analogue Scale (VAS);
- Daily Hot Flash (HF Diary).
- Beck Depression Inventory (BDI)
- Perceived Stress Scale (PSS; Cohen at al., 1983).
- The Speilberger State-Trait Anxiety Scale (STAI; Spielberger et al.,1983)
- Hamilton Depression Scale (HAM-D; Hamilton, 1960);
- Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989)—test days 2 and 4 only;

5. Data Management

Research data will be collected and recorded on case report forms specifically designed for this study. Data will be entered into a study database on a non-portable computer which is password protected. All of the information obtained from subjects is quoted by number and kept in locked confidential files. This information is not available to anyone except the investigators and study staff, and is not identified by the subject's name. The investigators and anyone else involved with this project are not allowed to reveal any identifying characteristics about the patients who participate in this study without their written consent. In the case of published reports from this study, care will be taken so that no individual subject will be identifiable.

All data will be stored in a locked file cabinet in a secure area of the clinic. Only those investigators directly involved with the project have access to this information. The researchers and study personnel involved with this project will abide by all regulations to keep subject's participation in this study confidential. However, a limit to confidentially exists in the possibility that a subject may discuss with friends and family their participation in this study. In addition, we as the investigators and study personnel are legally required to report abuse of children, elders or that the subject is a danger to themselves or others.

When the research is complete, the data will be stored in a locked file cabinet for up to 7 years. After 7 years, the study data will be archived with a professional medical records archive company.

6. Management of Information for Multi-Site Research where a Penn Investigator is the Lead Investigator of a Multi-Site Study, or Penn is the Lead Site or Coordinating Center in a Multi-Site Study.

Not applicable.

7. Subject Follow-up

Multiple subject visits are a required for this study. Efforts will be made to engage the subjects in the importance of the project, their participation, and what they are gaining from participating (i.e., psychiatric work up, medical testing, and free short-term estrogen treatment). If a subject is lost to the study before all the data is collected, we will make every attempt to contact that person via telephone and mail. However, these attempts sometimes fail and subjects are permanently lost to follow up.

If the subject is imprisoned, committed to a mental hospital, hospitalized for long term care, admitted to a drug/alcohol residential program, or a residential living facility or alike during the course of their participation in the study, then the subject would no longer be eligible to participate in the study because these circumstances would indicate that the subject no longer met study criteria (i.e., due to onset of psychiatric or drug/alcohol problem or incarceration which would prevent them from participating).

STUDY PROCEDURES

1. Detailed Description

This study involves TRP depletion, evaluation of cognition, and the use of fMRI during cognitive and affective task performance both before and after estrogen or *placebo treatment (PT).* There will be four successful* TRP depletion test sequences: two test sequences one week apart prior to ET *or PT*, and two sequences one week apart following approximately *6-weeks of double-blind ET or PT*. Each TRP depletion test day will involve ingestion of 70 capsules. During one of each pair of tests, the 70 capsules will contain 31.5 g of lactose (sham depletion; for participants who are not lactose intolerant) or 31.5 g of microcellulose (sham depletion; for participants who have mild to moderate lactose intolerance), while during the other test they will contain a total of 31.5 g of amino acids, but no tryptophan (see below; Neumeister et al., 2004; Neumeister et al., 2005). The order of the tests (sham vs. active depletion) will be randomly assigned in double blind fashion by the research pharmacist. Imaging procedures will take place on each of the test days. Subjects will be required to remain at the Center for Human Phenomic Science (CHPS) for the entire Test Day except during the fMRI scan.**

TRP Depletion Tests:

Each of the four test days will begin in the morning, no later than 7:30 AM, following an overnight fast that begins at midnight and subjects will remain fasting until the end of the test session, approximately 8-hours. Subjects will be admitted to a specially designated testing room on the CHPS/PCWBW and will remain in the test room for most of the session, but will be free to walk about the room and the CHPS/PCWBW, use the facilities, and drink water. They will be continuously monitored throughout the day by a trained research nurse, with a medical research investigator available at all times.

