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Ovarian Steroid Regulation of 5-HT$_{1A}$ Receptor Binding and G protein Activation in Female Monkeys

Nick Z. Lu, M.S., and Cynthia L. Bethea, Ph.D.

Serotonin 5-HT$_{1A}$ receptors play an important role in serotonin neurotransmission and mental health. We previously demonstrated that estradiol (E) and progesterone (P) decrease 5-HT$_{1A}$ autoreceptor mRNA levels in macaques. In this study, we questioned whether E and P regulate 5-HT$_{1A}$ binding and function and G$_{\alpha}$ subunit protein expression. Quantitative autoradiography for 5-HT$_{1A}$ receptors and G proteins using [^{3}H]8-OH-DPAT and [^{35}S]GTP-γ-S, respectively, was performed on brain sections of rhesus macaques from four treatment groups: ovariectomized controls (OVX), E (28 d), P (28 d), and E (28 d) plus P (the last 14 d) treated. Western blot analysis for G$_{\alpha}$ subunits was performed on raphe extracts from cynomolgus macaques that were OVX or OVX treated with equine estrogens (EE, 30 months). In the hypothalamus, E or E + P but not P alone decreased postsynaptic 5-HT$_{1A}$ binding sites. In the dorsal raphe nucleus (DRN), E, P, and E + P treatments decreased 5-HT$_{1A}$ autoreceptor binding. The Kd values for 8-OH-DPAT were the same for each treatment group. Both the basal and the R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding were decreased during hormone replacement whereas the coupling efficiency between the receptor and G proteins was maintained. Finally, EE treatment reduced the level of G$_{\alpha i3}$, but not G$_{\alpha i1}$, G$_{\alpha o}$, and G$_{\alpha z}$ in the DRN. In conclusion, these observations suggest that ovarian hormones may increase serotonin neurotransmission, in part, by decreasing 5-HT$_{1A}$ autoreceptors, 5-HT$_{1A}$ postsynaptic receptors, and the inhibitory G proteins for intracellular signal transduction. ***[Neuropsychopharmacology 27:12–24, 2002]***
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A body of evidence suggests that actions of the ovarian hormone, estradiol (E), on mood and cognitive function may be mediated by the serotonin neural system (McEwen 1999; Mann 1999). We have found that E, with or without progesterone (P), acts on gene expression in serotonin neurons in a manner that could increase serotonin neurotransmission. That is, E ± P treatment of ovariectomized (OVX) macaques increased tryptophan hydroxylase gene and protein expression (Pecins-Thompson et al. 1996; Bethea et al. 2000), but decreased the expression of serotonin reuptake transporter (Pecins-Thompson et al. 1998) and 5-HT$_{1A}$ autoreceptor mRNAs (Pecins-Thompson and Bethea 1999) in the dorsal raphe nucleus (DRN) of macaques. It is necessary, however, to know whether these documented changes in gene expression have functional consequences in the serotonin neural system.

Serotonin 5-HT$_{1A}$ receptors, either pre- or postsynaptically, play a pivotal role in serotonin neurotransmission (Raymond et al. 1999). The presynaptic autoreceptor, on

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the soma and dendrites of serotonin neurons, binds serotonin in the extracellular space and decreases neuronal firing and serotonin release (Sprouse and Aghajanian 1987; Blier and de Montigny 1987; Azmitia et al. 1996b). This ultra-short loop feedback mechanism is thought to cause a delay in the onset of efficacy of antidepressant drugs (Hjorth and Auerbach 1996; Hjorth et al. 2000). In depressed patients, 5-HT_{1A} autoreceptor levels are elevated (Stockmeier et al. 1998) and supplementation of antidepressant therapy with 5-HT_{1A} antagonists, such as pindolol, outperforms selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine alone (Artigas et al. 1996; Sacristan et al. 2000).

The reported effects of ovarian hormones on 5-HT_{1A} receptor binding in the rodent are not consistent. Depending on the area examined and the treatment regimen, postsynaptic 5-HT_{1A} receptor binding sites have been reported to increase (Flugge et al. 1999), decrease (Osterlund et al. 2000), or not change (Clarke and Maayani 1990; Frankfurt et al. 1994). Studies on ovarian steroid regulation of 5-HT_{1A} autoreceptor binding in rodents are lacking. We previously demonstrated that E $\pm$ P treatment of OVX macaques for one month decreased 5-HT_{1A} autoreceptor mRNA in the DRN (Pecins-Thompson and Bethea 1999). However, no change was observed in the postsynaptic 5-HT_{1A} receptor mRNA level in the hypothalamus (Gundlah et al. 1999). The effect of E or P on the binding activity of 5-HT_{1A} receptors in primates is unknown.

The 5-HT_{1A} receptor causes activation of the inhibitory G protein of $G_{i/o/z}$ families (Raymond et al. 1999). The association of guanosine triphosphate (GTP) molecules with α subunits of the G protein complex subsequently triggers cytosolic processes that decrease cAMP levels whereas $\beta\gamma$ subunits cause the opening of G protein-gated inwardly rectifying K^+ channels on cell membranes and thereby inhibit cell-firing (Raymond et al. 1999). E administration decreases the inhibition of 8-OH-DPAT, a potent 5-HT_{1A} agonist, on serotonergic cell firing in the DRN of rats (Lakoski 1988). Other studies demonstrate that E desensitizes 5-HT_{1A} mediated release of stress hormones such as corticosteroids by decreasing the level of subunits of G proteins (Raap et al. 2000). Clinical data indicate that subunits of G proteins are dysfunctional in psychiatric patients with bipolar disorders, depression, and anxiety (Vawter et al. 2000; Manji and Lenox 2000). However, it is not known whether E and P affect 5-HT_{1A} function and/or coupling to G proteins in primates. Therefore, we questioned whether E, P, or E + P would alter: (1) post- and presynaptic 5-HT_{1A} receptor binding; (2) basal and 8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding; and (3) G_α protein expression.

In this study, quantitative autoradiography was used to measure 5-HT_{1A} receptor-binding sites in hormone treated macaques and untreated OVX controls. Postsynaptic 5-HT_{1A} binding sites were determined in the hypothalamus and presynaptic 5-HT_{1A} binding sites were determined in the DRN. In addition, the effect of E and P on 5-HT_{1A} autoreceptor function was examined by determining the level of [^{35}S]GTP-γ-S binding with or without stimulation by R-(+)-8-OH-DPAT. Lastly, the generous donation of fresh-frozen midbrains from cynomolgus macaques maintained on long-term hormone replacement therapy enabled the examination of G protein expression.

