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ORIGINAL ARTICLE

Soy and social stress affect serotonin neurotransmission in primates

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ABSTRACT
Stress and sex steroidal milieu can each influence mood in women. The purpose of this study was to compare the effect of long-term conjugated equine estrogen (CEE), soy phytoestrogen (SPE), and social subordination stress on dorsal raphe serotonin neurotransmission of ovariectomized cynomolgus monkeys. Tryptophan hydroxylase (TPH) and serotonin reuptake transporter (SERT) protein content were determined, and the *in vitro* degradation of macaque SERT protein was examined in the presence and absence of protease inhibitors, serotonin (5-HT), and citalopram. Like CEE, SPE increased TPH protein levels. Social subordinates had markedly lower TPH protein levels than dominants regardless of hormone replacement. Therefore, these two variables had independent and additive effects. CEE and SPE increased SERT, and social status had no effect. Thus, the hormone-induced increase in SERT was accompanied by increased 5-HT synthesis and neuronal firing, which appears biologically reasonable as 5-HT prevented SERT degradation *in vitro*.
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INTRODUCTION

Both stress and sex steroids can influence mood in women; however, the mechanisms of these effects are not well understood. The available data suggest that serotonin (5-HT) is one of the key neurotransmitters modulating mood, and that both stress and steroids may influence 5-HT neurotransmission.

5-HT neurotransmission is governed by various regulatory proteins in both 5-HT neurons and their targets. Two pivotal control proteins are tryptophan hydroxylase (TPH), the committal enzyme in 5-HT synthesis, and the 5-HT reuptake transporter (SERT), which is blocked by the widely prescribed class of antidepressants known as selective 5-HT reuptake inhibitors (SSRIs). Changes that occur in either of these proteins because of stress or steroid hormones could have widespread repercussions in 5-HT neurotransmission and, in turn, mood or behavior.

Macaques have a menstrual cycle similar to that of women, substantial cortical inputs, and a complex social existence. Thus, they are exceptionally good models for social neuroscience. Female cynomolgus monkeys (*Macaca fascicularis*) form social status hierarchies, and low social status (or social subordination) appears stressful. Socially subordinate female cynomolgus monkeys (intact or ovariectomized [OVX]) secrete more cortisol than their dominant counterparts. They also receive more aggression and are involved in less affiliative behavior than dominants.[1] Thus, they appear socially stressed. In the follicular phase of the menstrual cycle, prolactin release in response to the 5-HT releaser, fenfluramine,

is higher in subordinates than dominants, suggesting greater central serotonergic capacity in subordinates.[2] However, in ovariectomized monkeys, the relation between the hormone response to fenfluramine and social status is reversed. Among OVX females, dominants have higher prolactin responses than subordinates.[3] Thus, some aspect of ovarian function appears to mediate the relation between social status and 5-HT neurotransmission in females. Furthermore, since 5-HT modulates corticosteroid hormone release in response to stress,[4,5] and cortisol may effect 5-HT neurotransmission,[6] the hypercortisolemia characteristic of subordinate females may be involved in social status differences in 5-HT neurotransmission.

Sex steroids clearly modulate 5-HT neurotransmission by actions at TPH, SERT, and 5-HT 1A autoreceptor gene and protein levels.[7] Previously, we showed that TPH protein levels in the dorsal raphe of macaques are low in OVX females, but TPH increased with 17β-estradiol and conjugated equine estrogens (CEE), a common hormone replacement therapy (HRT) for menopausal women.[8]

An opposing progestin in combination with an estrogen is necessary to reduce the risk of reproductive system cancers in women, but recent reports suggest deleterious health effects of combination HRT.[9] Alternative strategies not requiring an opposing progestin are under investigation. One of these is soy phytoestrogen (SPE). SPEs have either no agonist or, perhaps, antagonist effects in the mammary gland and uterus,[10] but little is known about SPE effects in brain. We questioned whether SPE would act on TPH protein like CEE in macaques.