Baseline behavioral ratings, blood samples, and vital signs will be taken from 7:30 - 8:00 AM. Initial assessments include measurement of sleep quality (PSQI; test days 2 and 4 only), and *hot flash severity (daily diary completed for previous 7 days)*. Mood will be evaluated using the HAM-D, BDI, POMS, STAI, PSS and VAS. Blood sampling in the morning on each test day will measure estradiol, progesterone, serum hormone binding globulin, DHEA, and baseline tryptophan (total and free) levels. Additionally, on the first test day only, one additional tube of blood will be drawn to examine genetics of estrogen synthesis and metabolism and genes for neurochemicals/systems modulated by estrogen and

other sex hormones. Functional polymorphisms to be examined include but are not limited to TPH2-703 G/T, 5HTTLPR SLC6A4, HTR1A-1019 C/G, rs6295, HTR2A-rs7997012. These functional polymorphisms are hypothesized to be linked to regulation of mood and/or aspects of cognition in human subjects. However, estrogen has pronounced effects upon multiple neurotransmitter systems and receptors involved in the regulation of cognition and mood including, acetylcholine, dopamine, glutamate and norepinephring in addition to serotonin. Therefore, we stipulate herein and in the subject consent form that we will limit all genetic analyses to those specifically related to estrogen and its neurotransmitter system targets as they relate to the regulation of mood and cognition. Following these baseline measurements, subjects will begin ingesting the 70 capsules of amino acid mixture or lactose/microcellulose sham. They will have approximately 30 minutes to ingest the capsules (8:00 – 8:30 AM), which will help to minimize discomfort. However, we anticipate the ingestion of capsules will begin prior to 8:00 AM, thus given each subject longer than 30 minutes to complete this task. In addition, a nurse will be present to insure subjects' safety during the process.

Please note that although ingesting 70 capsules may sound taxing, *all 8* women who began the study to date at Yale from our pilot study completed it. Thus, each of them took 70 capsules on four different test days. Because the capsules are ingested with a research nurse and experimenter present, participants tend to pace themselves by engaging in conversation between swallows and find the unpleasantness of the situation minimized. Even so, the average time needed to ingest all capsules has been only 25 minutes. According to one participant, the idea of 70 capsules is worse than actually taking them. Feelings of fullness and transient, but mild, nausea were the most common complaints. We switched to this particular method of TRP-D from that used in our previous studies (Epperson et al., 2006; Amin et al., 2006b) as our previous method required that women ingest 20 capsules in addition to 8 ounces of an amino acid drink. Despite flavoring the drink with chocolate syrup, it was gritty and unpleasant.

Approximately 5.5 hours following ingestion of the 70 capsules, participants will repeat evaluations of mood and undergo cognitive testing (1:30 – 2:00 PM). Immediately prior to the fMRI scan (2:30 PM), a second blood draw will be conducted to measure changes in free and total tryptophan. Participants will then be escorted to CAMRIS for performance of cognitive tasks while in a 3T MR scanner. Immediately prior to entering the scanner, all participants will submit a urine sample for toxicology and urine pregnancy tests, as required by CAMRIS. The scan will begin approximately 6.5 hours following ingestion of capsules. After the end of the test session, a regular diet will be resumed. We will provide a light lunch to all participants prior to discharging them at the end of the test day.

The second TRP depletion test will be administered 1-week after the first. If subjects were randomized to receive the capsules filled with 31.5 g of lactose/microcellulose (sham depletion) during the first depletion sequence, they will receive capsules filled with amino acids (active depletion) during the second sequence (and vice versa). The clinicians conducting the study and the subjects in the study will be blind to which mixture the subject is receiving in the capsules. Following approximately *6-weeks* of ET, another pair of test days will be scheduled one week apart, one sham and one active.

*In cases where participants do not fully complete a test day before being randomized to the study drug, they will have the option to repeat the unsuccessful test day in full.

**If subjects cannot commit to completing test days Monday through Friday, there is an option to complete scans on weekends. These participants will not be utilizing the services of the CHPS and will instead be monitored by a study MD during their test day instead of CHPS nurse. These women will report to 3535 Market Street and will complete the test day exactly as described above, with the

exception of spending the test day at the CHPS. They will be asked to remain in the PCWBW office until they are brought to CAMRIS for the afternoon testing session. A private room with a comfortable chair for resting during the test day will be provided to the subject who will then have their vital signs and bloodwork completed by the onsite study MD. A meal of an apple, turkey sandwich, and drink will still be provided to all participants upon completion of their test day. These items will be purchased at Subway or Wawa.