METHODS

Reagents

[^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 124.9-135 Ci/mmol) and [^{35}S]Guanosine 5′-(γ-thio)triphosphate (GTP-γ-S, 1250 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA). Rabbit anti-$G_{\alpha i3}$ polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-$G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$, polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat-anti rabbit antibody conjugated to horseradish peroxidase (HRP) was purchased from Chemicon International (Temecula, CA). All other reagents, unless otherwise stated, were from Sigma (St. Louis, MO).

Animals

This study was approved by the Institutional Animal Care and Use Committees of the Oregon Regional Primate Research Center (ORPRC) and the Bowman Gray School of Medicine. Animals were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association.

Twenty adult female rhesus monkeys *(Macaca mulatta)* were OVX and twelve out of the twenty were also hysterectomized in other research programs according to standard veterinary procedures by the surgical personnel of ORPRC. Approximately two to six months after finishing other protocols, the animals were assigned to this study. The rhesus macaques were born and reared in Oregon and were in good health. The animals were all young adults from 7 to 12 years old and weighed between 4.5 and 7.5 kg.

The OVX rhesus macaques received either an empty Silastic capsule for 28 days (OVX controls), an E-filled capsule for 28 days (E treated), a P-filled capsule for 28 days (P treated) or an E capsule for 14 days supplemented with a P capsule for an additional 14 days (E + P treated). Each treatment group contained five animals.

Treatment with E and P as described above has been shown to cause uterine endometrium differentiation in a manner similar to the normal 28-day menstrual cycle (Brenner and Maslar 1988). The E regimen has also been shown to induce nuclear progestin receptor expression

in numerous target organs including the brain (Bethea et al. 1992; Bethea 1994) and the addition of P to the E regimen stimulates prolactin secretion (Williams et al. 1985; Sprangers et al. 1990; Bethea et al. 1992).

Cynomolgus monkeys (*Macaca fasicularis*) were imported directly from Indonesia (Institut Pertanian Bogor, Bogor, Indonesia) to the Department of Comparative Medicine, Wake Forest University, Winston-Salem, NC. For a total of 34 months, all animals were fed a moderately atherogenic diet (40% of calories were from fat and 0.28 mg cholesterol/kcal, Adams et al. 1990). Monkeys lived in social groups consisting of four to six animals. They were ovariectomized and consumed the atherogenic diet for a 4-month pre-experimental period. Monkeys were then assigned to various hormonal treatment groups according to a stratified randomization scheme. For this study, the midbrain was obtained from three OVX control animals and three OVX animals treated with conjugated equine estrogen (EE, Premarin, Wyeth-Ayerst, Princeton, NJ) in the diet for 30 months (Adams et al. 1997). The dosage of EE was adjusted for body size and metabolic rate to approximate a serum concentration of EE in the treated monkeys to that of women taking the same compound (Clarkson et al. 1990).

Rhesus and cynomolgus macaques are closely related species. They have similar menstrual cycles and are cross-fertile. Immunocytochemistry studies have reported that nuclear steroid receptors are present in serotonin neurons of both species (Bethea 1993).

Surgery and Steroid Treatments of Rhesus Macaques

Silastic capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, I.M.; Fort Dodge Laboratories, Fort Dodge, IA). The E treated monkeys were implanted (S.C.) with one 4.5 cm E-filled Silastic capsule (inner diameter, 3.35 mm, outer diameter, 4.65 mm; Dow Corning, Midland, MI). The capsule was filled with crystalline estradiol (1,3,5(10)-estratrien-3,17-β-diol, Steraloids, Wilton, NH). The P treated monkeys were implanted with one 6.0 cm Silastic capsule containing crystalline progesterone (4-pregnen-3,20 dione, Steraloids). The E + P treated group received a 4.5 cm E-filled capsule, and 14 days later, received one 6.0 cm P-filled capsule for an additional 14 days. OVX monkeys implanted with empty 4.5 cm Silastic capsules were used as the control group.

Tissue Harvest and Cryosection for Autoradiography

The rhesus monkeys were euthanized at the end of the treatment period according to the procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine (10 mg/kg I.M.), given an overdose of pentobarbitol (25 mg/kg, I.V.), and exsanguinated by severance of the descending aorta. The left ventricle of the heart was cannulated and the head of each monkey was perfused with 1 L of 0.25 M sucrose in 0.05 M Tris buffer, pH 7.4, containing 5000 unit/L heparin and 2 L of 0.5 M sucrose in 0.05 M Tris buffer, pH 7.4. The brain was removed and dissected. Hypothalamic and midbrain pontine blocks were frozen in isopentane cooled to −55°C and stored at −80°C until sectioning that occurred within two months of storage. Coronal sections (10 μm) were cut on a cryostat at −20 to −22°C, thaw-mounted on Superfrost Plus Slides (Fisher Scientific, Santa Clara, CA), dehydrated under vacuum at 4°C for 2 h and then frozen at −80°C until processing for binding studies. Every seventeenth section at an interval of 170 μm was stained with thionin for morphological references and anatomical orientation (Paxinos et al. 2000).

Tissues from Cynomolgus Macaques for G protein Western Blot Analysis

Dorsal raphe tissue extracts were obtained from six OVX adult female cynomolgus macaques for Western blot analyses of G protein subunits. At the end of the protocol described above, the monkeys were anesthetized deeply with pentobarbital (30 mg/kg, I.V.); the cranium was 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. Detailed tissue preparation for Western analysis has been published (Bethea et al. 2000).

[^{3}H]-8-OH-DPAT Binding

5-HT_{1A} binding experiments were performed according to Verge et al. (1986). Briefly, sections were brought to room temperature in a desiccator and preincubated in the preincubation buffer (170 mM Tris and 4 mM $CaCl_2$, pH 7.6) at 22°C for 30 min. Then, sections were incubated with 2 nM [^{3}H]-8-OH-DPAT in assay buffer (the preincubation buffer supplemented with 0.01% L–ascorbic acid, 10 μM pargyline, and 10 μM fluoxetine) for 1 h followed by two rinses in the preincubation buffer for 4 min each at 4°C. Slides were then dipped in distilled water at 4°C for 3 s and dried rapidly with cold air. Non-specific binding was assessed on adjacent sections with the addition of 2 μM serotonin. Plus, saturation studies were performed by incubating serial sections with [^{3}H]-8-OH-DPAT at seven concentrations ranging from 0.1 nM to 8 nM.

Matching sections from OVX controls, E treated, P treated and E + P treated monkeys were processed in the same experiment and exposed to ^{3}H-sensitive ultra films along with ^{3}H autoradiographic micro-scales

(Amersham, Arlington Heights, IL) for 30 days. Films were developed in Kodak developer for 5 min and fixed for 8 min. Autoradiograms were digitized with a SONY CCD video camera. Densitometry was performed using NIH image on a Macintosh computer. Each film was calibrated with ^{3}H autoradiographic micro-scales.