The SERT is responsible for the recycle and clearance of 5-HT in the brain.[11] Dysfunction of SERT has been implicated in several psychopathological conditions such as depression and anxiety disorders. For example, SERT protein levels in the midbrain of patients with major depression are reduced, compared to those of healthy individuals,[12,13] while blunted 5-HT release and uptake are associated with various depressive disorders.[14–18] We recently reported that 1 month of ovarian steroid replacement increased binding of citalopram (an SSRI) to SERT, increased SERT immunofluorescence in fiber tracts and increased [^{3}H]5-HT uptake in several brain regions that receive 5-HT projections.[19] However, there was no change in citalopram binding or SERT immunofluorescence in the cell bodies with 1 month of treatment. The tissue in this study provided an opportunity to determine the effects of social status and long-term treatment (36 months) with estrogenic ligands on SERT protein in the somatodendritic portion of 5-HT neurons.

The objective of the experiment reported here is to compare the effect of long-term CEE, and SPE treatment on serotonin neurotransmission, and to examine the relation between social status and serotonin neurotransmission in the dorsal raphe of OVX cynomolgus monkeys. The protein content of TPH and SERT was determined, and the *in vitro* degradation of macaque SERT protein was examined in the presence and absence of protease inhibitors, 5-HT, and citalopram.

RESULTS

Quantitative Analysis of TPH Protein

The specific methodology used in Western analysis of TPH has been previously described and validated against protein density measured with the NIH Image Analysis software. There was a linear increase in the optical density of the TPH band with an increase in sample protein loaded on the gel.[8] Unfortunately, TPH was degraded in two of the control group samples and two of the CEE group samples; thus, there was no signal. This resulted in a sample size of $n=8$ for CEE, and $n=3$ for the control group. In order to determine if this small sample was representative of OVX cynomolgus monkeys in general, these data were compared with those from a prior study by Bethea *et al*,[8] uncorrected, and

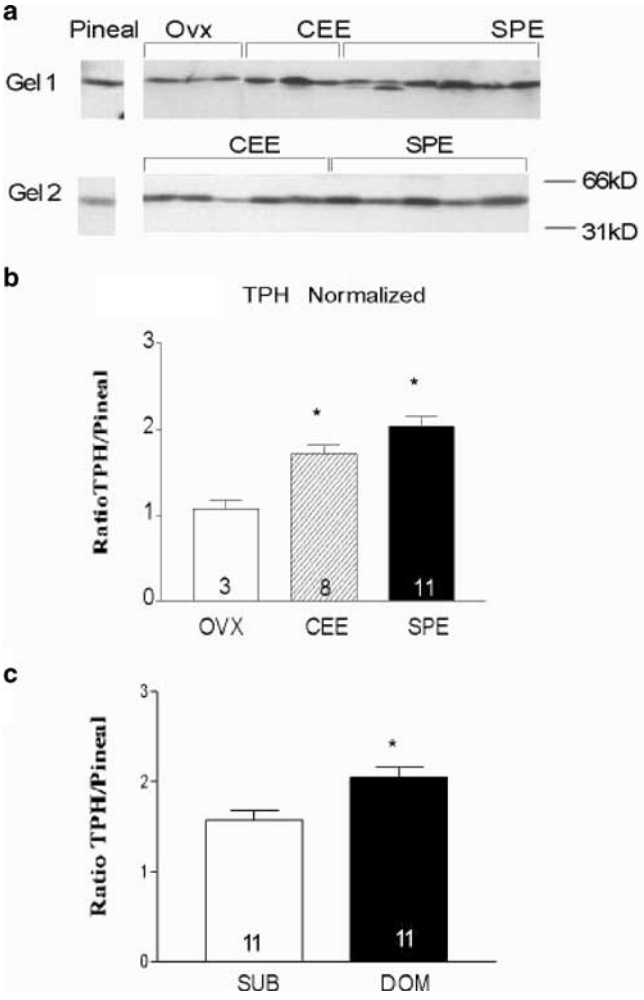

Figure 1 (a) Protein bands in two gels run simultaneously and probed with antibody to human tryptophan hydroxylase (TPH); (b) means (±SEM) of TPH/pineal protein levels in ovariectomized (OVX) cynomolgus monkeys treated with conjugated equine estrogens (CEE) and soy phytoestrogens (SPE) *vs* untreated controls (OVX). *: Different from OVX controls ($P<0.05$); (c) means (±SEM) of TPH/pineal protein levels from subordinate and dominant females within each treatment group ($P<0.04$). Numbers on bars represent sample size.

corrected for pineal density. In the prior study, the mean optical density was determined for two social groups of OVX females ($n=5$ and 4) in the same way as in the study reported here. Although there was variation among these groups, all three of these OVX control groups (two from the prior study and one from the current study) have lower values than their hormone-treated counterparts in either the prior study[8] or the current study (see Figure 1). Also the social status differences appeared to be quite similar in all the three treatment groups in the study reported here (see Figure 2). For these reasons, the data from the control group in the current study were deemed acceptable for comparison to the treatment groups.