Amino Acid Mixture:

According to a protocol successfully employed at the National Institutes of Health (NIH) with no reported adverse effects (Neumeister et al., 2004; Neumeister et al., 2005), an amino acid mixture will be prepared by combining amino acid powders into 70 capsules to be swallowed by the subject on the morning of each Test Day. Ingesting this tryptophan-free mixture stimulates protein synthesis, which depletes plasma TRP levels and causes the amino acids to compete with tryptophan to cross the blood-brain barrier, resulting in an overall decrease in brain tryptophan levels and, therefore, serotonin. In order to maintain the double-blind condition of each TRP depletion test, identical-appearing capsules containing either the amino acid mixture or placebo (31.5 g of lactose or microcellulose) will be given. The amino acids in this mixture are:

L-Isoleucine (4.2 g), L-Leucine (6.6 g), L-Lysine (4.8 g), L-Methionine (1.5 g), L-Phenylalanine (6.6 g), L-Threonine (3.0 g), L-Valine (4.8 g)

A pharmacist affiliated with the CHPS prepares the amino acid mixture. The pharmacist prepares the mixtures (both active and placebo) in advance batches, which will be kept in a temperature-controlled location (15-30 degrees Celsius) at Investigational Drug Service until a participant is scheduled for a study test day. The 70 capsules are then sent as a kit to the principal investigator/CRC/study nurse to dispense as part of the study challenge. The amino acids fall under guidelines for which there are adequate data demonstrating their safety. The amino acids are manufactured by the following companies:

- L-Isoleucine, L-Leucine, L-Phenylalanine, L-Threonine, L-Valine: Fagron
- L-Methionine: Spectrum (we also have L-Phenylalanine and L-Threonine from Spectrum)
- L-Lysine: Research Products International
- Capsules: Total

Each of the seven amino acids comes with a certificate of analysis indicating that the substance is nonpyrogenic, and that it has been inspected and meets the company's quality assurance standards prior to shipping. In addition, we have received an IND (#74,972) from the U.S. Food and Drug Administration, which approved the use of amino acids in this study. A separate IND specifically for the amino acids was not necessary.

Imaging Parameters

All fMRI scans will take place at the Center for Advanced Magnetic Resonance Imaging & Spectroscopy (CAMRIS) at the University of Pennsylvania School of Medicine. Whole-brain imaging data will be

acquired on a Siemens 3.0 Tesla Trio system. For structural whole brain images, the first scan is a sagittal localizer, followed by a T1 scan oriented in the axial-oblique dimension, parallel to the anterior commissure-posterior commissure (AC-PC) line (24 slices, repetition time = 300 ms, matrix size = 256 x 256, field of view = 220 x 220 mm). A three-dimensional high-resolution spoiled gradient scan will also be conducted (176 slices, repetition time = 2530 ms, matrix size = 256 x 256, field of view = 256 x 256 mm). Functional whole-brain images will be acquired using a gradient echo T2*-weighted echoplanar imaging scan (repetition time = 1500 ms; echo delay = 30 ms; flip angle = 80°; field of view = 220 x 220 mm). Measurements of cerebral blood flow at rest will involve scanning using the MRI arterial spin labeling (ASL) perfusion technique. Ten AC-PC aligned slices will be acquired for both the top and bottom parts of the brain (10 slices; 4 x 4 x 6 mm^3; echo delay = 20 ms; 80 images). A proton density (PD) weighted image will be acquired using the same sequence as perfusion.