Two measurements were generated from the autoradiograms, which required the operator to circle the anatomical area of interest. The first measurement was the average gray scale optical density, obtained by subtracting the background optical density value from the total optical density value of the region of interest. This indicates the relative intensity of the signal. The second measurement yielded the average positive pixel area, obtained by setting a threshold for positive signals above the background level (the same setting was used for all animals). The positive pixels indicate the area covered by the signal. Thus, a decrease in this parameter may represent a decrease in the number of cells above the threshold for detection. The optical density and positive pixel area reflect different aspects of protein binding signals although they should, in general, change in the same direction for a defined area.

In separate experiments, midbrain sections from four OVX control animals were preincubated with the preincubation buffer containing 1 to 100 nM E, P, or E + P for 30 min before [^{3}H]-8-OH-DPAT binding to determine the effect of steroids in vitro. In addition, twelve concentrations ranging from 0.1 nM to 300 μM of unlabeled serotonin, Way-100635 (5-HT_{1A} antagonist), L-694247 (5-$HT_{1B/D}$ antagonist; Tocris, Ballwin, MO), and ketanserin (5-HT_{2A} antagonist) were used to compete for [^{3}H]-8-OH-DPAT binding on serial sections. In these competition studies, sections were scraped off the slides, after washing, with GF/C Glass Microfiber filters (Whatman, England), equilibrated with 3 ml of BD ScintiVerse (Fisher Chemicals, Fair Lawn, NJ) for 3 h, and counted on a Tri-carb 1500 scintillation counter (Packard Instrument Co., Meridan, CT).

G protein Autoradiography Using [^{35}S]GTP-γ-S

[^{35}S]GTP-γ-S binding experiments were performed according to Dupuis et al. (1999) and Sim et al. (1997). Briefly, sections were brought to room temperature in a desiccator and preincubated in the assay buffer (in mM, 50 HEPES, 50 $NaCl_2$, 3 $MgCl_2$, and 0.2 EGTA, pH 7.4) at 25°C for 10 min. Then, sections were incubated with 2 mM GDP in the assay buffer at 25°C for 15 min followed by incubation with 0.04 nM [^{35}S]GTP-γ-S in the assay buffer supplemented with 2 mM GDP, 0.2 mM dithiothreitol (Roche Molecular Biochemicals, Indianapolis, IN), and 10 mU/ml adenosine deaminase at 25°C for 90 min. At the end of the incubation, reagents were washed off the sections by rinsing with 50 mM HEPES, pH 7.4 twice for 2 min each and dipping in distilled water at 4°C. Sections were rapidly dried with cool air. The basal binding and stimulated binding were defined in the absence or presence of 1 μM of R-(+)-8-OH-DPAT, respectively, during the incubation with [^{35}S]GTP-γ-S. Non-specific binding was defined on adjacent sections by the addition of 10 μM of unlabeled GTP-γ-S. Saturation studies were performed by incubating serial sections with [^{35}S]GTP-γ-S at concentrations ranging from 4 to 400 pM. Also, 0.1 and 1 μM Way-100635 and phentolamine (α adrenergic blocker) were used to block the R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding. In addition, nine concentrations of R-(+)-8-OH-DPAT up to 3 μM were used to stimulate [^{35}S]GTP-γ-S binding on serial sections to evaluate the potency of this 5-HT_{1A} agonist in the macaque.

Matching sections from OVX control animals, E treated, P treated and E + P treated monkeys were processed in the same experiment and exposed to Kodak Biomax MR films along with ^{14}C autoradiographic micro-scales (Amersham, Arlington Heights, IL) for three to six days. Films were developed in Kodak developer for 2 min and fixed for 5 min. Autoradiograms were digitized with a SONY CCD video camera. Densitometry was performed using NIH Image software as described above. Each film was calibrated with ^{14}C autoradiographic micro-scales.

Western Analysis for G protein Subunits

Monkey midbrain blocks containing the DRN were microdissected and hand homogenized in 50 mM Tris and 20 mM β-mercaptoethanol, pH 7.5 (Bethea et al. 2000). 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 μg/ml), Trypsin inhibitor (1 mg/ml), O-phenanthroline (1 mM), iodoacetamide (1 mM), PMSF (250 mM), and pepstatin A (1 μM) and further homogenized with a hand-held pestle and mortar (Fisher Scientific). The concentrations of the total protein in the homogenates were determined with the Bio-Rad (Hercules, CA) protein determination reagent according to Bradford (1976). 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 blot analyses were performed according to the modified procedures of Mullaney and Milligan (1990) with blotting buffer containing 25 mM of Tris base and 192 mM of glycine. The nitrocellulose membranes (Osmonics, Westborough, MA) were blocked in 5% non-fat dry milk for 45 min before incubating with antibodies for G_{α} subunits at 4°C overnight. The dilutions for rabbit anti-$G_{\alpha i3}$, $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$, were 1:1600, 1:150, 1:700, and 1:200, respectively. The following morn-

ing, the blots were washed in saline and 0.05% Tween-20 (Bio-Rad) and incubated with 1:2000 of goat anti-rabbit antibody conjugated to HRP at room temperature for 2 h and then developed with Supersignal chemiluminesence kits (Pierce, Rockford, IL) followed by exposing to Kodak X-OMAT AR films. Densitometric analysis of signal bands was performed with NIH Image Gel Plotting software.

Hormone Assays

Concentrations of serum E, P, and prolactin were measured in samples obtained at necropsy of the rhesus macaques. Radioimmunoassays were performed in the Endocrine Service Core at ORPRC as described previously (Resko et al. 1974, 1975; Bethea and Papkoff 1986). Concentrations of serum E in cynomolgus macaques were measured at Wake Forest University (Bethea et al. 2000).

Statistics

For autoradiography, measurements from four to six levels of each area examined were used to yield an average for each animal. There were five animals in each treatment. The optical density values and positive pixel areas of signals on autoradiograms were within the linear range of standards. Average values from each treatment group were compared with unpaired 1-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc pairwise comparison. Competition and saturation binding data were analyzed with nonlinear regression curve-fitting programs using GraphPad Prism 3.0 (San Diego, CA). In Western blot analysis, the density of signal bands from OVX and EE treated groups were compared with 2-tailed Student's *t*-test. Steroid hormone concentrations were compared with 1-way ANOVA and Student-Newman-Keuls. Statistical comparisons between treatments were conducted with Prism 3.0. A confidence level of $p < .05$ was considered significant.

