Steroid Regulation of Tryptophan Hydroxylase Protein in the Dorsal Raphe of Macaques

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Background: *Tryptophan hydroxylase (TPH) is the rate-limiting enzyme for the synthesis of serotonin, and serotonin is a pivotal neurotransmitter in the regulation of mood, affective behavior, pituitary hormone secretion, and numerous autonomic functions. We previously demonstrated that estradiol (E) and progesterone (P) increase TPH mRNA levels in the dorsal raphe of macaques.*

Methods: *This study employed western blotting and densitometric quantitation to determine whether the changes observed at the level of gene expression were manifested by changes in TPH protein expression and whether modified estrogens or progestins had actions similar to the native ligands. In addition, the effect of the antiestrogen tamoxifen was examined. Ovariectomized (ovx) rhesus and cynomolgus macaques were untreated or treated with E, P, E+P, equine estrogens (EE), medroxyprogesterone (MPA), EE+MPA, or tamoxifen. The dorsal raphe region was subjected to Western analysis.*

Results: *E treatment for 28 days increased TPH protein mass four to six fold over ovariectomized controls. Addition of P to the E regimen or treatment with P for 28 days after E priming did not alter TPH from E treatment alone. Treatment of ovx macaques with a low dose of P caused a two-fold increase in TPH protein. Treatment of ovariectomized macaques for 30 months with EE alone or MPA alone significantly increased TPH protein; however, unlike P, the addition of MPA to the EE regimen blocked the stimulatory effect of EE. Tamoxifen treatment significantly reduced TPH protein compared to EE and ovariectomized control animals.*

Conclusion: *The stimulatory effect of E and P on TPH protein in the dorsal raphe of macaques correlates with the previously observed effect at the level of mRNA expression. P had no effect on the stimulatory action of E, whereas MPA blocked the stimulatory effect of EE. Tamoxifen acted as a potent antiestrogen on TPH protein expression. If TPH protein mass influences serotonin synthesis, then these steroids will impact many autonomic systems that are regulated by serotonin.* Biol Psychiatry 2000;47:562–576

Key Words: Tryptophan hydroxylase, serotonin, dorsal raphe, depression, estrogen, progesterone, medroxyprogesterone acetate, macaque

Introduction

The lifetime prevalence of depression in women has been estimated to be as high as 21%, which is about twice the rate of men. Several lines of evidence suggest an association between depression and reproductive function (Halbreich et al 1984; MacKenzie 1986; Pearlstein 1995; Schwartz 1991; Weissman and Klerman 1985; Williams et al 1995; Yonkers and Chantilis 1995). A gender difference in depression incidence emerges in adolescence. Depression is associated with the late luteal phase of the menstrual cycle, the perimenopause state, and the postpartum period, all of which are physiological states characterized by declining estradiol and progesterone concentrations (Gitlin and Pasnau 1989; Halbreich et al 1992; Pearlstein 1995). Estrogen therapy may alleviate depression or anxiety in women when given alone; it may also provide adjunct therapy to prevent nonresponse to conventional antidepressants (Gregoire et al 1996; Halbreich 1997; Oppenheim 1983). Several authors have suggested that depression associated with changes in the level of ovarian steroids is related to serotonin neural function (Eriksson et al 1995; Halbreich and Tworek 1993; Halbreich et al 1995; Mortola 1994; Steiner et al 1995).

Ovarian steroids could affect the serotonin system at multiple points. One pivotal point is at the level of tryptophan hydroxylase (TPH). TPH is the rate-limiting enzyme in serotonin (5HT) synthesis. We previously demonstrated that TPH mRNA levels increase in the dorsal raphe of macaques treated with estradiol (E) alone or estradiol + progesterone (E+P) (Pecins-Thompson et al 1996). Although addition of P to the E regimen had little effect on TPH mRNA levels, E+P treatment markedly increases prolactin secretion (Sprangers et al 1990; Wil-

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liams et al 1985), and prolactin is frequently cited as a neuroendocrine marker for serotonin function (Cleare et al 1996; Mitchell and Smythe 1990; O'Keane and Dinan 1991; Siever et al 1984; Sommers et al 1994). In rats, a difference in the translational efficiency of TPH mRNA between the pineal gland and the dorsal raphe has been reported (Dumas et al 1989). In macaques, E and E+P produce a similar increase in TPH mRNA in the raphe (Pecins-Thompson et al 1996), but in light of the rodent data, it seemed possible that P could further increase TPH translational efficiency, thereby producing higher levels of TPH protein. This may in turn lead to an increase in serotonin production. We therefore tested the hypothesis that TPH protein mass would be higher in macaques treated with E+P compared to macaques treated with E alone due to P-induced changes in translational efficiency. This hypothesis does not rule out alternative regulatory mechanisms, such as changes in TPH activity via phosphorylation mechanisms or changes in enzymes responsible for the degradation of serotonin.

The extremely low expression level of TPH mRNA in spayed macaques led to the speculation that this may be one of the molecular mechanisms by which surgical menopause increases negative affect. The question then arises as to why many women transition through natural menopause with little or no symptomology, whereas some women become markedly depressed (Sherwin 1996). There is currently no evidence that monkeys manifest depressive behavior after ovariectomy. We therefore tested the hypothesis that there may be a gradual adjustment in TPH protein expression in macaques that had been spayed for different lengths of time.

The development of modified or conjugated estrogens and progestins that are effective after oral ingestion has lead to their widespread use in hormone replacement therapy of menopausal women. There is substantial evidence that estrogen is beneficial to the function of the cardiovascular system (Adams et al 1990; Clarkson et al 1990, 1997; Grodstein et al 1997; Wagner et al 1996), the skeletal system (Marcus, 1997), and the central nervous system, particularly cognition and affect (Bimonte and Denenberg 1999; Fink et al 1996). Unopposed estrogen increases the risk of both uterine cancer and breast cancer, however. Thus, hormone replacement therapy includes a progestin to antagonize the action of estrogens in the uterus and breast. Currently, reports are accumulating that medroxyprogesterone acetate (MPA), the progestin most commonly used in the United States in hormone replacement regimens, antagonizes the beneficial effects of estrogens in the cardiovascular system and on mood, whereas natural progesterone does not (Adams et al 1990; Clarkson et al 1990; de Lignieres and Vincens 1982; Hulley et al 1998; Miyagawa et al 1997; Sherwin 1991). In this study, we tested the hypothesis that the addition of MPA to estrogen replacement therapy would decrease TPH protein mass in the dorsal raphe of macaques.

A large clinical trial is currently underway to determine whether tamoxifen, a mixed estrogen agonist and antagonist, can be of prophylactic benefit to women at high risk for breast cancer (Yeomans-Kinney et al 1995). Tamoxifen acts as an estrogen antagonist in breast and as an estrogen agonist in the skeletal and cardiovascular systems (Costantino et al 1997; Love et al 1992; Webb et al 1995; Williams et al 1997b). Tamoxifen also inhibits estrogen-induced hippocampal dendritic spine growth in vitro (Murphy and Segal 1996). A greater percent of cancer patients on tamoxifen reported depression than those not taking tamoxifen (Cathcart et al 1993) raising the question of how tamoxifen acts in serotonin neurons. In this study, we tested the hypothesis that tamoxifen would act as an estrogen antagonist on TPH protein mass in the dorsal raphe of macaques.

In summary, TPH protein mass was examined qualitatively with immunocytochemistry and measured quantitatively with western blotting in the dorsal raphe region of monkeys treated with E and P as previously described for measurement of TPH mRNA. In addition, the use of western blotting also enabled examination of TPH from frozen brain tissues that had been banked from long-term preclinical trials. Rhesus macaques (*Macaca mulatta*) were treated in various paradigms with natural E and P. Cynomolgus macaques (*Macaca fasicularis*) were treated with equine estrogens (EE), plus and minus MPA, or tamoxifen in a long-term treatment paradigm. Because TPH requires phosphorylation for activation (Kuhn et al 1997; Kumer et al 1997), the results from these studies will provide a foundation for further examination of the effect of ovarian steroids and modified steroids on TPH enzyme activity.

Methods and Materials

These experiments were approved by the Institutional Animal Care and Use Committees of the Oregon Regional Primate Research Center and the Bowman Gray School of Medicine. All monkeys were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Unless otherwise stated, reagents were ordered from Sigma Chemical Co. (St. Louis, MO).