Randomization to Estrogen Treatment (ET) or Placebo Treatment (PT)
After completing the first two TRP depletion test sequences, participants will be randomized to receive either treatment with 17β-estradiol (Vivelle Dot® 0.10 mg/day) or a look-alike placebo patch for a total of 8 weeks. The research pharmacist who is responsible for dispensing the estrogen or placebo patches will also be responsible for the randomization of all subjects together with the statistician on the study. Randomization will be blocked and stratified. We will use a block size of 3 to be able to maintain a 2:1 ratio of estrogen to placebo within each stratum. To stratify randomization based upon time since last menstrual period by tertiles (1^{st}=0-40 mos; 2^{nd}=40-80 mos; 3^{rd}=80-120 mos), we will prepare separate randomization tables for each stratum. The research pharmacist, PI, and research project manager will know to which group the subject has been randomized. The research pharmacist, PI or research project manager can be reached at any time should it become necessary to unblind a study participant immediately. The subject and all other study-related personnel (clinicians, raters, fMRI technicians, etc.) will be blind to subject assignment.

For the peri- and post-menopausal women participating in our previous tryptophan depletion/estrogen study (Amin et al., 2006b), mean baseline estradiol level was 31.72 pg/ml. With the 0.10 - 0.15 mg/d estradiol dose, mean estradiol levels were 96.33 pg/ml. Thus, our goal is to achieve a similar increase in estradiol in subjects participating in the present study. These doses are higher than the lowest available and may be higher than the lowest effective dose, which is the standard in clinical practice. However, in this experimental context, it is necessary to have a significant increase in plasma estradiol level in order to evaluate estrogen's effects. In addition, the standard dose in clinical practice is recommended for long-term ET. The proposed short-term ET regimen (chosen to more closely mimic the cyclic regimens currently recommended) is not likely to be associated with as significant risks as long-term ET. In our previous study, although women with greater time since LMP were found to have lower pre-treatment estradiol levels, the relationship between time since LMP and estradiol was less negative following ET, suggesting increased similarity among participants. Nevertheless, we will control for both age and plasma estradiol level when examining effects of time since LMP.

Following completion of approximately 6 weeks of ET or PT, the subjects will participate in the last two TRP depletion test sequences (one sham and one active depletion). Upon completion of the 4^{th} test day (or use of estrogen for at least 2 weeks in those who drop out), all subjects will be debriefed regarding their active vs placebo patch status and subjects randomized to ET will take Prometrium (micronized progesterone USP encapsulated with peanut oil) 200 mg daily or medroxy progesterone 5 mg/d (for women with peanut allergies) for 12 days. Prometrium and medroxy progesterone are USFDA approved for antagonism of estrogen effects on the endometrium in women taking hormone treatment (HT). According to a review of studies of oral progesterone in menopausal women, use of progesterone,

the progestational steroid produced by the human corpus luteum, minimizes side effects seen with synthetic progestins (McAuley et al., 1996). Long-term protection of the endometrium by Prometrium given as 200 mg/day for 12 days/month or medroxy progesterone given as 5 mg/d has been established (Gibbons and Thorneycroft, 1999). Oral micronized progesterone at a dose of 200 mg/ day is well-tolerated, with the only specific side effect being mild and transient drowsiness. Those women who would like to continue hormone therapy will be prescribed 3 additional months of estrogen (as well as an additional Prometrium treatment for women who have an intact uterus) and then referred to their gynecologist for continued treatment. The participant or her insurance company will be responsible for covering the cost of the additional treatment. Those women who wish to discontinue their HT will do so after cycling themselves with Prometrium or medroxy progesterone for 12 days. After the 12 days of Prometrium or medroxy progesterone with the Vivelle patch, women will discontinue both hormones.

Steroid Assays:
Blood for estradiol and progesterone will be obtained on each test day and sent to as yet to be determined laboratory for assaying via HPLC with Tandem Mass Spectrometry. The lower limit of detection is 1 pg/ml for estradiol and 0.1 ng/ml for progesterone. Intra-assay coefficient of variation (CV) for samples with mean estradiol level of 1.06 ng/dl is 2.27%, for inter-assay precision, the CV is 4.36% for mean estradiol level of 1.08 ng/d.

2. Specimens

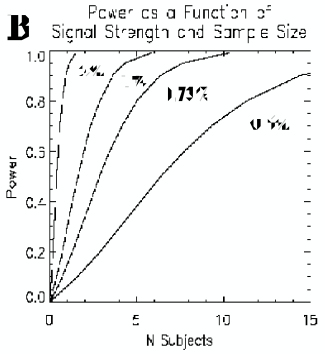
Not applicable.