RESULTS

Distribution Pattern and Regulation of [^{3}H]8-OH-DPAT Binding in the Monkey Hypothalamus

The anatomical distribution of hypothalamic postsynaptic 5-HT_{1A} receptors labeled with [^{3}H]8-OH-DPAT matched the distribution pattern of 5-HT_{1A} mRNA in the hypothalamus observed previously (Gundlah et al. 1999). A discrete pattern of [^{3}H]8-OH-DPAT binding in the hypothalamus was observed in the vertical limb of diagonal band of Broca (DB), preoptic area (POA), supraoptic (SON), paraventricular (PVN), periventricular (PEV), ventromedial nucleus (VMN), and the dorsal medial hypothalamus (DMH) (Figure 1).

The optical density was averaged from four to six levels of each hypothalamic nucleus and compared with ANOVA (5 animals/treatment). 5-HT_{1A} binding sites were significantly decreased by E or E + P, but not by P alone compared with the OVX control animals in the DB, PVN, PEV, VMN, and DMH (Figure 2). Positive pixel areas reflected optical density values in each hypothalamic region examined. In the SON and POA, optical density values and pixel areas of [^{3}H]8-OH-DPAT binding were not different among treatment groups.

Distribution Pattern and Regulation of [^{3}H]8-OH-DPAT Binding in the Monkey Raphe

[^{3}H]8-OH-DPAT labeling on monkey midbrain sections matched the distribution pattern of 5-HT_{1A} autoreceptor mRNA (Pecins-Thompson and Bethea 1999), i.e., robust in the dorsal raphe, moderate in the median raphe, and light in the periaqueductal gray (Figure 3). Specific [^{3}H]8-OH-DPAT binding was saturable (Figure 4). Kd values (nM ± S.E.M.) for the radioligand on monkey midbrain sections equaled 3.06 ± 2.26, 1.86 ± 2.25, 3.21 ± 2.88, and 2.66 ± 2.68 in OVX, E, P, and E + P treated groups, respectively (not different, $p > .05$). Unlabeled serotonergic compounds blocked the binding of [^{3}H]8-OH-DPAT with the following IC_{50} rankings: Way-100635 < 5-HT <

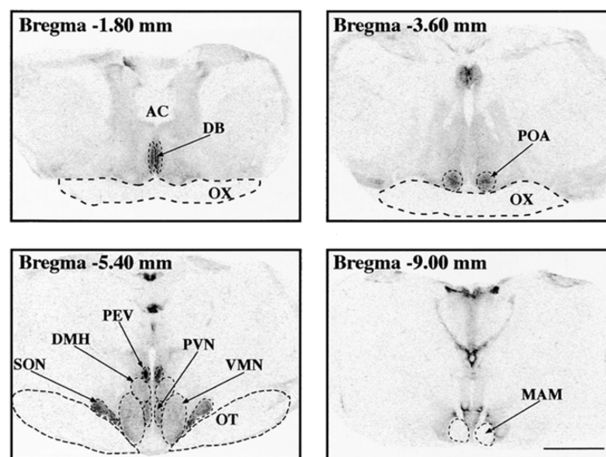


Figure 1. Autoradiograms of [^{3}H]8-OH-DPAT binding on coronal monkey hypothalamic sections (10 μm) in a rostral to caudal direction. The dark areas are autoradiographic signals representing 5-HT_{1A} receptor density in different hypothalamic nuclei. Significant signal intensity was detected in the vertical limb of the diagonal band of Broca (DB), preoptic area (POA), supraoptic (SON), paraventricular (PVN), periventricular (PEV), ventromedial nucleus (VMN), and dorsal medial hypothalamus (DMH). For anatomical orientation, the following landmarks are indicated. AC (anterior commissure), MAM (mammillary body), OT (optic tract), and OX (optic chiasm). Scale bar = 5 mm.

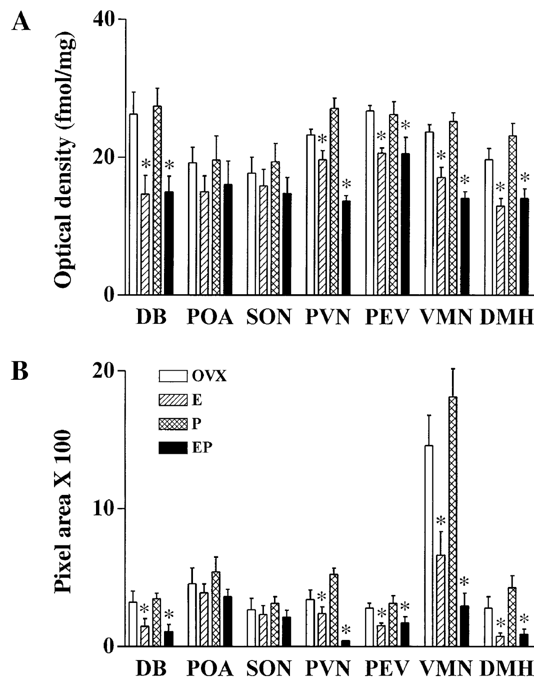


Figure 2. Comparison of [^{3}H]8-OH-DPAT binding sites in the monkey hypothalamic sections from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A: Average optical density values from four to six levels of each hypothalamic nucleus (n = 5 animals/treatment). Compared with the OVX control animals using ANOVA, 5-HT_{1A} binding sites were significantly decreased by E, or E + P but not by P alone in the DB (p = .004, F = 6.9, df = 18), PVN (p < .0001, F = 24, df = 18), PEV (p = .0254, F = 4.1, df = 18), VMN (p < .0001, F =19, df = 18), and DMH (p = .0005, F =11, df = 18). B: Positive pixel areas reflecting the [^{3}H]8-OH-DPAT binding in the hypothalamus. Compared with the OVX control animals, [^{3}H]8-OH-DPAT labeled positive pixel areas were significantly decreased by E, or E + P but not by P alone in the DB (p = .0155, F = 4.8, df = 18), PVN (p < .0001, F = 20, df = 18), PEV (p = .042, F = 3.5, df = 18), VMN (p < .0001, F =17, df = 18), and DMH (p = .0025, F = 7.6, df = 18). [^{3}H]8-OH-DPAT binding sites were not changed by any of the treatments in the POA or SON. * significantly different from the OVX control group.