Treatment of cynomolgus monkeys for 36 months significantly increased TPH protein levels ($F[2,16]=9.16$, $P=0.002$; Figure 1b). Values for the CEE and the SPE groups were similar (Tukey's test $P>0.10$), whereas both CEE (Tukey's test $P<0.05$) and SPE (Tukey's test $P<0.05$) increased TPH relative to controls.

Socially subordinate females had lower TPH protein levels than dominant monkeys ($F[1, 16]=5.27$, $P<0.04$; Figure 1c). Inspection of Figure 2a suggests that TPH levels were lower in subordinates than dominants regardless of treatment. Figure 2b illustrates that the relation between social status and TPH was linear, and that there was a significant correlation between social status and TPH protein mass ($r=0.51$, $P<0.02$).

Figure 3b demonstrates that CEE and SPE treatment for 36 months significantly increased SERT protein (78 kDa) levels

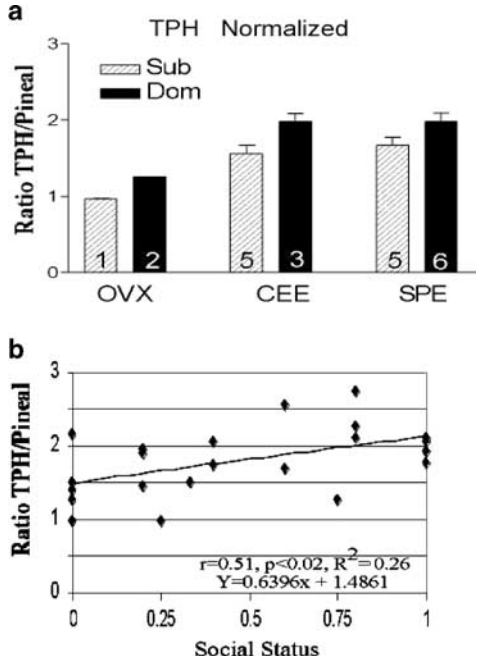

Figure 2 (a) TPH/pineal protein levels in subordinate and dominant females within each treatment group; (b) linear regression and correlation between social status and the ratio of TPH/pineal protein levels.