3. Genetic Testing

Per above, we will obtain 10 cc whole blood for the study of genes related to estrogen metabolism and the neurochemical targets of estrogen. These include, but may not be limited to serotonin, acetylcholine, dopamine, glutamate, and norepinephrine neurotransmitter systems. Our goal is to interrogate functional polymorphisms which are linked to the regulation of mood and cognition and link these to possible effects of estrogen on these behaviors. We are not examining genes in relation to psychiatric disorders in this sample as all subjects included in this study are without previous or present psychopathology.

4. Statistical Analysis

<u>Power analysis for determining number of subjects</u>:



To determine the number of subjects to run in this functional imaging study, we first considered the effect of sample size on the stability of the average. This was investigated with a simulation study in which the variance in averaged t-test values was computed at different sample sizes, ranging from 2 to 39. Figure 1 Panel A illustrates that this variance approaches asymptotic minimum after about 10 subjects. Panel B reveals that with 10 subjects there should be excellent power for signals as low as 0.75%. A similar figure of 10-12 subjects has been recommended for group analyses using a random effects approach (Friston, Holmes, & Worsley, 1999), as will be used in our group analyses.

Although we will enroll women who are perimenopausal based last menstrual period being within 12 months, it is not a primary aim of the study to compare outcomes based upon peri versus post menopausal status. Hence, we do not aim to enroll a specific number of perimenopausal women.

fMRI Analysis: Functional scans will be realigned, normalized, time-corrected, and spatially smoothed by an 8 mm FWHM Gaussian kernel. The time series will be high-pass filtered to correct for drift across the scan. For blocked design experiments (verbal and figural 2-back, emotional go/no-go), regressors will represent trial conditions; for the event-related design experiments (verbal and figural recognition), regressors will represent individual trials and trial outcomes (forgotten/remembered).

At the individual level, voxel-wise fixed-effects contrast analyses (Friston, 1994) will be performed for each subject to assess the magnitude of pair-wise difference in blood oxygen level dependent (BOLD) signal between the emotional and the neutral control condition. At the group-level, a second stage of the process will use a random effects model (Holmes & Friston, 1998) to create SPM (Mosso, 1881) maps depicting loci that are active across subjects. Movement parameters derived from the realignment correction (for all six possible directions) will be entered into the design matrix as covariates of no interest. For a priori regions of interest, clusters will be reported if they are significant at P=.05, corrected, using a small volume correction (SVC). Clusters will also be reported if they are significant at a threshold of P=0.001, uncorrected, with an extend threshold of 10 voxels.

Repeated measures analysis of variance (ANOVA) will then be performed to test interaction of TRP depletion and ERT on brain activation patterns and simple effects analyses will reveal overall effects of estrogen or TRP depletion.

Other Analyses: Effects of TRP depletion and ERT on cognitive performance and mood will be assessed using random effects, mixed models. Repeated measures ANOVA will be used to assess the effects of TRP depletion on free and total plasma TRP levels. The relationship between behavioral ratings and cognitive performance and plasma TRP levels will be assessed using bivariate correlational methods.

RISK/BENEFIT ASSESSMENT

1. Risks

Treatment with Estrogen: The reported adverse reactions to estrogen treatment include nausea, vomiting, breast tenderness or enlargement, enlargement of benign tumors of the uterus, retention of excess fluid, spotty darkening of the skin, and vaginal bleeding. An increased risk of stroke or blood

clots is present from the beginning of estrogen administration, but long-term estrogen treatment (more than 5 years) may increase the risk of cardiovascular events, gallbladder disease, and dementia. Most recently, estrogen treatment alone was not associated with an increased risk of breast carcinoma. Serious side effects will be minimized by short-term treatment, exclusion of women who would be at heightened risk and careful monitoring of the treatment.

Progesterone Treatment: Adverse effects of progesterone (Prometrium) or medroxy progesterone include breast tenderness, bloating, fluid retention, and depression. In addition, findings from recent studies suggest that long-term (at least 5 years) progesterone treatment increases the risk of breast cancer, possibly more so than estrogen. Women who participate in this study must have had a normal mammogram within the previous 12 months. In addition, short-term treatment will limit the risks associated with progesterone administration.