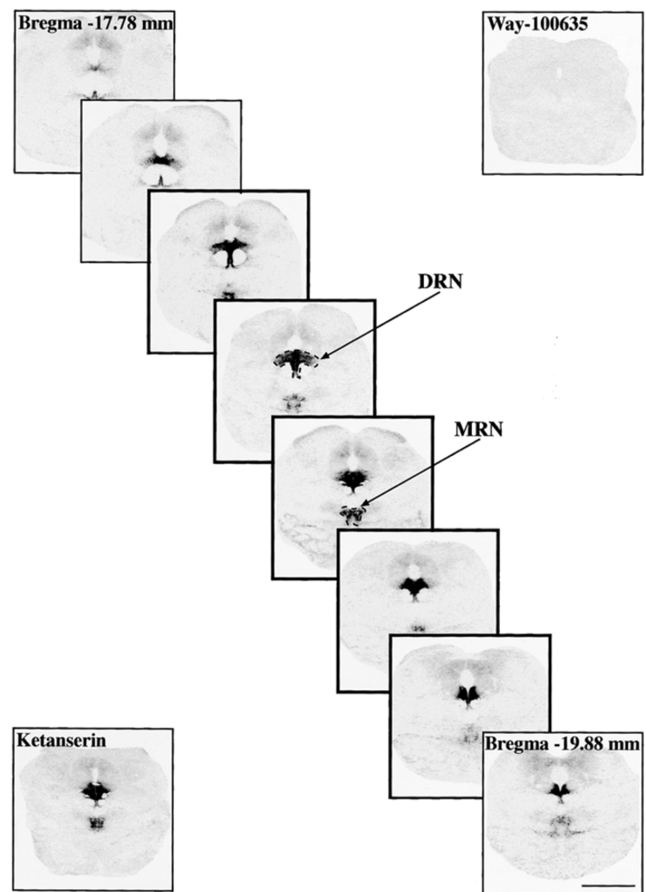


Figure 3. Autoradiograms of [^{3}H]8-OH-DPAT binding on monkey midbrain sections (10 μm). Midbrain sections through eight levels of the DRN with an interval of 300 μm are shown. The five levels yielding the average numbers of 5-HT_{1A} binding sites are in dark frames. [^{3}H]8-OH-DPAT binding was dense in the DRN, moderate in the median raphe nucleus (MRN) and light in the periaqueductal gray. Way 100635 (1 μM) effectively blocked 5-HT_{1A} labeling. Ketanserin (1 μM) did not affect 5-HT_{1A} labeling. Scale bar = 5 mm.

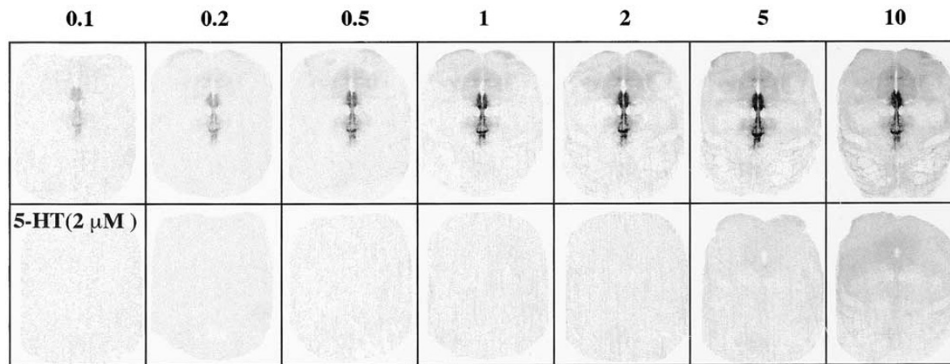
L-694,247 << Ketanserin, agreeing with the literature (Boess and Martin 1994; Nelson 1991).

E, P, and E + P treatments all significantly reduced the level of 5-HT_{1A} autoreceptor binding sites in the DRN compared with the OVX controls (Figure 5). Average optical density values from five levels of the DRN (n = 5/treatment) were significantly decreased by E, P, and E + P. Pixel areas reflected the optical density values. Compared with the OVX control animals, the positive pixel area representing [^{3}H]8-OH-DPAT binding in the DRN was significantly decreased by all three treatments.

In addition, in vitro treatment with E and P of midbrain sections from OVX control animals did not change the level of [^{3}H]8-OH-DPAT binding.

[^{35}S]GTP-γ-S Binding in the DRN

The distribution pattern of [^{35}S]GTP-γ-S binding sites in the DRN matched with that of [^{3}H]8-OH-DPAT binding (Figure 6). Average optical density values and pixel area measurements of [^{35}S]GTP-γ-S binding sites on five levels of the DRN are shown in Figure 7. Compared with OVX control animals, the optical density values representing the basal and stimulated [^{35}S]GTP-γ-S binding were both significantly decreased by E, P, and E + P compared with the control group. The positive pixel areas reflected the optical density values and were significantly decreased in all treatment groups.



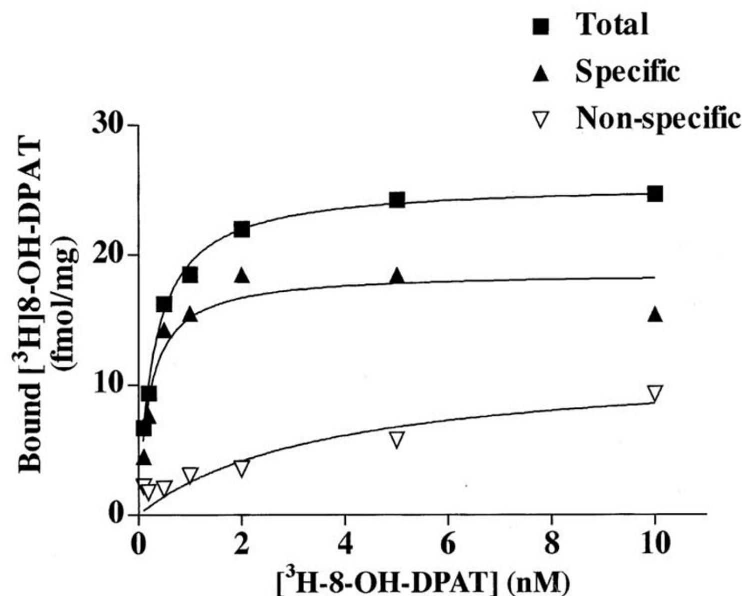


Figure 4. Saturation studies of [^{3}H]8-OH-DPAT binding in the DRN on monkey midbrain sections (10 μm). Serial sections were incubated with seven concentrations of [^{3}H]8-OH-DPAT ranging from 0.1 nM to 10 nM. Densitometric analysis of [^{3}H]8-OH-DPAT binding sites was performed with NIH Image software. One site curve fitting was performed with Prism 3.0. [^{3}H]8-OH-DPAT binding in the monkey DRN reaches equilibrium at 1 to 2 nM. The non-specific binding was below 15% of the total binding.

The apparent Kd values (nM ± S.E.M.) for the basal [^{35}S]GTP-γ-S binding equaled 0.148 ± 0.022, 0.130 ± 0.019, 0.153 ± 0.024, and 0.106 ± 0.017 whereas the Kd values for the stimulated binding equaled 0.122 + 0.016, 0.115 ± 0.015, 0.119 ± 0.016, and 0.093 ± 0.015 in OVX, E, P, and E + P treated groups, respectively (not different, $p > .05$). These values indicate that the affinity of the radioligand with G proteins on monkey midbrain sections was not affected by any of the treatments.