Animals and Experimental Groups: TPH Immunocytochemistry (ICC)

Female rhesus monkeys were ovariectomized (ovx) and hysterectomized (spayed) according to previously described proce-

dures 3–6 months before assignment to this project (Sprangers et al 1990). Animals ranged in age from 6–15 years old. For TPH ICC, nine previously spayed rhesus monkeys were assigned to treatment groups as follows ($n = 3$/group):

1) Spayed–ovariectomized and hysterectomized controls
2) E-treated—spayed female adults treated for 28 days with an E-filled Silastic capsule
3) E+P-treated—spayed female adults treated for 28 days with an E-filled Silastic capsule supplemented with a P-filled Silastic capsule for the last 14 of the 28 days

These nine animals were part of a previous study on the regulation of TPH mRNA in which details of their treatments, perfusion, and processing of the brain tissue were described (Pecins-Thompson et al 1996). Sections for immunocytochemistry (ICC) were thaw-mounted on Superfrost Plus slides (Fischer Scientific, Santa Clara, CA) and stored at −80° C. Adjacent sections from these animals were also used for measurement of tryptophan hydroxylase (TPH) mRNA, serotonin reuptake transporter (SERT) mRNA and 5HT1A mRNA using in situ hybridization (ISH). The TPH, SERT, and 5HT1A mRNA results have been published elsewhere (Pecins-Thompson and Bethea 1998; Pecins-Thompson et al 1996, 1998).

Animals and Experimental Groups: Western Analysis

TREATMENT WITH NATURAL HORMONES: RHESUS MACAQUES (*MACACA MULATTA*). The pontine midbrain (fresh, not perfused) was obtained from 29 female adult (5–16 years old) rhesus monkeys undergoing necropsy for other experimental programs at the Oregon Regional Primate Research Center (ORPRC). The rhesus monkeys were sedated with Ketamine HCl (10 μg/kg IM) and then anesthetized deeply with pentobarbital (30 μg/kg IV). The brain was removed from the cranium, and the midbrain was dissected, wrapped in foil, and immediately dropped in liquid nitrogen. It was then stored at −80° C until sufficient numbers of animals were collected to warrant western analysis. The time from cutting the diaphragm (cessation of oxygen to the brain) and freezing of the midbrain was on the order of 10 min. Each necropsy is performed in a routine and consistent manner. Blood samples were obtained at necropsy for radioimmunoassay to verify serum levels of E and P.

The experimental treatments of the rhesus monkeys included the following:

1) Spayed—eight female adults were ovariectomized and hysterectomized for various lengths of time (2–40 months).
2) E replacement—three adult females were spayed and treated for 28 days with a 3.0 cm E-filled Silastic capsule implanted subcutaneously.
3) Artificially cycled—three female adults were spayed and implanted with a 3.0 cm E-filled Silastic capsule. Fourteen days after implantation of the E capsule, animals received one 6.0 cm P-filled Silastic capsule. The P capsule was removed and reimplanted every 14 days for 7 cycles. The animals were euthanized during the E+P treatment period.
4) E primed, then P—four spayed female adults received a 3.0 cm E-filled Silastic capsule for 7 days, then a 6.0 cm P-filled capsule was implanted. After 7 days of E+P, the E capsule was removed, and the animals were maintained with only the P capsule in place for an additional 4 weeks.
5) Intact—one female adult exhibiting normal menstrual cycles was euthanized during the luteal phase.
6) Low dose P—five female adults were spayed and treated with P only for 2 weeks using a 3.0 cm P-filled Silastic capsule implanted subcutaneously.
7) Pregnant—due to other experimental paradigms, five rhesus monkeys were euthanized at various stages of gestation or postpartum ($n = 1$/per time point, 48, 52, 97, 152 days gestation and 30 days postcesarean).

TREATMENT WITH MODIFIED HORMONES: CYNOMOLGUS MACAQUES (*MACACA FASICULATA*). The subjects of the study were female cynomolgus monkeys imported directly from Indonesia (Institut Pertanian Bogor, Bogor, Indonesia). For a total of 34 months, all animals were fed a moderately atherogenic diet (40% of calories were from fat and .28 mg cholesterol/kcal) (Adams et al 1990). Monkeys lived in social groups consisting of four to six animals. They were ovariectomized (ovx) and consumed the atherogenic diet for a 4-month pre-experimental period. Monkeys were then assigned by use of a stratified randomization scheme to one of four experimental groups ($n = 5$/group):

1) untreated ovx controls
2) ovx treated with conjugated equine estrogens (EE)(Premarin,Wyeth-Ayerst, Princeton, NJ)
3) ovx treated with medroxyprogesterone acetate (MPA)(Cycrin, Wyeth-Ayerst)
4) ovx treated with EE plus MPA.
5) ovx treated with tamoxifen (Nolvadex, ICI Pharmaceuticals)

Hormones were administered continuously in the diet for 30 months (Adams et al 1997; Williams et al 1997a). As in previous studies, the difference in caloric intake between monkeys and human beings was used to calculate the appropriate dose for the monkeys; it was adjusted for differences in both body size and metabolic rate (Clarkson et al 1990). On this basis, a 4-kg monkey received .17 mg EE and/or .65 mg MPA. Plasma concentrations of estradiol, estrone, MPA, and tamoxifen were measured by radioimmunoassay of samples collected 2 hours after administration to verify that dosing resulted in plasma concentrations similar to those of women taking these compounds (Adams et al 1997; Williams et al 1997a, 1997b).

After 30 months of treatment, the monkeys were anesthetized deeply with pentobarbital (30 mg/kg IV); the cranium was then retracted, and the brain was removed for dissection. 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 of TPH.

TPH Immunocytochemistry

Frozen sections mounted on slides were immediately immersed in cold 85% ethanol, followed by 10 mmol/L PBS buffer (pH 7.3). The sections were blocked with normal rabbit serum (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and then incubated at 4 C overnight with affinity purified sheep anti-human TPH (1:100, Chemicon International Inc., Temecula, CA). The next day, the sections were washed in 10 mmol/L PBS, reblocked with normal rabbit serum, drained, incubated with biotinylated rabbit anti-sheep second antibody for 30 minutes, and then washed in PBS followed by a 60-minute incubation in the Vector ABC reagent. The slides were then rinsed .05 mmol/L Tris (pH 6.5) and placed in 200 mL of .05 mmol/L Tris containing 140 mg diaminobenzidine tetrahydrochloride (DAB)(Wako Chemical, Richmond, VA), 40 μL of 30% hydrogen peroxide, and 2 mL of 20% nickel chloride. The reaction was stopped in .05 mmol/L Tris, and the sections were dehydrated in a graded series of ethanols, infiltrated with xylene, and coverslipped.

Midbrain Dissection for TPH Protein Assay

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 the major portion of the dorsal raphe. Each piece was immediately homogenized in 500 μL of 50 mmol/L Tris (pH 7.5; ICN Biomedicals, Aurora, OH) and 20 mmol/L 2-β-mercaptoethanol and centrifuged at 10,000 × *g* for 2 min. The supernatant was removed and stored at −80° C until TPH western blot analysis. 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 (Bradford 1976).

Midbrain TPH Western Analysis

Raphe extracts containing 300 μg of protein were loaded on a 10% SDS polyacrylamide gel. Aliquots of a large homogenate of monkey pineals were maintained at −80° C. The same volume and protein concentration (129 μg) of pineal homogenate were included on each gel as a positive control. Molecular weight markers (Biorad) were also included. Western blotting was performed according to the modified procedures of Dumas et al (1989) with blotting buffer containing 25 mmol/L Tris base (ICN) and 192 mmol/L glycine. The nitrocellulose membrane was initially stained with 2% Ponceau-S, which displayed the molecular weight markers and multiple 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) containing the sample lanes was blocked with 5% aqueous Carnation nonfat dry milk (Nestle Food Company, Glendale, CA) for 45 min. Affinity purified sheep anti-TPH (Chemicon International Inc., Temecula, CA) was used at a dilution of 1/500 in buffer containing 50 mmol/L Tris and 150 mmol/L NaCl (pH 7.5). Rabbit anti-sheep antibody conjugated with horseradish peroxidase (Chemicon) was used as second antibody at a dilution of 1/7,000. TPH signal was detected by exposing the blot to chemilumenescent film after developing with Supersignal substrate reagents (Pierce, Rockford, IL). Scientific imaging film (Eastman Kodak) was used for development of signal. The NIH image software program was used for quantitative analysis of the TPH band.

Densitometric Analysis of Western Blotting Results

The TPH band on the film was captured using an XC-77 CCD video camera (Sony, Towada, Japan). 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.

Assay Validation

The quantitative analysis for TPH protein was confirmed by analyzing increasing concentrations of monkey pineal gland extracts and monkey raphe extracts. Pineal samples (50 μg, 100 μg, and 150 μg of protein) were electrophoresed and blotted as described above and used for quantitative analysis of TPH. One rhesus monkey raphe was processed with the same method and samples (100 μg, 200 μg, and 300 μg of protein) were electrophoresed and blotted as described above and used for quantitative analysis of TPH.

Steroid Hormone Determinations

Serum concentrations of estradiol-17-β and progesterone in the rhesus monkeys were determined by RIA in the blood sample obtained at necropsy in the Hormone Assay Core of the Oregon Regional Primate Research Center as described by Resko et al (1974, 1975). Serum concentrations of estradiol-17-β, MPA and tamoxifen in the cynomolgus monkeys were determined in blood samples obtained 2 hours after dosing by RIA in the Hormone Assay Core of the Department of Comparative Medicine, Bowman Gray School of Medicine (Adams et al 1997; Williams et al 1997a).