Tryptophan depletion test: The risks associated with ingestion of the amino acid mixture include increased depression, nausea, vomiting, and diarrhea. The symptoms are transient and usually resolve within 24 hours.

Fasting: Subjects are required to abstain from eating from mid-night to approximately 3:30 pm on test days. Individuals will take the capsules in the morning per above and may drink clear fluids and coffee or tea without dairy or sugar. During fasting individuals are likely to experience hunger, and possibly a mild headache and irritability. The headache usually improves upon eating after the scan session but may continue into the evening, particularly if no anti-inflammatory medications are taken.

Lactose: Subjects who suffer from extreme lactose intolerance will not be allowed to participate in this study. Extreme lactose intolerance is described as a history of severe abdominal discomfort or cramping up after eating or drinking food products that contain lactose. However, subjects who describe themselves as having mild to moderate lactose intolerance will be given microcellulose capsules instead of lactose when randomized to sham depletion.

fMRI: fMRI conducted at 3 T has not been associated with any adverse medical effects. The principal anticipated difficulty is feelings of claustrophobia associated with all MR imaging of the head. All subjects will be screened for the presence of any metallic objects that they may be holding or have implanted in their bodies, and all potential subjects with metallic implants will be excluded. This questionnaire will be repeated prior to imaging to insure that they are not bringing any metallic materials into close proximity of the magnet, where they might be pulled toward the magnet or heated by the magnet. It is also possible participants may experience a slight metallic taste in their mouths either during or after the scan. They may also experience slight dizziness after coming out of the scanner or have a slight headache following their scan.

EXPERIMENTAL DEVICE CLAUSE: Some of the pulse sequences and/or RF coils are not FDA approved but are considered non-significant risk investigational devices.

Incidental Findings: There is also a risk that during the course of this study, an unexpected finding may be observed in MRI scans, even though the scans are not intended for clinical purposes. These findings or abnormalities are termed “incidental findings”. Most incidental findings have no significant health consequences, but in a small percentage of cases further evaluation or treatment may be indicated. If an incidental finding is noted in any image data, we will consult with experts, and it might be recommended that the participant obtain a full clinical MRI scan. It will then be up to the participant to

pursue it with your physician. Although study personnel may be able to provide some advice, the decision of whether and how to pursue an incidental finding can only be made by the participant's physician who has knowledge of her medical history.

Use of venipuncture for blood sampling: Inserting a needle into a vein is safe when done by professionals under clean conditions. Sometimes a bruise will occur at the puncture site and on rare occasions fainting, a blood clot, or an infection may form in the vein. If this occurs, appropriate treatment will be instituted immediately.

Behavioral Assessments: Subjects may feel upset when answering questions on the TLEQ and ACE. Should a subject report or appear distressed about the feelings that arise from answering these questions, then arrangements will be made for the subject to see the study doctor for an assessment immediately. All subjects reporting significant distress, homocidality, or suicidality, will be evaluated by a trained psychiatrist (P.I. and co-P.I.) and a decision about emergency treatment versus referral will be made. If participants opt to complete the ACE over the phone and experience distress the same arrangements will be made for participants to speak with a study doctor about their distress.

Nonspecific risks: As a result of their participation in these studies, subjects will have more blood drawn than would be the case in usual clinical practice. The maximal amount of blood subjects will have drawn will be less than one blood donation (85 cc), spread out over a period of 1-8 weeks.

2. Benefits

A better understanding of the effects of estrogen on 5-HT function, mood, and cognition is a potential benefit to the society at large. In light of the recent findings from the Women's Health Initiative it is particularly important that we begin to clarify who should receive estrogen treatment (peri and/or postmenopausal women) and what kind of cognitive and mood benefits can be expected from such treatment. Knowledge of the impact of ovarian hormones on brain function is important to understanding and treating menopausal symptoms.

Another potential benefit is that subjects will receive ERT and an extensive psychiatric and medical evaluation.

3. Subject Confidentiality

How will confidentiality of data be maintained? Check all that apply.