In addition, adjacent midbrain sections were incubated with increasing concentrations of R-(+)-OH-DPAT to stimulate [^{35}S]GTP-γ-S binding. The concentration of R-(+)-OH-DPAT (nM ± S.E.M.) producing half-maximal stimulation equaled 3.09 ± 0.33, 1.96 ± 0.46, 3.51 ± 0.78, and 3.55 ± 1.10 in OVX, E, P, and E + P treated groups, respectively (not different, $p > .05$). Thus, the potency of R-(+)-OH-DPAT to stimulate [^{35}S]GTP-γ-S binding was the same in each treatment group.

Lastly, the coupling efficiency between 5-HT$_{1A}$ and G proteins was calculated as (Stimulated – Basal) / Basal × 100% and averaged for each treatment group. In OVX, E, P, and E + P treated groups, the percentage increase from basal to stimulated [^{35}S]GTP-γ-S binding as derived from optical density values equaled 54.64 ± 3.42, 62.02 ± 8.09, 57.04 ± 4.42, and 48.75 ± 3.151, respectively (not different, $p > .05$). The percentage increase equaled 59.15 ± 13.82, 65.48 ± 15.84, 87.02 ± 19.71, and 81.58 ± 9.644, respectively (not different, $p > .05$) when

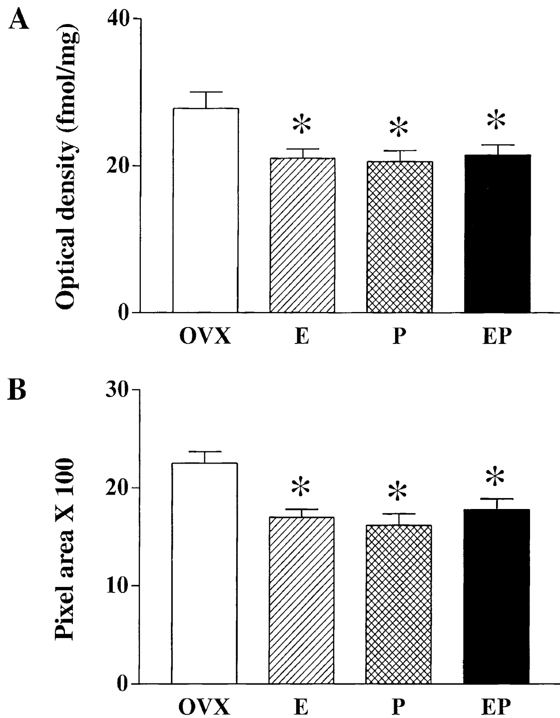


Figure 5. Comparison of [^{3}H]8-OH-DPAT binding levels in the monkey DRN from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A: Average optical density values from six levels of the DRN (n = 5/treatment). Compared with the OVX control animals, 5-HT_{1A} binding sites were significantly decreased by E, P, and E + P (ANOVA, $p = .027$, F = 4.1, df = 18). B: Positive pixel areas reflecting [^{3}H]8-OH-DPAT binding in the DRN. Compared with the OVX control animals, [^{3}H]8-OH-DPAT generated pixel areas in the DRN were significantly decreased by all three treatments (ANOVA, $p = .006$, F = 6.2, df = 18). * significantly different from the OVX control group.

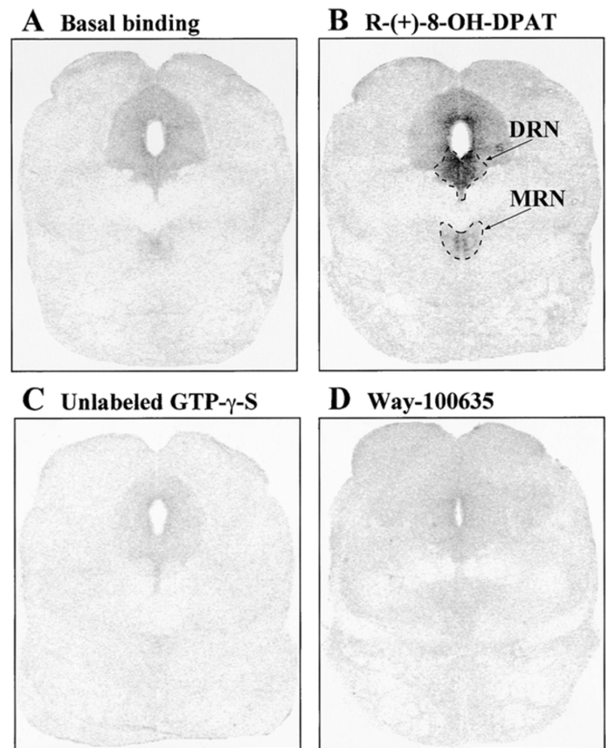


Figure 6. Autoradiograms of [^{35}S]GTP-γ-S labeling in the monkey midbrain sections (10 μm). A: The basal binding of [^{35}S]GTP-γ-S was diffuse. B: The stimulated binding of [^{35}S]GTP-γ-S in the presence of 1 μM of R-(+)-8-OH-DPAT was robust in the DRN, moderate in the MRN, and light in the periaqueductal gray. C: Non-specific binding was defined in the presence of 10 μM unlabeled GTP-γ-S. D: The R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding in the DRN can be blocked by the selective 5-HT_{1A} receptor antagonist Way 100635 (1 μM) but not by phentolamine (data not shown). Scale bar = 5 mm.

derived from the positive pixel areas. Therefore, there was no change in the coupling efficiency between the receptor and G proteins with steroid treatment.

Western Blot Analysis of $G_{\alpha i3}$, $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$

Figure 8 shows that EE treatment for 30 months significantly reduced $G_{\alpha i3}$ protein levels in the macaque dorsal raphe compared with the OVX controls by unpaired 2-tailed Student's *t*-test. Levels of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ proteins in EE treated animals were not different from those in the control group.