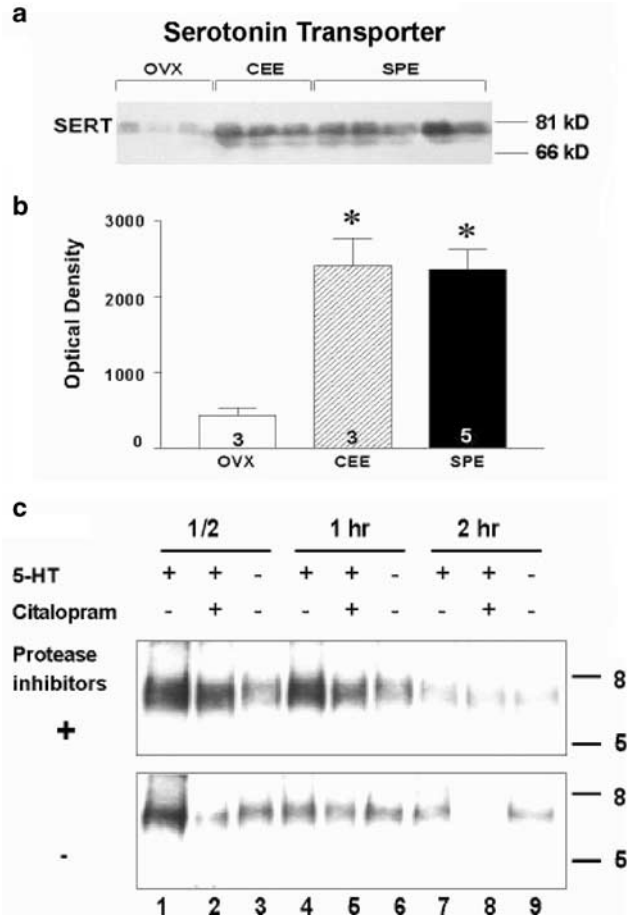

Figure 3 (a) Protein bands in Western blot probed with antibody to human serotonin reuptake transporter (SERT); (b) the means (±SEM) of SERT protein levels in OVX cynomolgus monkeys treated with conjugated equine estrogens (CEE) and SPE *vs* untreated controls (OVX). *: Different from OVX controls ($P<0.05$); (c) degradation SERT at 37°C by serotonin (5-HT, 0.4 nmol/mg total protein) and inhibition of degradation by citalopram (0.4 nmol/mg) at 30 min, 1 h, and 2 h.

in the macaque dorsal raphe compared with the OVX controls ($F[2,5]=12.11$; $P=0.01$; Tukey's test, CEE *vs* OVX, and SPE *vs* OVX $P<0.05$). There was no effect of social status on SERT protein levels ($F[1,5]=0.11$, $P=0.75$), and no correlation between social status and SERT protein mass ($r=0.02$, $P=$NS). There was a positive, although nonsignificant, association between SERT and TPH protein levels ($r=0.45$, $P>0.10$). As shown in Figure 3c, SERT degradation was inhibited by the addition of 5-HT (0.4 nmol/mg total protein) *in vitro*. The protective effect of 5-HT was blocked by citalopram (0.4 nmol/mg).

DISCUSSION

Available data suggest that 5-HT plays a major role in mood determination and that both stress and steroids may influence 5-HT neurotransmission. Previously, we demon-

strated that TPH protein content is low in the raphe region of OVX macaques, and that 17β-estradiol and CEE treatment each increase TPH protein, while tamoxifen reduces it. TPH remains elevated when natural progesterone is added to 17β-estradiol treatment, but is reduced to OVX levels when medroxyprogesterone acetate is added to CEE treatment, suggesting that this progestin analog blocked the beneficial effects of CEE.[8]

This report replicates the CEE effect observed in the previous study,[8] and extends the previous observations by demonstrating that SPEs increase dorsal raphe TPH levels, similar to 17β-estradiol and CEE. This result suggests that the positive effects on mood associated with estradiol and CEE may extend also to SPEs. Since SPEs have no proliferating effects on mammary and uterine tissue, combination therapies with an opposing progestin are unnecessary.

The available evidence suggests SPEs act in the brain, but whether they act like an estrogen agonist or antagonist depends on the particular neural system under study, and this may reflect the involvement of different isoforms of the estrogen receptor. For example, coumestrol blocked the ability of 17β-estradiol to induce progestin receptors in wild-type mice.[20] In addition, soy isoflavone supplements are reported to antagonize reproductive behavior and estrogen receptor alpha- and beta-dependent gene expression in the rat hypothalamus.[21] Like estradiol, SPEs appear to increase choline acetyltransferase mRNA levels[22] and brain-derived neurotrophic factor mRNA levels in frontal cortex.[23] SPEs also increased hippocampal nerve growth factor, although not as much as estradiol in OVX monkey frontal cortex.[22] In male rats, phytoestrogens decreased medial basal hypothalamic, preoptic, and amygdaloid calcium-binding proteins without altering androgen metabolizing enzymes.[24] In male and female rats, phytoestrogens appear anxiolytic, and have sexually dimorphic effects on visual–spatial memory.[25] To date, only estrogen receptor beta (ERβ) has been detected in 5-HT neurons of macaques,[26,27] so our study indicates that SPEs are agonists at ERβ in the context of 5-HT neurons.

Low social status has been described as stressful in several animal models, and people of low socioeconomic status experience more stress than their higher socioeconomic counterparts.[28,29] Low social status in primates is accompanied by increased risk of many diseases. Exactly how social stressors are transduced by the brain into perceptions of stress, physiological stress responses, and deleterious effects on mood and many other health outcomes is not well understood. Previously, we have observed that socially subordinate female cynomolgus monkeys are more socially isolated and experience more hostility than their dominant counterparts. They have physiological responses typically associated with stress, such as increased cortisol secretion, and they are preferentially susceptible to behavioral depression.[1,30] Dominant females are typically more aggressive than subordinates. In the presence of ovarian cyclicity, in the early follicular phase, dominants have lower 5-HT transmission, as indicated by prolactin responses to fenfluramine, than subordinates. This is consistent with the known relation between low 5-HT and high aggression.[31,32] However, when the ovaries are removed, this relation changes. After ovariectomy, dominants are still more aggressive than subordinates; however, OVX dominants have higher indices of 5-HT neurotransmission than OVX subordinates, the opposite of what is observed in intact females.[3,33] These results suggest that the relation among 5-HT, ovarian hormones, and behavior is not invariant. Among female monkeys, ovarian steroids may mediate the relation between social status and 5-HT. Since the publication of these studies, a direct relation between low socioeconomic status and low serotonergic activity (also measured by the prolactin response to fenfluramine) has been observed in a study of 270 men and women 24–60 years of age.[34]