Statistical Analysis

Midbrain TPH protein mass (OD = area under optical density curve of the band on the film in arbitrary units) and serum hormone concentrations were compared using analysis of variance (ANOVA), followed by the Student-Newman-Keuls pairwise post hoc comparison. In order to assay sufficient numbers of animals for statistical comparisons and to include control samples and molecular weight markers, multiple gels were needed on two occasions (natural hormone treatments and synthetic hormone treatments). If an animal was assayed more than once, then the mean of the multiple OD measurements was used for further statistical analysis (one number for each animal). Otherwise, the individual OD of the band was entered. Data analysis was conducted using the Instat Statistic Program (GraphPad, San Diego, CA) on a Macintosh computer. A confidence level of $p < .05$ was considered significant.

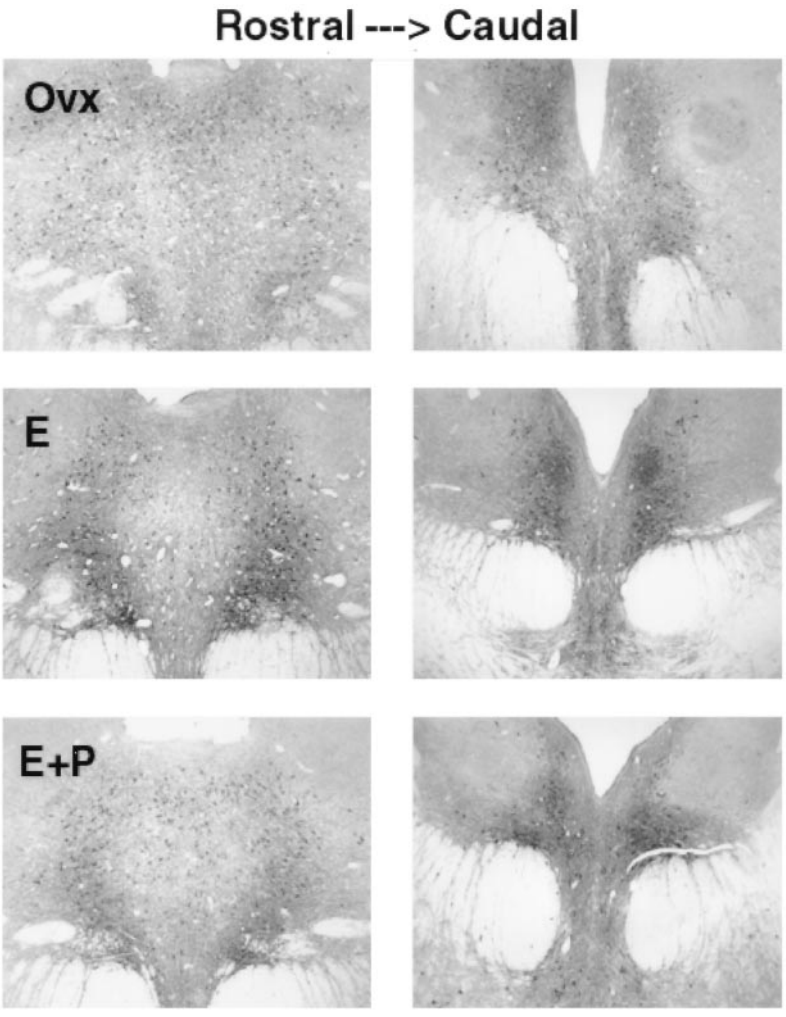


Figure 1. Representative photomicrographs of TPH immunohistochemical staining in the dorsal raphe of rhesus macaques that were spayed and then untreated (top), treated with E for 28 days (middle), or treated with E for 28 days and supplemented with P for the last 14 days of the 28-day period (bottom). There is a visual increase in the intensity of the label for TPH upon treatment with E or E+P, but it is difficult to accurately quantitate. At higher magnifications, the reaction deposit can be observed to extend into the axons and dendrites in the E- and E+P-treated animals, and the number of cells per field with very darkly staining cytoplasm is increased with E and E+P.

Results

TPH Immunocytochemistry

Representative photomicrographs of the dorsal raphe region of a spayed control, an E-treated, and an E+P-treated monkey are shown in Figure 1. These animals were treated, euthanized, and processed at the same time. Then the sections were processed within the same immunocytochemical assay. Moreover, the immunostained sections are adjacent to sections previously used for TPH mRNA analysis (Pecins-Thompson et al 1996). The antibody to TPH labeled cell bodies in the dorsal raphe. The density of the label appears darker in the animals treated with E or E+P. In addition, TPH immunoreaction product appears to spread into axons of the E- and E+P-treated animals. Thus, the relative amount of the immunoreaction product reflects the previously reported level of TPH mRNA (Pecins-Thompson et al 1996). Quantitation of immunoreaction product in tissue sections is difficult because the avidin–biotin bridging reaction is not linear, and the density of label may vary depending on the depth of the cell in the section. Therefore, western analysis was developed using the same antibody to TPH.

Assay Validation for Quantitative Analysis of TPH Enzyme

Comparison of the TPH band on the films to the molecular weight markers on the nitrocellulose membranes indicated that the molecular weight of monkey TPH is approximately 55–56 kD. The density of the TPH enzyme signal from the western analysis is positively correlated with the concentration of protein loaded on the gel (Figure 2). The density (area under peak) of 50, 100, and 150 μg of pineal TPH protein extracts was 596, 2243, and 3369, respectively. The density of 100, 200, and 300 μg of raphe protein extracts was 3399, 5395, and 9102, respectively. Thus, analysis of TPH enzyme with Western analysis and the NIH Image Analysis software was linear and quantitative in the range of 50 to 300 μg of protein loaded.

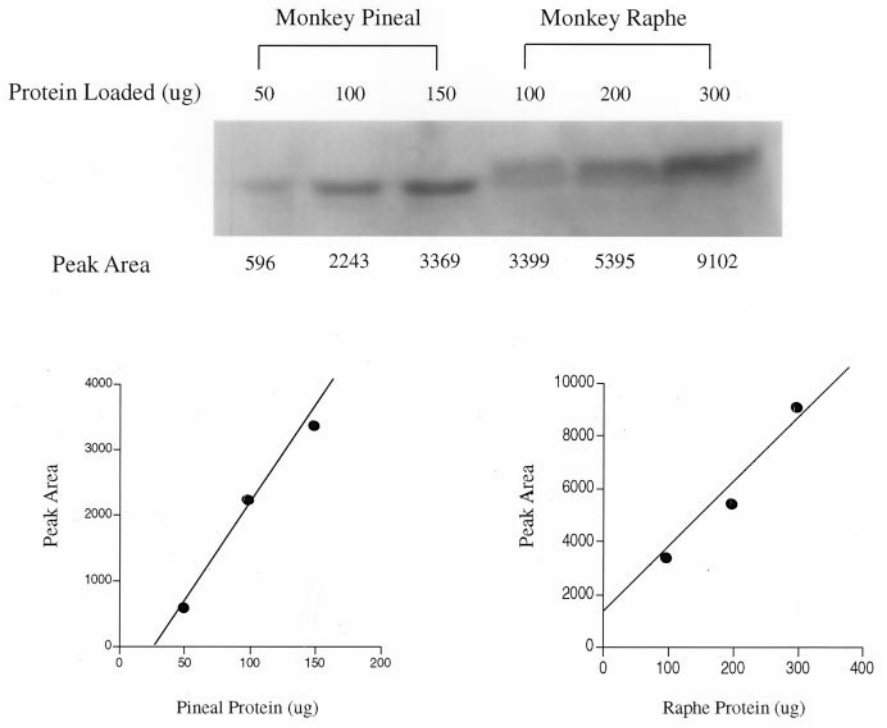


Figure 2. Demonstration of the linearity of the western blotting procedure. Increasing concentrations of extracts from the pineal gland and the dorsal raphe produced a linear increase in band density as determined with the NIH Image software subroutine for gel analysis.

Natural Hormone Treatment of Rhesus Macaques

Figure 3 contains a comparison of TPH protein mass (mean OD ± SEM) in rhesus monkeys that had been spayed for various lengths of time. There was a significant increase in TPH levels from 6 to 40 months after spaying ($n = 5$) compared with 2 to 4 months after spaying ($n = 3$). It may be noted that spayed or ovx monkeys have a low level of E in the serum. In the series of animals used here, E ranged from 2 to 20 pg/mL in the absence of ovarian secretion. This low level of E is attributed to the production of E by fat and adrenal tissue.