Hormone Levels

Serum samples were collected from rhesus macaques at necropsy and assayed for E, P, and prolactin. The assays exhibited a less than 9% intra-assay coefficient of variation. The sensitivity of the assays equaled 5 pg/ml for E and 0.1 ng/ml for P and prolactin. The mean (± S.E.M.) concentration of E in the serum of E and E + P treated groups was 105.6 ± 20.02 pg/ml. The mean (± S.E.M.) concentration of P in the serum of P and E + P treated groups was 6.82 ± 1.56 ng/ml. The E level was within the range reported for the mid to late follicular phase and P level was within the range reported for the mid-luteal phase of the primate menstrual cycle (Hotchkiss and Knobil 1994). The mean (± S.E.M.) concentrations of E and P in the serum of the untreated OVX control group were 9.8 ± 4.8 pg/ml and 0.15 ± 0.05 ng/ml, respectively. The mean (± S.E.M.) concentrations of prolactin in the serum of OVX, E, P, and E + P treated groups were 48.53 ± 6.07, 222.55 ± 66.17, 101.60 ± 17.35, and 551.52 ± 120.52, respectively. E + P treatment significantly increased the serum prolactin level compared with OVX control, E, or P treated groups (ANOVA, $p <$

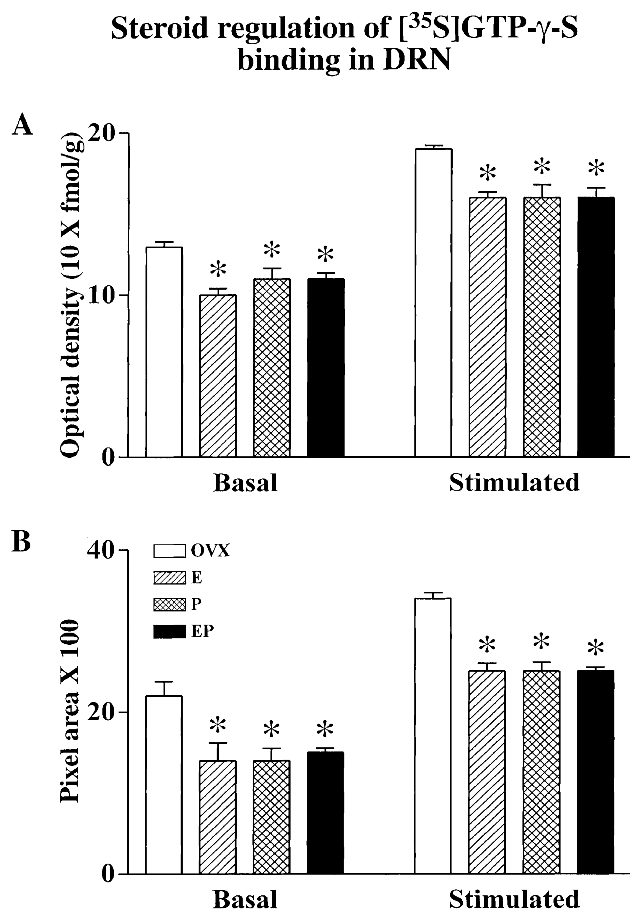


Figure 7. Average number of [^{35}S]GTP-γ-S binding sites in the midbrain sections from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A: Average optical density values from five levels of the DRN (n = 5/treatment). Compared with the OVX control animals with ANOVA, basal [^{35}S]GTP-γ-S binding sites were significantly decreased by E, P, or E + P (p = .0087, F = 5.8, df = 17). R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding sites in all treatment groups were also decreased (p = .0011, F = 9.6, df = 17). B: Pixel areas reflected the optical density values for both the basal and stimulated [^{35}S]GTP-γ-S binding in the DRN. Compared with the OVX control animals, positive pixel areas representing the basal [^{35}S]GTP-γ-S labeling were significantly decreased by E, P, or E + P (p = .0135, F = 5.1, df = 17). The positive pixel areas representing the stimulated binding were also decreased in all treatment groups ($p < .0001$, F = 23, df = 17). * significantly different from the OVX control group.

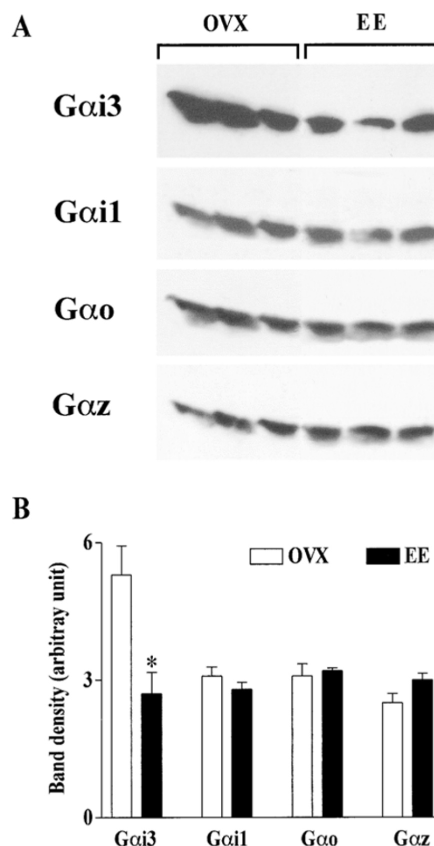


Figure 8. Equine estrogen (EE, 30 months) significantly reduced $G_{\alpha i3}$ protein levels. A: G protein subunit signal bands detected on Western blots. B: Densitometric analysis showed a significant decrease of the $G_{\alpha i3}$ band density by EE treatment compared with the control group with unpaired 2-tailed t-test (p = .0297, t = 3.3, df = 3). The levels of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ proteins were not different between EE treated and OVX control groups. * significantly different from the OVX control group.

.05), consistent with previous reports (Williams et al. 1985; Sprangers et al. 1990; Bethea et al. 1992).

DISCUSSION

Results from these experiments demonstrated that one month of E and P significantly decreased 5-HT$_{1A}$ receptor binding sites and G protein activation in macaques. Specifically, postsynaptic 5-HT$_{1A}$ in the hypothalamus was downregulated by E and E + P but not by P alone. The 5-HT$_{1A}$ autoreceptor in the DRN was downregulated by all three hormone treatments. Also in the DRN, the basal and R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding were reduced by each ovarian hormone treatment as well. The expression of $G_{\alpha i3}$ protein, but not of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$, in the DRN on Western blots was significantly reduced by conjugated EE.

Postsynaptic 5-HT$_{1A}$ receptor binding in the hypothalamus was down-regulated by E and E + P but not by P alone. The location of 5-HT$_{1A}$ receptor binding was consistent with that of 5-HT$_{1A}$ mRNA expressed in discrete regions in the hypothalamus (Gundlah et al. 1999). However, the steady-state mRNA level of the postsynaptic 5-HT$_{1A}$ in the same regions was not decreased by hormone replacement. Thus, mRNA levels for this receptor do not reflect the functional capacity of the re-

ceptor in the postsynaptic target cells. This discrepancy between 5-HT_{1A} mRNA and binding levels may be attributed to the modification of 5-HT_{1A} translational efficiency by hormone treatments. In rats treated with E, postsynaptic 5-HT_{1A} mRNA downregulation occurs days before the downregulation of receptor proteins and then the receptor mRNA level recovers (Osterlund et al. 2000). Alternatively, therefore, the decreased postsynaptic 5-HT_{1A} receptor protein with one month of hormone replacement in monkeys might result from a reduced receptor message somewhat earlier in the treatment.