One mechanism for sex steroid mediation of the social status – 5-HT relation is by modulation of 5-HT availability. In this report, we demonstrate that among OVX females, social subordinates have lower dorsal raphe TPH levels than dominants. This is consistent with our prior observation of reduced indices of serotonergic neurotransmission in OVX subordinates *vs* dominants as measured by the prolactin response to fenfluramine.[3] Our results suggest that in the absence of ovarian function or exogenous selective estrogen receptor modulators (SERMs) therapy, OVX subordinate females have exceedingly low TPH levels. The effects of social status and the exogenous replacement therapies examined here are independent and additive. Perhaps, this apparent additive nature of stress and lack of ovarian function contribute to the increased incidence of mood disorder among susceptible women at the time of menopause.

SERT protein was not regulated by social status. While social subordination appears stressful and is accompanied by increased stress hormone levels, it is a chronic stress of low enough intensity that subordinates tolerate it for years. In contrast, the acute and comparatively high intensity stress of repeated social defeat in mice is associated with increased SERT gene expression.[35] Duration and intensity of the social stressor may have different neurochemical effects, or this may be a species difference. More research is needed to clarify this issue.

SERT protein increased with estrogenic ligands. In a previous study, 1 month of estrogen treatment did not change the density of citalopram-binding sites or immunofluorescent SERT in the raphe of macaques compared to the OVX controls. However, after 1 month of treatment, SERT increased in terminal fields.[19] In this study, SERT protein mass, as measured by Western blot analysis, was significantly higher in the raphe extracts from monkeys treated with CEE or SPE for 36 months compared to OVX controls. It is possible that the difference we observed between SERT-binding sites or immunofluorescence[19] and SERT protein mass during hormone replacement is because of the difference in the duration of the treatments. It is attractive to speculate that with hormone replacement there is first an increase in SERT trafficked to the projection areas, and then the increase in SERT extends back to the cell body region.

There is, nonetheless, an overall increase in SERT with estrogenic treatment that occurs coincident with an increase

in TPH. The increase in TPH is likely to increase 5-HT synthesis. We have also shown that ovarian hormones decrease 5-HT 1A binding to [^{3}H]8-hydroxy-2-(di-*n*-propylamine) tetralin ([^{3}H]8-OH-DPAT) and decrease the ability of 8-OH-DPAT to stimulate glutamate triphosphate gamma subunit (GTPγS) binding.[36] Together, these changes should increase 5-HT neurotransmission. Thus, it seems incongruent that SERT would also increase and thereby increase removal of 5-HT. However, it has been suggested that protein expression and activity of SERT are regulated by the level of extracellular 5-HT.[37] Functional SERT proteins on the cell membrane exist in a dynamic multimeric complex with phosphatase 2A and the stability of this complex is usage-dependent.[38] When the level of extracellular 5-HT is high, SERT will continuously pump 5-HT to the inside of cells. The translocation of 5-HT through the portal of SERT strengthens the interaction between SERT and phosphatase 2A, thus preventing SERT phosphorylation and SERT internalization. According to this model, the level of functional SERT protein should positively correlate with 5-HT levels in the extracellular space. Our data indicate that 5-HT does indeed preserve SERT in an *in vitro* paradigm in either the presence or absence of protease inhibitors.