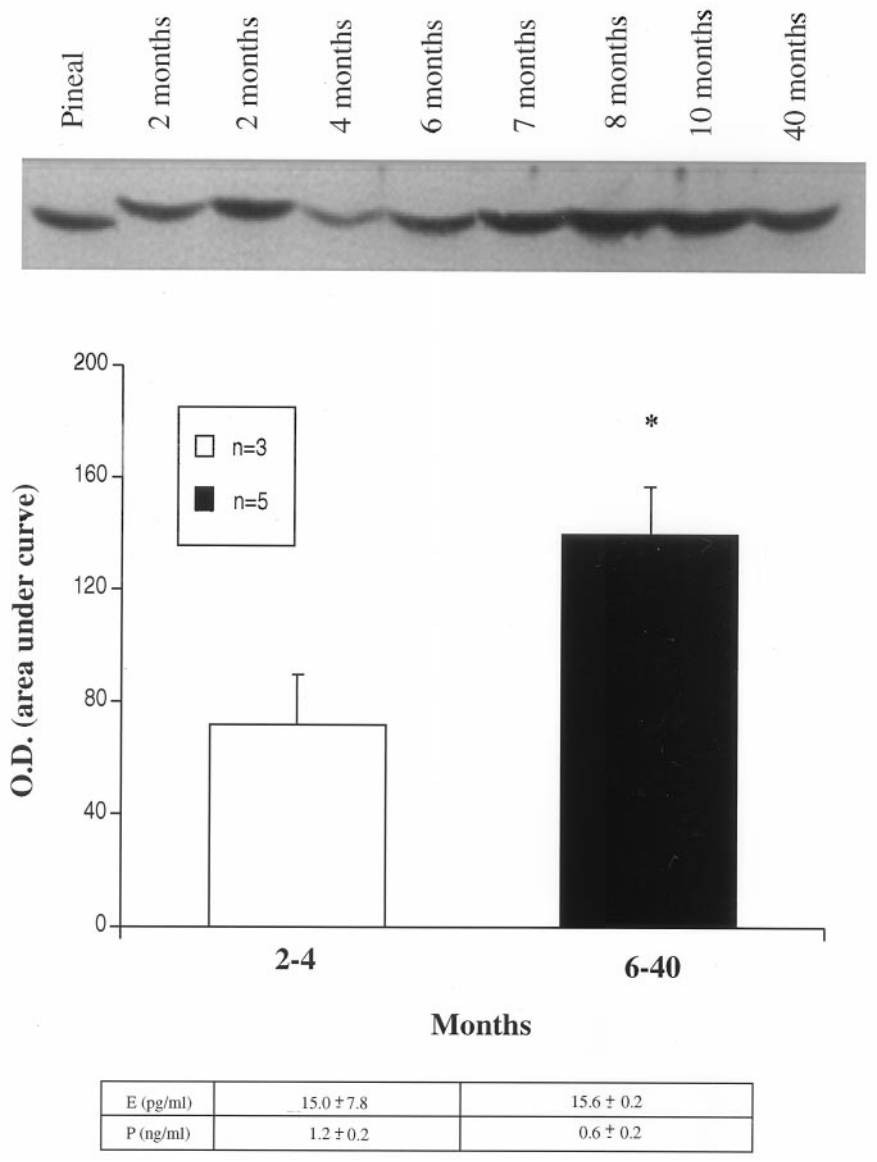
Figure 4 shows that E treatment increased TPH protein mass greater than three fold compared to the 2-month spayed control. The Intact animal was combined with the artificially cycled group (E then E+P) due to similar levels of serum E and P. There was no statistical difference between TPH protein mass in the animals treated with E alone compared to animals in which P was present, either due to the natural cycle or due to hormone supplementation (Artificial cycle). Moreover, TPH protein levels remained elevated when the E capsule was removed and the P capsule was left in place for 4 weeks prior to necropsy. Nonetheless, it should be noted that there was modest level of E present in the serum of the 4-week P-maintained animals (ca. 20 pg/mL).

Treatment of spayed animals with a low dose of P for 2 weeks approximately doubled the mass of TPH protein detected compared to a 2-month spayed animal; however, on this gel, TPH protein was six fold higher in the E-alone treated animal than in the spayed animal. It should be noted that E was present at a low level in the serum of the P-only treated animals (ca. 10 pg/mL) (Figure 5).

Figure 6 illustrates the TPH protein levels in a series of animals that were euthanized at various stages of gestation (complete gestation = 176 ± 3 days in rhesus macaques) or 30 days after a Caesarean (C-) section was performed. It is rare for a pregnant monkey to go to necropsy, and thus there is only one animal at each time point. Nonetheless, the serum E concentrations were nearly constant in this series of pregnant animals, whereas the serum P concentrations varied depending on the day of gestation. A linear regression analysis between serum P and TPH optical density yielded a correlation coefficient r = .932. TPH levels declined in accordance with the modest serum E concentration and very low serum P concentration 30 days after C-section,

Hormone Replacement Therapy in Cynomolgus Macaques

Administration of EE alone or MPA alone to cynomolgus macaques for 30 months significantly increased the TPH protein levels compared to ovx controls. By contrast, administration of EE plus MPA significantly reduced the TPH protein levels compared to EE alone, such that TPH protein levels in ovx controls and EE+MPA-treated monkeys were not different (Figure 7).



E (pg/ml)	15.0 ± 7.8	15.6 ± 0.2
P (ng/ml)	1.2 ± 0.2	0.6 ± 0.2

Figure 3. Western blot comparison of TPH mass in rhesus macaques that had been ovx for various lengths of time. There is a significant increase in TPH signal with time after ovariectomy (ANOVA $p < .05$; $F = 4.3262$). The serum levels of E and P at necropsy in the respective groups are shown at the bottom.

Long-term treatment with tamoxifen also significantly decreased TPH protein levels. TPH protein levels were lower in animals treated for 30 months with tamoxifen than in animals treated for 30 months with EE and also lower than in animals that had been ovx for 30 months (Figure 8). The levels of estradiol 17β, estrone, MPA, and tamoxifen achieved in the serum of the animals shown in Figures 7 and 8 have been reported elsewhere (Adams et al 1997; Williams et al 1997a). Table 1 summarizes in a relative fashion the mass of TPH observed across the different treatment paradigms. Overall, the comparison is consistent with ovarian steroids augmenting TPH protein expression, whereas the loss or antagonism of ovarian steroids is associated with lower TPH protein expression.

Discussion

Serotonin is only one of the neurotransmitters modulating mood and pituitary hormone secretion, but its involvement is undisputed (Owens and Nemeroff 1994; Van de Kar 1989). Serotonin neurotransmission can be regulated at many points. Within the serotonin neuron, complex mechanisms govern precursor uptake, 5HT synthesis, release, reuptake, degradation, and autoreceptor inhibition of firing. Each of these mechanisms involves highly specific proteins which, minimally, are transcribed as RNA, translated into amino acid sequences, and then frequently subjected to further posttranslational trafficking and modifications such as phosphorylation.