☒ Paper-based records will be kept in a secure location and only be accessible to personnel involved in the study.
☒ Computer-based files will only be made available to personnel involved in the study through the use of access privileges and passwords.
☒ Prior to access to any study-related information, personnel will be required to sign statements agreeing to protect the security and confidentiality of identifiable information.
☒ Whenever feasible, identifiers will be removed from study-related information.
☐ A Certificate of Confidentiality will be obtained, because the research could place the subject at risk of criminal or civil liability or cause damage to the subject's financial standing, employability, or liability.
☐ A waiver of documentation of consent is being requested, because the only link between the subject and the study would be the consent document and the primary risk is a breach of confidentiality. (This is not an option for FDA-regulated research.)
☐ Precautions are in place to ensure the data is secure by using passwords and encryption, because the research involves web-based surveys.
☐ Audio and/or video recordings will be transcribed and then destroyed to eliminate audible identification of subjects.
☐ Other *(specify):* _____

All of the information obtained from subjects is quoted by number and kept in locked confidential files. Any identifiable information that is obtained in connection with this study will remain confidential and will be disclosed only with the subject's permission or as required by U.S. or State law. Examples of information that we are legally required to disclose include abuse of a child or elderly person, or certain reportable diseases. This information is not available to anyone except the investigators and is not identified by the subject's name. The subject's clinical records are protected by the procedures of University of Pennsylvania School of Medicine. The investigators and anyone else involved with this project will not be allowed to reveal any identifying characteristics about the patients who participate in this study without their written consent. In the case of published reports from this study, care will be taken so that no individual subject will be identifiable.

Representatives from the Penn Office of Human Research may inspect study records during internal auditing procedures. However, these individuals are required to keep all information confidential. The U.S. Food and Drug Administration (FDA) and National Institutes of Health may also inspect study records during external auditing procedures. These individuals are also required to keep all information confidential.

4. Subject Privacy/Protected Health Information

The data to be collected for this study contain protected health information (PHI). The following PHI identifiers will be collected:

- Name
- Street address, city, county, precinct, zip code, and equivalent geocodes
- All elements of dates (except year) for dates directly related to an individual and all ages over 89
- Telephone numbers

- Fax numbers
- Electronic mail addresses
- Social security numbers

The PHI obtained in this study will be quoted by number and kept in locked confidential files. Any identifiable information that is obtained in connection with this study will remain confidential and will be disclosed only with the subject's permission or as required by U.S. or State law.

5. Compensation

Subjects will receive $200 for each test sequence ($800 if they participate in the four test sequences). Those subjects who complete all four-test sequences will receive an additional $250, making the total possible compensation for participation in this project $1050. Subjects will also receive ERT and an extensive psychiatric and medical evaluation, free of charge, which will also be of some value. Subjects will be paid for each test session for which they appear regardless of whether they successfully complete the test. No other direct benefits are associated with the tests.

6. Data and Safety Monitoring

This protocol presents moderate risks to the subjects, and these risks are defined as due to the study procedures and treatments although there are adequate protections to minimize these risks. In order to minimize the risks of participating in this study, we will carefully monitor all subjects and all serious unanticipated and anticipated adverse events. Any unanticipated adverse events will be reported to the IRB within 10 days of their occurrence. In the case of death, it will be reported to the IRB within 3 days. The principal investigator (N. Epperson) is responsible for making these reports to the IRB. Dr. Epperson or another study doctor will review all adverse events as they occur. Dr. Epperson will evaluate frequency and severity of the adverse events and determine if modification to the protocol or consent forms is required. In addition, Dr. Epperson will code the severity of adverse events and determine attribution of the event. These events will be summarized in our annual request for reapproval application to the IRB.

In addition, all subjects who sign the consent form are required by the protocol to submit mammogram, Pap smear, and breast exam results from their doctor (results must be within the past year) prior to beginning the study. This insures that all subjects have been examined by their gynecologists prior to enrolling in the study. We also send a courtesy letter to the gynecologist (after obtaining a signed release of information form) informing him or her of their patient's intentions to participate in this study.

Vivelle-Dot® is manufactured by Noven Pharmaceuticals, Inc., and is being purchased from Novartis using study funds through Investigational Drug Services as needed. . These companies ask that in the event of an adverse event that we submit an adverse event report immediately.