Clinical studies with positron emission tomography scans (PET scans) and post-mortem tissue are consistent with our data. In depressed patients, 5-HT and SERT levels are both low compared to healthy individuals.[12,13,17,39] In SERT knockout mice, brain 5-HT concentrations are also reduced to 60–80%.[40] In HEK-293 cells expressing SERT, when there is more 5-HT available, there is also more uptake.[37,38] Furthermore, estrogen treatment increases the level of both SERT binding and steady-state mRNA in the dorsal raphe nucleus of rats.[41] In other words, an increase in 5-HT neurotransmission is accompanied by an increase in transporter levels and function. However, if SERT only functions to remove 5-HT, then this correlation is paradoxical. It is worth considering the possibility that SERT transports 5-HT in both directions and that under conditions of increased 5-HT synthesis and neuronal firing, there will be a proliferation of transporters that will function to export 5-HT as well. SERT exports 5-HT with other pharmacological manipulations such as fenfluramine treatment and 3,4-methylenedioxy-methamphetamine (MDMA) administration.[42,43] Along this line of reasoning, the efficacy of SSRIs that block only the uptake, but not release, of 5-HT via SERT would increase in the presence of ovarian steroids.

In conclusion, SPE increases TPH protein in the serotonergic cell body region of nonhuman primates in a manner similar to CEE, a common estrogenic compound utilized in HRT. However, SPE has a more benign peripheral profile than CEE and does not cause proliferation of the endometrium. In addition, social status also affects TPH protein with subordinate females exhibiting lower levels than dominant females regardless of hormone replacement. The combination of hormone treatment and social status yielded the lowest TPH protein in the OVX subordinate females and the highest TPH protein in the hormone-replaced dominant females. If TPH protein predicts 5-HT synthesis, then low ovarian hormones and low social status could produce low 5-HT synthesis and, in turn, increased vulnerability to depression or anxiety. In addition, we show that CEE and SPE increase SERT protein in the raphe region, but social status had no effect. Moreover, 5-HT prevents SERT degradation. The hormone-induced increase in SERT is believed to accompany an increase in 5-HT synthesis and neuronal firing.

MATERIALS AND METHODS

Animal Subjects

The subjects of this study were a part of a larger study investigating the effects of sex steroid exposure on coronary artery disease risk, and the experimental methodology has been described in detail.[44] Throughout the study, the monkeys were housed in small social groups of three to six animals each. All animals within a group underwent the same treatment. Briefly, adult female cynomolgus monkeys were obtained from Institut Pertainian Bogor, Bogor, Indonesia, and for 26 months were fed a moderately atherogenic diet that contained 17% of calories from protein, 45% of calories from fat, 38% of calories from carbohydrate, and 0.28 mg cholesterol/cal. At the end of 26 months, the monkeys were OVX to make them surgically postmenopausal. All procedures involving animals were conducted in compliance with state and federal laws, standards of the US DHHS, and guidelines established by the Wake Forest University animal care and use committee.

Study Design

This was a three-group, parallel arm design, with a 36-month treatment period. Monkeys were assigned to a treatment group using stratified randomization which accounted for premenopausal social group. The diet for all monkeys contained soy protein isolate. The soy protein was extracted with ethanol to remove phytoestrogens for the control group diet and the diet fed to the CEE group. The treatments used were either Premarin, an oral preparation of CEE (Wyeth-Ayerst Laboratories, Inc.), at a dose comparable to a dose of 0.625 mg/day for women, or soy protein isolate containing SPE at a dose approximately equivalent to 129 mg/day for women, expressed as aglycone units. The doses of CEE and SPE fed to the monkeys were based on the assumption that women in the US eat an average of 1800 cal/day. Thus, a dose of 0.625 mg CEE was added to 1800 cal of diet. Monkeys were fed 120 cal of diet/kg body weight (BW) and, therefore, took in about 0.042 mg CEE/kg BW. There were 129 mg of phytoestrogens in 1800 cal of the SPE diet; thus, the monkeys were fed about 8.6 mg SPE/kg BW. This type of caloric adjustment of dose accounts for differences in metabolic rates between monkey and human subjects. The unextracted soy protein contained an average of 1.105 mg genistein, 0.365 mg daidzein, and 0.08 mg glycitein/g soy protein. The ethanol-extracted soy protein contained an average of 0.04 mg genistein, 0.01 mg daidzein, and 0.01 mg glycitein/g soy protein isolate. The monkeys were fed one-third of their diet in the morning and two-thirds of their diet in the afternoon. Hormone

concentrations measured three times during the postmenopausal phase revealed that CEE increased 17β-estradiol concentrations, whereas SPE had no effect. SPE increased androstenedione concentrations whereas CEE treatment did not. The groups were not different in testosterone or dihydroepiandrosterone sulfate levels. The mean (±SEM) total plasma isoflavone concentrations of the SPE group was 776±85 nmol/l (459±45 equol, 161±32 genistein, 112±18 daidzein).[44]