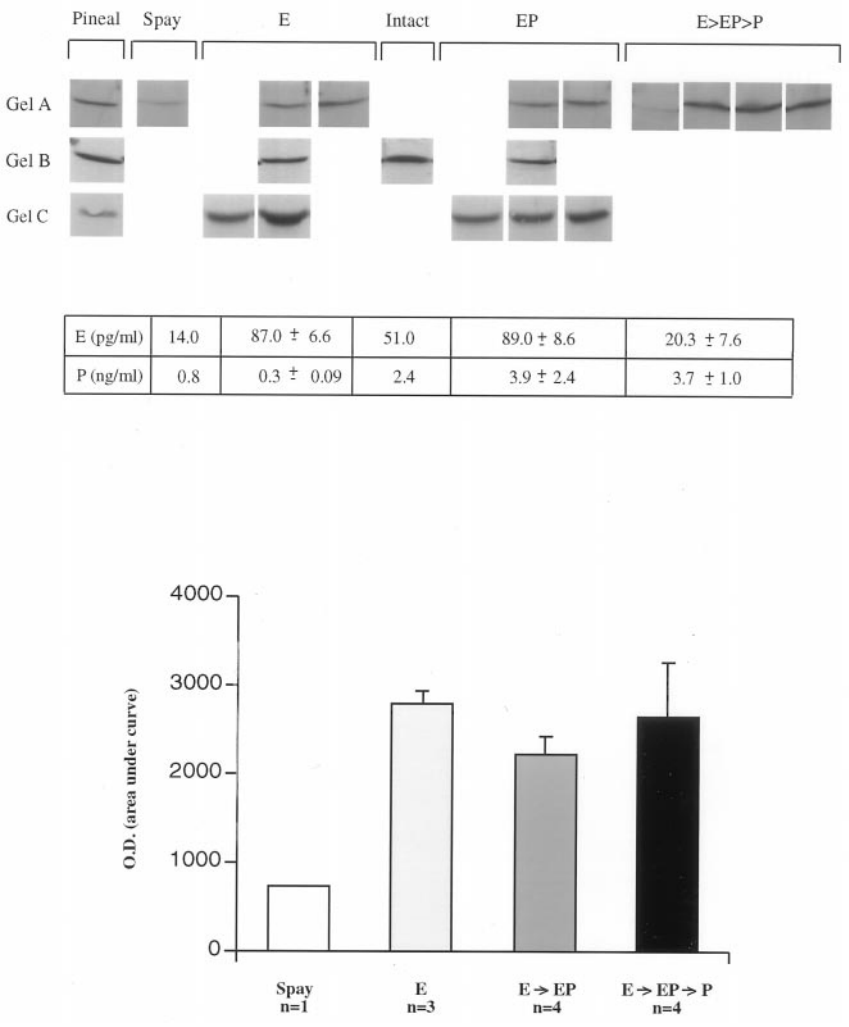
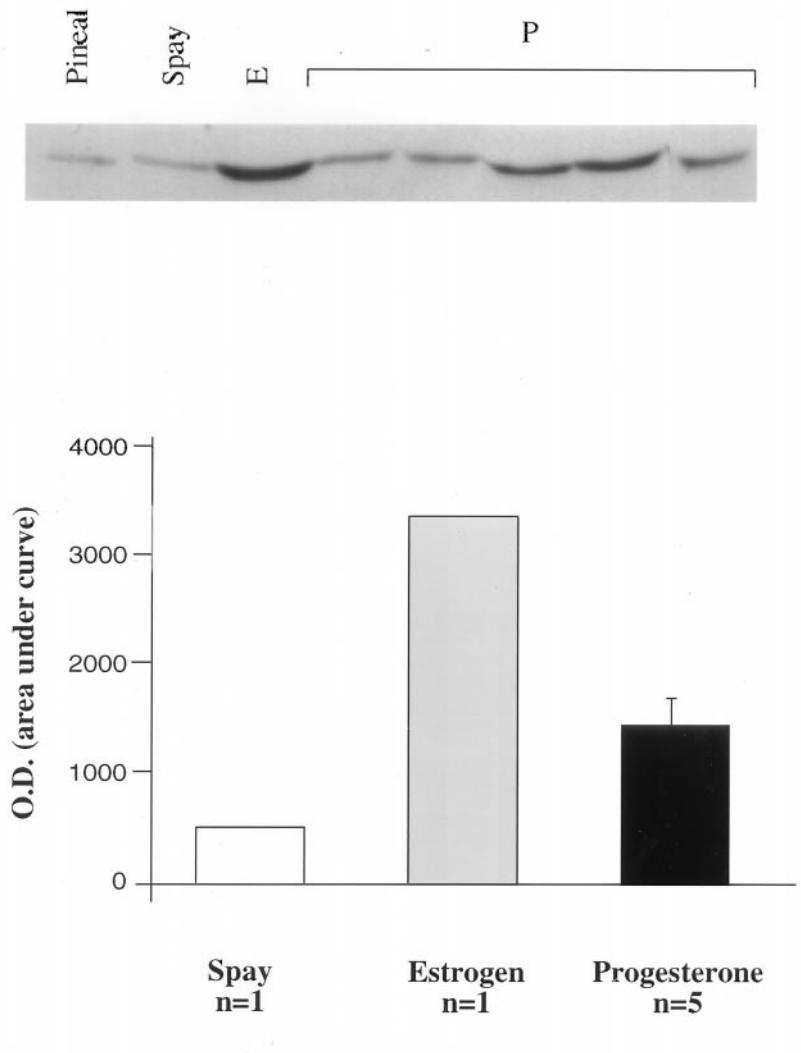


Figure 4. Western blot comparison of TPH protein in animals exposed to E and P. Three gels were needed to analyze sufficient numbers of animals for statistical comparisons and to include control wells and molecular weight markers. Each row contains a complete gel, but the bands were cut apart to line up in labeled columns. Each column represents one animal. Note that samples of one E-treated animal and one E+P-treated animal were included on each gel (columns 4 and 8). An additional E+P-treated animal was assayed twice (column 9). The average OD of the different measurements on these animals was used to obtain the group means. The pineal gland bands represent three different aliquots of a frozen pool. One spay animal from the 2–4 month group shown in Figure 3 was included for comparison. The E group contains three animals that were treated with one E-filled capsule for 28 days. The intact animal was euthanized during the late luteal phase. The EP group contains three animals that were artificially cycled and euthanized during the E+P treatment phase. The "E then E+P then P" group contains four animals that were primed with E and then maintained for 28 days with P treatment only. The serum levels of E and P at necropsy are shown under the gels. Due to similar levels of E and P, the intact animal was combined with the artificially cycled animals for statistical comparisons. ANOVA with post hoc pairwise comparison was performed on the "E only group" vs. the "E+P group" vs. the "E then P only group." There was no statistical difference between the groups treated with E alone or E combined with P, although the steroid treatments markedly elevated TPH compared to the spayed control animal (ANOVA $p = .11$; $F = 2.8252$).

In questioning the effect of ovarian hormones, E and P, on serotonin neural function, we initially focused at the level of transcription using nonhuman primates. E and P act largely through nuclear receptors that are transcription factors. Previously, we reported that E or E+P increase mRNA expression of TPH (Pecins-Thompson et al 1996) and decrease the expression of mRNAs that code for the serotonin reuptake transporter, SERT, and the 5HT1A autoreceptor (Pecins-Thompson and Bethea 1998; Pecins-Thompson et al 1998). It is currently unknown whether these changes in mRNA are manifested by changes in protein and/or if they result in functional consequences. Therefore, starting with TPH, we sought to determine whether the increase in TPH mRNA observed with E and E+P treatment would lead to a concomitant increase in TPH protein mass.

Although this issue seems straightforward, there are several corollaries that impact the question. TPH mRNA increased with E treatment, and addition of P had no additional effect (Pecins-Thompson et al 1996); however, two presumed endpoints of serotonin neurotransmission, prolactin secretion (Sprangers et al 1990; Williams et al 1985) and the 5HT/5HIAA ratio in cerebral spinal fluid (Schutzer et al 1997), were not affected by E alone but instead increase with E+P. Thus, there is a discrepancy between steroid regulated TPH mRNA and two indices of serotonin neurotransmission.

This discrepancy is not completely untoward given the various processes intervening between TPH mRNA and serotonin neurotransmission; however, the fact that P causes this discrepancy suggests that we do not yet understand what P is doing and where it acts. The potential answers to this question could be numerous. In combination with E, we found that P does not have a unique action



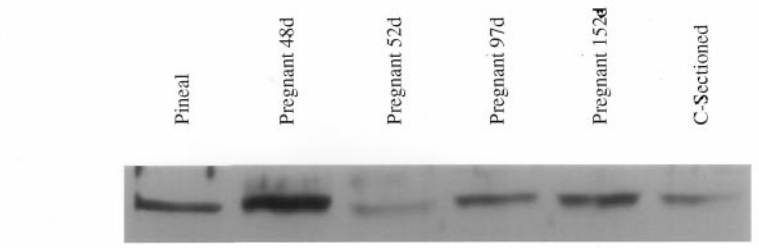
E (pg/ml)	2.0	95.0	9.4±4.4
P (ng/ml)	1.2	0.3	2.3±0.5

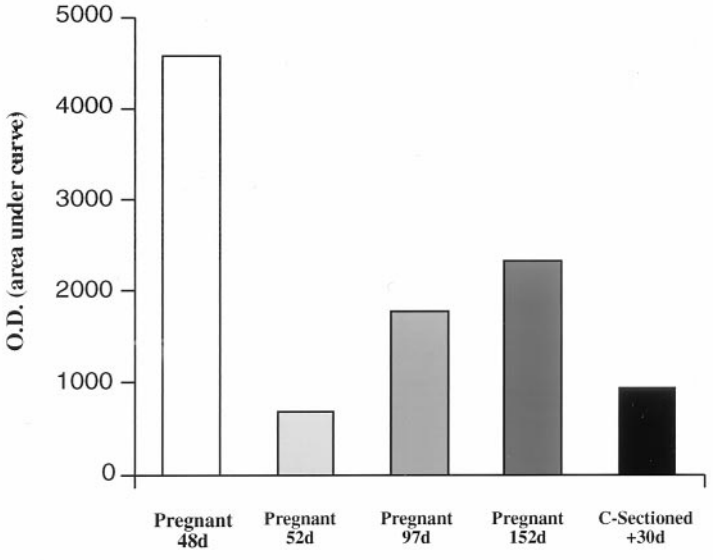
Figure 5. Western blot comparison of TPH mass in a spayed control and an E-treated animal vs. five animals that had been spayed and then treated for 14 days with a low dose of P. P alone caused a two-fold increase in TPH over the 2 month spayed control. No statistical comparison was performed on these results.

at the level of SERT gene expression (Pecins-Thompson et al 1998), but P has an additive effect at the level of 5HT1A autoreceptor gene expression (Pecins-Thompson and Bethea 1998). The next question is whether P acts on translation or posttranslational mechanisms of pivotal proteins, such as TPH, SERT, or the 5HT1A autoreceptor.

In the experiment reported here and using a sound statistical test, we did not detect any difference in TPH protein expression with western blotting when P treatment was added to a moderate E regimen in spayed macaques. Rather, similar levels of TPH protein were measured in animals treated with E alone or E+P. These animals were treated in the same manner as the animals in which we previously observed no statistical difference in the level of TPH mRNA with E or E+P treatment (Pecins-Thompson et al 1996). In addition, immunocytochemical examination of TPH protein reflected TPH mRNA expression in adjacent sections (Pecins-Thompson et al 1996). Hence, for this particular model, we can tentatively conclude that TPH protein levels reflect TPH mRNA levels and that there is no indication of P action on translation.