The research pharmacist, PI, and research project manager will know to which group the subject has been randomized. The research pharmacist, PI or research project manager can be reached at any time should it become necessary to unblind a study participant immediately. The subject and all other study-related personnel (clinicians, raters, fMRI technicians, etc.) will be blind to subject assignment.

7. Investigator's Risk/Benefit Assessment

This study presents moderate risk to the participant. Effective screening will be used to eliminate subjects who will be placed at a greater risk. This includes a medical and psychiatric history, physical

examination, and laboratory studies. No subject will be enrolled in the study unless they provide written documentation of having had a normal gynecologic examination, PAP smear and mammogram within the past year. This will be required of all women taking estrogen while they are participating in the tryptophan depletion study. The careful psychiatric evaluation will rule out any psychiatric conditions that could affect the subject's suitability for the study.

In addition, if a subject is pregnant, or planning to become pregnant, they will not be allowed to participate in this study. Hormone replacement therapy and the tryptophan depletion paradigm are not approved for use and administration during pregnancy and breastfeeding. Women who can have children must have a negative pregnancy test prior to enrolling in this study and must practice a medically accepted method of contraception during the entire study; this includes abstinence. Medically accepted methods of contraception will be discussed with each subject.

Subjects will be closely followed during the study by an experienced clinical research team. Careful monitoring of cardiovascular and behavioral parameters repeatedly and frequently during each test session will allow objective evaluation of the effects of the test. Prior experience with this test includes no significant adverse effects. In addition, all tests are done on a specially designed and staffed research unit with constant medical and nursing supervision. This unit has completed a large number of studies of this type and is specifically designed and equipped to support such studies. The principal investigator will be on call at all times to evaluate any problems that arise.

INFORMED CONSENT

1. Consent Process

The nature of the project, the procedures, the relative risks and benefits, and the alternative to participation in the project will be discussed with the subject initially over the telephone in order to determine their interest. If interested, subjects will be read a verbal consent document in order for them to provide their consent to participate in a telephone interview designed to determine eligibility. If upon completing this telephone interview, the subject continues to meet study criteria and continues to express interest in participating in the project, then a face-to-face meeting will be scheduled. At this meeting, the nature of the project, the procedures, the relative risks and benefits, and the alternative to participation in the project will be discussed in great detail with the subject. If following this discussion, the subject continues to be interested in the project, and screening procedures confirm eligibility (this will only be done if the subject did not consent to the telephone interview as the assumption is that almost all subjects presenting to the office will have completed the screening procedures to be here), informed written consent will be obtained from the subject on the consent form approved by the University of Pennsylvania Office of Human Research IRB. A copy of the consent form will be provided to all subjects. The investigators and anyone else involved with this project will not be allowed to reveal any identifying characteristics about the patients who participate in this study without their written consent.

Competency of subjects will be assessed using the MMSE.

Each subject must provide her own consent to participate in this project.

Following informed written consent, the subject will then participate in the in-office screening procedures that comprise visits 1 and 2 in order to further determine their eligibility for this project (visits 1 and 2 are the admission visits which typically occur on two different days for ease of scheduling).

2. Waiver of Informed Consent

We have requested a waiver of informed in order to complete a brief phone interview with participants before asking them to present for the study admission visit. After explaining the study, all participants who express interest in participating will be read a document stating the types of questions they will be asked and will be given the option to complete the interview over the phone. All participants who meet criteria will then be given the option to complete the ACE over the phone in order to further determine eligibility. Participants who continue to meet criteria will then be scheduled for an in-office screening visit.

RESOURCES NECESSARY FOR HUMAN RESEARCH PROTECTION

The PI and her research study personnel have over 14 years experience conducting studies like the study described herein. Additionally the PI is a well-established investigator who has published the results of previous studies similar to the study design proposed in this application.

The study staff and PI have experience working in psychiatric clinical research and have worked with women with postpartum depression, premenstrual dysphoric disorder, depression, post-traumatic stress disorder, and obsessive compulsive disorder in addition to the healthy control population to be used in this study.

There is sufficient time to conduct this research in a 5-year period.

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