Social Status

Social status was determined by recording the outcomes of agonistic interactions. The monkey that could elicit submissive behavior from all other monkeys in her group was ranked number one. The monkey that could elicit submissive behavior from all but the first ranking monkey was second in rank, and so on. Previously, we observed that social status is a stable characteristic of these animals over long periods of time,[45] and that was also the case in the current study. Social status was determined 6–12 months prior to necropsy and again within 2 weeks of necropsy. The ranks were corrected for social group size;[1] thus, they varied between zero (most subordinate) and one (most dominant). The Pearson r correlation between the two rankings was $r=0.99$, suggesting that these animals occupied the same social status in their groups over a long period of time.

Tissue Preparation

After 36 months, the monkeys were anesthetized with sodium pentobarbitol (100 mg/kg, i.v.) and exsanguinated. The cranium was retracted, and the brain was removed. The individual brain blocks, including a mid- and hindbrain section, were sealed in plastic bags, immersed in liquid nitrogen, and then stored at −80°C until microdissection of the midbrain for Western analysis. The brains from all members of a social group were used in the current study. Since illness is a stressor and treatment for illness can alter hormone absorption, social groups were chosen for study in which the members had minimal illness throughout the treatment period and had plasma hormone levels representative of their treatment group. The raphe nuclei from monkeys in one social group of controls ($n=5$), two social groups treated with CEE ($n=10$), and two social groups treated with SPE ($n=11$) were evaluated for TPH protein. The raphe nuclei from monkeys in the same social groups were also evaluated for SERT protein (control: $n=3$; CEE: $n=3$; SPE: $n=5$).

Midbrain Dissection for Western Assays

The dissected pontine midbrain section displayed the rounded central canal on its anterior surface and the wing-shaped canal on its caudal surface. This section was microdissected; a small square piece of tissue was harvested, which extended from the middle of the central gray to the decussation of the cerebellar peduncles. The piece was the width of the central gray and contained a major portion of the dorsal raphe. Each piece was immediately homogenized in 1 ml of 50 mM Tris (pH 7.5; ICN Biomedicals, Aurora, OH, USA) and 20 mM 2-β-mercaptoethanol and centrifuged at 12 000 *g* for 10 min. The supernatant was removed and stored at −80°C until TPH Western blot analysis. The pellet was stored at −80°C until solubilization and assay for SERT protein with Western blot analysis.

Total Protein

Immediately prior to loading the gel, protein assays were performed on the supernatant with the Bio-Rad protein determination reagent according to the method of Bradford.[46]

TPH Western Blot Analysis

Raphe extracts containing 300 g of protein were loaded on a 10% SDS polyacrylamide gel. Aliquots (129 μg) from the same pool of monkey pineal homogenate were included on each gel as a positive control. Molecular weight markers (Bio-Rad) were also included. Western blotting was performed according to the modified procedures of Dumas *et al*[47] with blotting buffer containing 25 mM Tris base (ICN) and 192 mM glycine. The nitrocellulose membrane was initially stained with 2% Ponceau-S, which displayed the molecular weight markers and multiple separate protein bands in the sample lanes. The lane containing the molecular weight markers was cut from the membrane, and the sample lanes were processed further.

The nitrocellulose membrane (Schleicher & Schuel, Keene, NH, USA) containing the sample lanes was blocked with 5% aqueous Carnation nonfat dry milk (Nestle Food Company, Glendale, CA, USA) for 45 min. Affinity-purified sheep anti-TPH (Chemicon International Inc., Temecula, CA, USA) was used at a dilution of 1 : 500 in 50 mM Tris and 150 mM NaCl (pH 7.5). Rabbit anti-sheep antibody conjugated with horseradish peroxidase (Chemicon) was used as secondary antibody at a dilution of 1 : 7000. TPH signal was detected by exposing the blot to autoradiographic film after developing with Supersignal substrate reagents (Pierce, Rockford, IL, USA). Scientific imaging film (X-OMAT AR film, Eastman Kodak) was used for development of the signal. TPH was degraded in two of the control group samples and two of the CEE group samples; thus, there was no signal. This resulted in a sample size of $n=8$ for CEE, and $n=3$ for the control group.