An apparent dose–response relationship was observed between P and TPH protein expression in pregnant macaques who had a higher concentration of circulating estrogens than the hormone-supplemented animals in Figure 3. This data is consistent with a recent report that P levels correlate with 5HT neuronal activity in rodents (Klink and Debonnel 1998). The pregnant monkeys are not completely comparable to the spayed monkeys, however, because many other factors are present during pregnancy. Furthermore, in spayed females, a low dose of P caused a two-fold increase in TPH protein on a back-





E (pg/ml)	214.0	217.0	181.0	218.0	29.0
P (ng/ml)	3.4	0.8	1.4	2.7	0.3

Figure 6. Western blot comparison of TPH mass in pregnant animals that were euthanized at different days of gestation and following a C-section for other clinical or experimental reasons. The serum levels of E and P at necropsy are shown at the bottom. The level of E was consistent in the pregnant animals, but the level of P varied. A linear regression analysis was performed on the results from the pregnant animals. There was a linear correlation between serum P and the optical density of the TPH signal ($r = .932$; $F = 13.233$).

ground of very low E. TPH mRNA expression has not been documented in the pregnant or P-only models. Nonetheless, the initial experiment with E and P replacement suggests that TPH protein reflects TPH mRNA and that the majority of regulation occurs at the level of transcription. An experiment in which the concentrations of E and P are carefully manipulated in a dose-related manner with sufficient numbers of animals at each dose and measurement of both TPH mRNA and TPH protein would be desirable, but prohibitively expensive. Unfortunately, serotonin neurons in rats lack nuclear receptors for E or P, and TPH is not regulated in rats by ovarian hormones (Alves et al 1997, 1998). This suggests that rats may not provide an adequate model for primates in this regard.

The data presented here do not support the hypothesis that P augments TPH translation. Therefore, the question still remains of precisely where P acts to increase serotonin neural function. TPH requires phosphorylation for activity (Johansen et al 1996; Kuhn et al 1997; Kumer et al 1997). Therefore, we need to determine if P affects the expression of kinases that facilitate TPH phosphorylation. Moreover, P could affect other biosynthetic and metabolic enzymes, such as amino acid decarboxylase or monoamine oxidase, or even impact cofactor availability.

Conjugated equine Es (EE) increased TPH protein levels in a manner similar to E (estradiol 17β); however, the results with the synthetic progestin MPA differed from those with natural P. Addition of MPA to the E regimen decreased TPH protein expression. Because TPH mRNA and protein correlated in the E and E+P replacement paradigm, we speculate that this effect is exerted at the level of transcription. MPA alone, however, increased TPH protein levels in a manner similar to the low dose of P administered to spayed macaques. Thus, natural P and MPA have

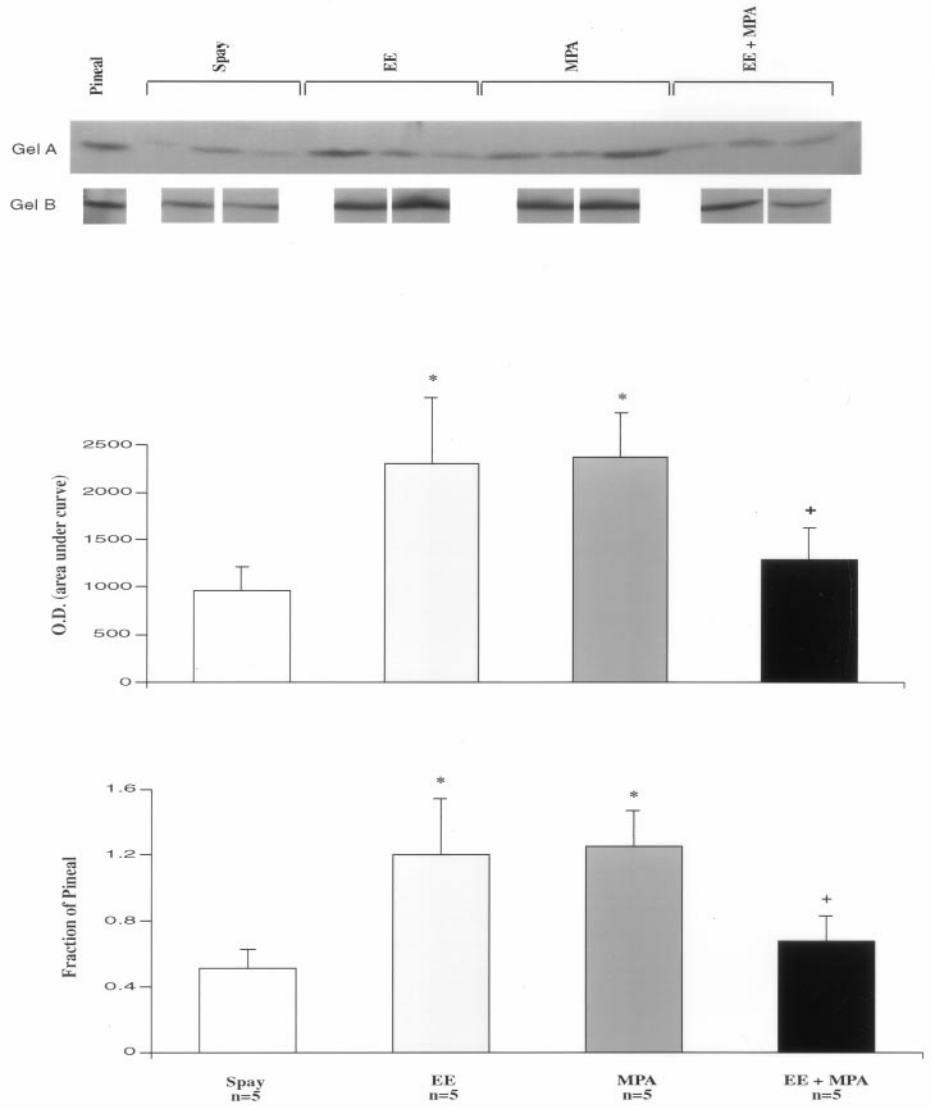


Figure 7. Western blot comparison of TPH mass in ovariectomized cynomolgus macaques that were either untreated or treated with conjugated equine estrogens (EE), with medroxyprogesterone acetate (MPA), or with EE + MPA for 30 months in their diet. The number of animals included in the study required two gels, which were run simultaneously. Each row illustrates one complete gel, but the lanes in gel B were separated to line up under the respective treatment labels. The top histogram represents the averages of the optical densities of the treatment groups. In the bottom histogram, the optical density of each animal was converted to a fraction of the pineal positive control band on the respective gel prior to obtaining group averages for statistical analysis. In each analysis, there was a significant increase in TPH mass with EE and MPA treatments alone (ANOVA $p < .02$; $F = 5.6265$); however, TPH mass was reduced to spayed control levels in the group treated with EE + MPA. * Different from spayed control group, $p < .05$, Student-Newman-Keuls post hoc comparison. $^{+}$ Different from EE group, $p < .05$, Student-Newman-Keuls post hoc comparison.

similar positive actions on TPH expression when administered alone on a low background of E. By contrast, in the presence of moderate levels of E, MPA acts as an antagonist, whereas natural P does not. A molecular mechanism to explain the different actions of P and MPA is lacking, although MPA has androgenic activity in addition to its progestin quality. These results may provide part of a biological explanation for the reports that MPA antagonized the beneficial effect of E on mood in menopausal women (Sherwin 1991; Sherwin and Gelfand 1989), but that natural P did not (de Lignieres and Vincens 1982). Similar results were observed with respect to parameters of cardiovascular function (Clarkson et al 1997; Hulley et al 1998; Miyagawa et al 1997).

Tamoxifen had a pronounced antiestrogenic action on TPH protein expression. This compound is used extensively in breast cancer treatment (Horwitz 1995), and it is currently under investigation as a prophylactic for women at risk of developing breast cancer (Yeomans-Kinney et al 1995). Tamoxifen is considered a mixed estrogen agonist and antagonist because it has estrogen-like action in the bone, but not in the breast or uterus (Love et al 1992; Webb et al 1995). The results presented herein indicate that tamoxifen is an estrogen antagonist with respect to TPH expression. The observation that TPH protein was lower in tamoxifen-treated animals than ovx animals suggests that tamoxifen is antagonizing the low level of E present in the ovx animals (from adrenal or fat tissue). These data are consistent with the observation that women taking tamoxifen experienced depression more frequently than women taking placebo (Cathcart et al 1993).