SERT western blot analysis

Monkey midbrain pontine blocks containing the dorsal raphe nucleus were microdissected and hand-homogenized in 50 mM Tris and 20 mM β-mercaptoethanol, pH 7.5, as described above. The homogenates were subjected to centrifugation at 12 000 *g* at 4°C for 10 min. Pellets containing membrane-bound proteins were obtained and resuspended in Tris (10 mM) and EDTA (1 mM), pH 7.2, containing leupeptin (1 mg/ml), trypsin inhibitor (1 mg/ml), *O*-phenanthroline (1 mM), iodoacetamide (1 mM), PMSF (250 mM), and pepstatin A (1 mM) and further homogenized with a handheld pestle and mortar (Fisher Scientific, Pittsburgh, PA, USA). Samples containing 50 μg of total protein from each animal were dissolved with 10% SDS

containing 4% β-mercaptoethanol at 80°C for 15 min and heated at 90°C for 10 min before loading onto a vertical mini gel system. Western blotting was performed according to the modified procedures of Qian *et al*[48] with blotting buffer containing 25 mM of Tris base and 192 mM of glycine. The nitrocellulose membranes (Osmonics, Westborough, MA, USA) were blocked in 5% nonfat dry milk for 45 min before incubating with primary antibodies at 4°C overnight. The anti-SERT primary antibody ST51-2 was used at a dilution of 1 : 600. The following morning, the blots were washed in saline and 0.05% Tween-20 (Bio-Rad), incubated with secondary antibody conjugated to HRP at 1 : 2000 at room temperature for 2 h, and then developed with Supersignal chemiluminesence kits (Pierce, Rockford, IL, USA) followed by exposure to Kodak X-OMAT AR film.

In addition, to examine the regulation of SERT by 5-HT *in vitro*, the same amount of midbrain extract was incubated with or without added 5-HT (0.4 nmol/mg total protein) for 30–120 min in the absence or presence of protease inhibitors listed above before processing for Western blot analysis. Citalopram (0.4 nmol/mg) was used to block the effect of 5-HT.

Densitometric Analysis of Protein Bands

The specific bands on the film were captured using an XC-77 CCD video camera (Sony, Towada, Japan) and a framegrabber board on a Mac G4. Densitometric analysis of signal bands was performed using NIH Image Gel Plotting software. The region of interest containing the sample band was marked. The image analysis program scans each lane and converts the size and intensity of each band to a peak. The area under each peak was calculated. The TPH band from monkey pineal extract was used as the positive control on the TPH blots.

Statistical Analysis

The optical density of the band on the film in arbitrary units was compared between treatment groups using analysis of variance (ANOVA). In addition, Pearson *r* correlations were calculated between social status and TPH, and between TPH and SERT. Owing to the number of samples, two gels were required for the TPH analysis. To correct for differences between the gels, the optical density of each TPH protein band was corrected with the optical density of the pineal gland band. All statistical analyses were run on both the average optical density and the corrected optical density ratio of TPH/pineal and no differences in the results of these analyses were found. Corrected optical density is used for analysis throughout this report. *Post hoc* tests were conducted using Tukey's test. One gel ($n = 11$) was run on selected samples for SERT analysis, thus the uncorrected optical densities are reported and were subjected to ANOVA. All reported *P*-values are the results of two-sided tests.

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DUALITY OF INTEREST

None declared.

ABBREVIATIONS

5-HT	*serotonin*
8-OH-DPAT	*8-hydroxy-2-(di-n-propylamine) tetralin*
ANOVA	*analysis of variance*
BW	*body weight*
CEE	*conjugated equine estrogen*
ERβ	*estrogen receptor beta*
GTPγS	*glutamate triphosphate gamma subunit*
HRT	*hormone replacement therapy*
MDMA	*3,4-methylenedioxy-methamphetamine*
OVX	*ovariectomized*
PET scans	*positron emission tomography scans*
SERMs	*selective estrogen receptor modulators*
SERT	*serotonin reuptake transporter*
SPE	*soy phytoestrogen*
SSRIs	*selective 5-HT reuptake inhibitors*
TPH	*tryptophan hydroxylase*

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