We also observed an increase in TPH protein with time after spaying (complete hysterectomy). We have not

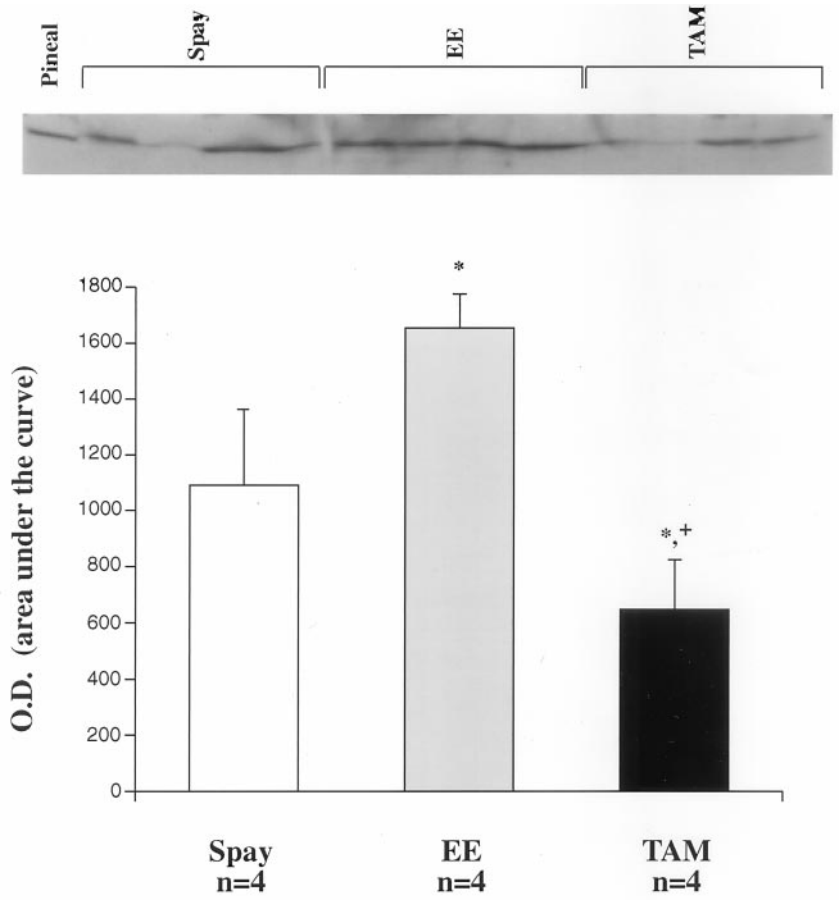


Figure 8. Western blot comparison of ovariectomized cynomolgus macaques that were either untreated (spay), ovx and treated with EE, or ovx and treated with tamoxifen for 30 months in their diet. There was a significant difference in TPH signal across the groups (ANOVA $p < .002$; $F = 19.864$). * Significantly different from spayed control group, $p < .05$, Student-Newman-Keuls post hoc comparison. $^{+}$Significantly different from EE-treated group, $p < .01$, Student-Newman-Keuls post hoc comparison.

examined TPH mRNA in this paradigm, but if TPH protein reflects TPH mRNA as suggested above, then a modest adjustment in transcription may occur after removal of ovarian hormones. This observation may relate to the highly individual experience of postmenopausal depression. Some women report depression upon menopause, whereas others transition with little or no symptomology.

Table 1. Comparison of Relative TPH Mass in the Dorsal Raphe Region of Macaques across Different Experimental Paradigms

Group	Relative TPH protein mass
Short-term spayed	*
Long-term spayed	**
Estrogen	****
Estrogen+progesterone	****
Estrogen then progesterone only	****
Progesterone only	**
Pregnant	***
Postcesarean section	*
Equine estrogens (EE)	****
Medroxyprogesterone (MPA)	****
Equine estrogens + MPA	**
Tamoxifen	*

One star represents the lowest level of TPH detected, and additional stars represent approximate fold increases over the lowest level of TPH detected.

It is attractive to speculate that a modest recovery in TPH may occur in some women but not others and moreover, that it may be a vulnerable process. Previous studies suggest that women who experience depression prior to menopause are more likely to experience depression after menopause as well (Stewart and Boydell 1993). Perhaps such women have an abnormal or defective TPH regulatory mechanism. In addition, negative affect is more likely to follow precipitous surgical menopause than slower natural menopause (Sherwin and Gelfand 1985). This further indicates that with time, there may be compensatory events in the serotonin neural system of which TPH gene expression may be one.

P could also act at many other points to facilitate serotonin neurotransmission. Although we did not observe any additional effect of P over E at the level of SERT gene expression, SERT protein requires phosphorylation for activation as well (Blakely et al 1998). Therefore, we need to ask whether P could also alter the activity of SERT via posttranslational mechanisms. We did find that P further decreased 5HT1A mRNA when added to a E regimen. If

the decrease in 5HT1A mRNA is manifested by a decrease in receptor protein, then this action of P would further disinhibit serotonin neural firing. These and other questions are awaiting investigation.

In conclusion, we have presented evidence that TPH protein expression increased similarly in monkeys treated with E alone versus E+P and that TPH protein regulation reflects TPH mRNA expression that was determined in a previous study. Thus, we have no evidence that P alters TPH translation. In other paradigms, P alone modestly increased TPH protein expression, and MPA alone increased TPH protein as well; however, MPA antagonizes the stimulatory effect of estrogens on TPH protein expression, unlike natural P. We also demonstrated that tamoxifen behaves as an estrogen antagonist on TPH protein expression. Finally, TPH protein expression increased with time after spaying. Changes in TPH protein caused by estrogens, anti-estrogens, or progestins probably reflect regulation at the level of gene transcription. TPH protein mass does not indicate enzyme activity, but if greater levels of TPH mRNA or TPH protein augment serotonin production, then these observations provide a biological basis for the ability of ovarian and modified steroids to alter mood and other autonomic functions regulated by serotonin. Specifically, the increase in TPH protein observed with E plus or minus natural P and with conjugated EE is predicted to elevate mood. MPA or P alone may also have a modestly positive effect on affect. By contrast, the decrease in TPH protein when MPA is added to estrogens or with tamoxifen administration is unlikely to improve affect and may even precipitate depressive symptoms.

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References

Adams MR, Kaplan JR, Manuck SB, et al (1990): Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys: lack of an effect of added progesterone. *Arteriosclerosis* 10:1051–1057.

Adams MR, Register TC, Golden DL, Wagner JD, Williams JK (1997): Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscler Thromb Vasc Biol* 17:217–221.

Alves SE, Weiland NG, Hastings NB, Tanapat P, McEwen BS (1997): Estradiol regulation of 5HT neurons in the dorsal raphe of the rat differs from the monkey: an analysis of tryptophan hydroxylase mRNA levels. *Soc Neurosci* 23:1222.

Alves SE, Weiland NG, Hayashi S, McEwen BS (1998): Immunocytochemical localization of nuclear estrogen receptors and progestin receptors within the rat dorsal raphe nucleus. *J Comp Neurol* 391:322–334.

Bimonte HA, Denenberg VH (1999): Estradiol facilitates performance as working memory load increases. *Psychoneuroendocrinology* 24:161–173.

Blakely RD, Ramamoorthy S, Schroeter S, et al (1998): Regulated phosphorylation and trafficking of antidepressant-sensitive serotonin transporter proteins. *Biol Psychiatry* 44:169–178.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

Cathcart CK, Jones SE, Pumroy CS, Peters GN, Knox SM, Cheek JH (1993): Clinical recognition and management of depression in node negative breast cancer patients treated with tamoxifen. *Breast Cancer Res Treat* 27:277–281.

Clarkson TB, Cline JM, Williams JK, Anthony MS (1997): Gonadal hormone substitutes: effects on the cardiovascular system. *Osteoporosis Int* 1(suppl 7):43–51.

Clarkson TB, Shively CA, Morgan TM, Koritnik DR, Adams MR, Kaplan JR (1990): Oral contraceptives and coronary artery atherosclerosis of cynomolgus monkeys. *Obstet Gynecol* 75:217–222.

Cleare AJ, Murray RA, O'Keane V (1996): Reduced prolactin and cortisol responses to d-fenfluramine in depressed compared to healthy matched control subjects. *Neuropsychopharmacology* 14:349–354.

Costantino JP, Kuller LH, Ives DG, Fisher B, Dignam J (1997): Coronary heart disease mortality and adjuvant tamoxifen therapy. *J Natl Cancer Inst* 89:776–782.

de Lignieres B, Vincens M (1982): Differential effects of exogenous oestradiol and progesterone on mood in postmenopausal women: individual dose/effect relationship. *Maturitas* 4:67–72.

Dumas S, Darmon MC, Delort J, Mallet J (1989): Differential control of tryptophan hydroxylase expression in raphe and in pineal gland: Evidence for a role of translation efficiency. *J Neurosci* 24:537–547.

Eriksson E, Hedberg MA, Andersch B, Sundblad C (1995): The serotonin reuptake inhibitor paroxetine is superior to the noradrenaline reuptake inhibitor maprotiline in the treatment of premenstrual syndrome. *Neuropsychopharmacology* 12: 167–176.

Fink G, Sumner BEJ, Rosie R, Grace O, Quinn JP (1996): Estrogen control of central neurotransmission: effect on mood, mental state, and memory. *Cell Mol Neurobiol* 16: 325–344.

Gitlin MJ, Pasnau RO (1989): Psychiatric syndromes linked to reproductive function in women: a review of current knowledge. *Am J Psychiatry* 146:1413–1422.

Gregoire AJP, Kumar R, Everitt B, Henderson AF, Studd JWW (1996): Transdermal oestrogen for treatment of severe postnatal depression. *Lancet* 347:930–933.

Grodstein F, Stampfer MJ, Colditz GA, et al (1997): Postmenopausal hormone therapy and mortality. *N Engl J Med* 336: 1769–1775.

Halbreich U (1997): Role of estrogen in postmenopausal depression. *Neurology* 48 (Sup7):S16–S20.

Halbreich U, Piletz J, Halaris A (1992): Influence of gonadal hormones on neurotransmitters, receptor, cognition and mood. *Clin Neuropharmacol* 15(suppl 1) Pt. A:590A–591A.

Halbreich U, Rojansky N, Palter S, Tworek H, Hissin P, Wang K

(1995): Estrogen augments serotonergic activity in postmenopausal women. *Biol Psychiatry* 37:434–441.

Halbreich U, Tworek H (1993): Altered serotonergic activity in women with dysphoric premenstrual syndromes. *Int J Psychiatry Med* 23:1–27.

Halbreich U, Vital-Herne J, Goldstein S, Zander K (1984): Sex-related differences in depression: A reappraisal Can gender differences in prevalence of depression be attributed to biological differences? *Psychopharmacol Bull* 20:472–474.

Horwitz KB (1995): Editorial:When tamoxifen turns bad. *Endocrinology* 136:821–823.

Hulley S, Grady DD, Bush T, et al (1998): Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA* 280:605–613.

Johansen PA, Jennings I, Cotton RG, Kuhn DM (1996): Phosphorylation and activation of tryptophan hydroxylase by exogenous protein kinase A. *J Neurochem* 66:817–823.

Klink R, Debonnel G (1998): Functional modulation of rat dorsal raphe serotonergic neruons by ovarian steroids. *Soc Neurosci* 24:1757.

Kuhn DM, Arthur R Jr, States JC (1997): Phosphorylation and activation of brain tryptophan hydroxylase: Identification of serine-58 as a substrate site for protein kinase A. *J Neurochem* 68:2220–2223.

Kumer SC, Mockus SM, Rucker PJ, Vrana KE (1997): Amino-terminal analysis of tryptophan hydroxylase: protein kinase phosphorylation occurs at serine-58. *J Neurochem* 69:1738–1745.

Love RR, Mazess RB, Barden HS, et al (1992): Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 326:852–856.

MacKenzie TB (1986): Lifetime prevalence of psychiatric disorders in women with perimenstrual difficulties. *J Affect Disord* 10:15–19.

Marcus R (1997): Use of estrogen for prevention and treatment of osteoporosis. *Endocrine* 6:207–211.

Mitchell P, Smythe G (1990): Hormonal responses to fenfluramine in depressed and control subjects. *J Affective Disord* 19:43–51.

Miyagawa K, Rosch J, Stanczyk F, Hermsmeyer K (1997): Medroxyprogesterone interferes with ovarian steroid protection against coronary vasospasm. *Nature Med* 3:324–327.

Mortola JF (1994): A risk-benefit appraisal of drugs used in the management of premenstrual syndrome. *Drug Safety* 10:160–169.

Murphy DD, Segal M (1996): Regulation of dendritic spine density in cultured hippocampal neurons by steroid hormones. *J Neurosci* 16:4059–4068.

O'Keane V, Dinan TG (1991): Prolactin and cortisol responses to d-fenfluramine in major depression: evidence for diminished responsivity of central serotonergic function. *Am J Psychiatry* 148:1009–1015.

Oppenheim G (1983): Estrogen in the treatment of depression: neuropharmacological mechanisms. *Biol Psychiatry* 18:721–725.

Owens MJ, Nemeroff CB (1994): Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Chem* 40:288–295.

Pearlstein TB (1995): Hormones and depression: what are the facts about premenstrual syndrome, menopause and hormone replacement therapy? *Am J Obstet Gynecol* 173:646–653.

Pecins-Thompson M, Bethea CL (1998): Ovarian steroid regulation of 5HT1A autoreceptor messenger ribonucleic acid expression in the dorsal raphe of rhesus macaques. *Neuroscience* 89:267–277.

Pecins-Thompson M, Brown NA, Bethea CL (1998): Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. *Mol Brain Res* 53:120–129.

Pecins-Thompson M, Brown NA, Kohama SG, Bethea CL (1996): Ovarian steroid regulation of tryptophan hydroxylase mRNA expression in rhesus macaques. *J Neurosci* 16:7021–7029.

Resko JA, Norman RL, Niswender GD, Spies HG (1974): The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in rhesus monkeys. *Endocrinology* 94:128–135.

Resko JA, Ploem JG, Stadelman HL (1975): Estrogens in fetal and maternal plasma of the rhesus monkey. *Endocrinology* 97:425–430.

Schutzer WA, Widmann AA, Bethea, CL (1997): Effect of estrogen (E) and progesterone (P) on CSF concentrations of biogenic amines in pigtail macaques. *Soc for Neurosci* 23:2039.

Schwartz S (1991): Women and depression: a Durkheimian perspective. *Soc Sci Med* 32:127–140.

Sherwin BB (1991): The impact of different doses of estrogen and progestin on mood and sexual behavior in postmenopausal women. *J Clin Endocrinol Metab* 72:336–343.

Sherwin BB (1996): Menopause, early aging and elderly women. In Jensvold MF, Halbreich U, Hamiltion JA editors. *Psychopharmacology and Women: Sex, gender and hormones.* Washington, DC: American Psychiatric Press, 225–240.

Sherwin BB, Gelfand MM (1985): Sex steroids and affect in the surgical menopause: a double-blind cross-over study. *Psychoneuroendocrinology* 10:325–335.

Sherwin BB, Gelfand MM (1989): A prospective one-year study of estrogen and progestin in postmenopausal women: effects on clinical symptoms and lipoprotein lipids. *Obstet Gynecol* 73:759–766.

Siever LJ, Murphy DL, Slater S, de la Vega E, Lipper S (1984): Plasma prolactin changes following fenfluramine in depressed patients compared to controls: an evaluation of central serotonergic responsivity in depression. *Life Sci* 34: 1029–1039.

Sommers DK, Van Wyk M, Snyman JR (1994): Dexfenfluramine-induced prolactin release as an index of central synaptosomal 5-hydroxytryptamine during treatment with fluoxetine. *Eur J Clin Pharmacol* 46:441–444.

Sprangers SA, West NB, Brenner RM, Bethea CL (1990): Regulation and localization of estrogen and progestin receptors in the pituitary of steroid treated monkeys. *Endocrinology* 126:1133–1142.

Steiner M, Steinber S, Stewart D, et al (1995): Fluoxetine in the treatment of premenstrual dysphoria. *N Engl J Med* 332: 1529–1534.

Stewart DF, Boydell KM (1993): Psychologic distress during menopause: associations across the reproductive life cycle. *Int J Psychiatry Med* 23:157–162.

Van de Kar LD (1989): Neuroendocrine aspects of the serotonergic hypothesis of depression. *Neurosci Biobehav Rev* 13:237–246.

Wagner JD, Martino MA, Jayo MJ, Anthony MS, Clarkson TB, Cefalu WT (1996): The effects of hormone replacement therapy on carbohydrate metabolism and cardiovascular risk factors in surgically postmenopausal cynomolgus monkeys. *Metab: Clin Exp* 45:1254–1262.

Webb P, Lopez GN, Uht PM, Kushner PJ (1995): Tamoxifen activation of the estrogen receptor/Ap-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* 9:443–456.

Weissman MM, Klerman GL (1985): Gender and depression. *Trends Neurosci* 8:416–420.

Williams JBW, Spitzer RL, Linzer M, et al (1995): Gender differences in depression in primary care. *Am J Obstet Gynecol* 173:654–659.

Williams JK, Honore EK, Adams MR (1997a): Contrasting effects of conjugated estrogens and tamoxifen on dilator responses of atherosclerotic epicardial coronary arteries in nonhuman primates. *Circulation* 96:1970–1975.

Williams JK, Wagner JD, Li Z, Golden DL, Adams MR (1997b): Tamoxifen inhibits arterial accumulation of LDL degradation products and progression of coronary artery artherosclerosis in monkeys. *Arterioscl Thromb Vascular Biol* 17:403–408.

Williams RF, Gianfortoni JG, Hodgen GD (1985): Hyperprolactinemia induced by an estrogen-progesterone synergy: quantitative and temporal effects of estrogen priming in monkeys. *J Clin Endocrinol Metab* 60:126–132.

Yeoman-Kinney A, Vernon SW, Frankowski RF, Weber DM, Bitsura JM, Vogel VG (1995): Factors related to enrollment in the breast cancer prevention trial at a comprehensive cancer center during the first year of recruitment. *Cancer* 76:46–56.

Yonkers KA, Chantilis SJ (1995): Recognition of depression in obstetric/gynecology practices. *J Amer Obstet Gynecol* 173: 632–638.