The downregulation of postsynaptic 5-HT_{1A} in the hypothalamus may affect several aspects of neuroendocrine functions and behaviors. The 5-HT_{1A} receptor is inhibitory and hence, a decrease in receptor availability would remove serotonergic inhibition of the target neurons. For example, 8-OH-DPAT inhibits lordosis and stimulates food intake in rats and E priming before 8-OH-DPAT administration reverses both behavioral effects (Trevino et al. 1999; Jackson and Uphouse 1996; Salamanca and Uphouse 1992). In addition, E treatment before 8-OH-DPAT administration also reduces the 5-HT_{1A} mediated release of stress hormones such as corticosterone (Raap et al. 2000).

The binding of [^{3}H]8-OH-DPAT in the DRN reflects the affinity and number of 5-HT_{1A} autoreceptors on the soma and dendrites of serotonin neurons (Verge et al. 1985; Azmitia et al. 1996a). The downregulation of 5-HT_{1A} binding sites in the monkey raphe areas by ovarian hormones largely reflected the downregulation of 5-HT_{1A} mRNA observed previously with a similar treatment paradigm (Pecins-Thompson and Bethea 1999). In contrast to the effect in the hypothalamus, P alone decreased 5-HT_{1A} autoreceptor binding in the DRN. This raises the possibility that P acts by different mechanisms in the hypothalamus and raphe. The region-specific regulation may be due to the phenotype difference between the cells expressing pre- and postsynaptic 5-HT_{1A} receptors. The neurons that express 5-HT_{1A} autoreceptors are serotonergic and contain estrogen receptor β (Gundlah et al. 2000, 2001) and progesterone receptor (Bethea 1993) whereas serotonin postsynaptic target cells are of numerous phenotypes including GABAergic (Mirkes and Bethea 2001), glutamatergic (Azmitia et al. 1996a), and oxytocin-producing (Raap et al. 2000). They also express different combinations of estrogen receptor α, β, or progesterone receptor (Bethea et al. 1992; Shughrue et al. 1997; Gundlah et al. 2000). In line with this finding, 5-HT_{1A} agonist mediated receptor desensitization (Kreiss and Lucki 1997), G protein activation (Sim-Selley et al. 2000), serotonin release (Kreiss and Lucki 1994), and antidepressant regulation of the 5-HT_{1A} receptor (Chaput et al. 1991) are all brain area specific as well, indicating that the characteristic of this receptor is different in different neuronal populations.

Serotonin 5-HT_{1A} receptors are coupled with inhibitory G proteins of $G_{i/o/z}$ families. They negatively regulate adenylate cyclase activity and cell firing (Raymond et al. 1999). This study demonstrated that R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding in the macaque dorsal raphe was reduced during hormone replacement, indicating that ovarian steroids downregulated 5-HT_{1A} autoreceptor mediated G protein activation. Our observation that E and P inhibit the initial step in the 5-HT_{1A} cell signaling is consistent with limited observations in rodents. In rats, E decreases the ability of 8-OH-DPAT to inhibit serotonergic cell firing in the DRN (Lakoski 1988). The downregulation of 5-HT_{1A} autoreceptor activity by E and P would disinhibit serotonin release in brain regions containing serotonergic projections. In addition, the basal [^{35}S]GTP-γ-S binding level was also decreased by E, P, or E + P. A likely explanation for this observation is a down-regulation of G protein levels by the hormones.

The generous donation of midbrains from cynomolgus macaques maintained on long-term EE enabled the preliminary examination of steroid regulation of G protein expression. Western blot analysis revealed that the level of $G_{\alpha i3}$ proteins extracted from the dorsal raphe of EE treated macaques was significantly lower than that of OVX controls, but $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ did not change. This differs slightly from the observation of Raap et al. (2000), who found that $G_{\alpha i3}$, $G_{\alpha i1}$, and $G_{\alpha z}$ protein levels are all downregulated by E in the rat hypothalamus. The rat hypothalamus contains a heterogeneous mixture of cell populations that express postsynaptic 5-HT_{1A} receptors, which could couple with a variety of G protein systems. Our observation indicates that in macaque dorsal raphe, $G_{\alpha i3}$ is especially sensitive to steroid modulation. Moreover, $G_{\alpha i3}$ has the highest affinity for the 5-HT_{1A} receptor (Raymond et al. 1999). Therefore, steroid regulation of this G protein subunit could alter the sensitivity of presynaptic 5-HT_{1A} autoreceptor signaling as reflected in our [^{35}S]GTP-γ-S binding results. The long-term treatment of the cynomolgus macaques with EE may limit a direct comparison to the [^{35}S]GTP-γ-S binding data in the rhesus. However, we previously observed a similar increase in tryptophan hydroxylase protein expression in OVX rhesus macaques treated with natural E for one month and OVX cynomolgus macaques treated with EE for 30 months (Bethea et al. 2000).

The mechanisms by which E and P decrease 5-HT_{1A} gene and protein expression are unknown. The steroid effects at the time point investigated in this study were obtained with prolonged treatments and could be attributed largely to the genomic actions of E and P. The promoter region of the 5-HT_{1A} gene contains glucocorticoid response elements, AP1 sites, and SP1 sites, but no response elements for E or P (Storring et al. 1999; Ou et al. 2000; Meijer et al. 2000; Ou et al. 2001). Transcription factor NF-κ-B stimulates the expression of 5-HT_{1A} gene in CV1-b cells (Meijer et al. 2000). Estrogen receptor β sequesters NF-κ-B through protein-protein interactions

(An et al. 1999) and thereby represses NF-κ-B driven gene expression(Stein and Yang 1995). We have found that estrogen receptor β but not α is expressed in macaque serotonin neurons (Gundlah et al. 2000, 2001) and that E treatment decreases the nuclear immunodetection of NF-κ-B in the DRN (Earl et al. 2001). These data indicate that steroid receptors may inhibit 5-HT_{1A} gene expression via protein-protein interactions with other transcription factors such as NF-κ-B.

Serotonin 5-HT_{1A} receptors, upon activation, undergo protein kinase C and A-mediated receptor phosphorylation and internalization (Raymond et al. 1999). Results from this study indicated that it is unlikely that steroids modified the phosphorylation state of 5- HT_{1A}. First, the ligand binding activity of 5-HT_{1A} in all treatment groups were the same as indicated by the similar Kd values between 8-OH-DPAT and the receptor. Second, the coupling efficiency of the receptor with G proteins, which is dependent on the phosphorylation state of the receptor, was not affected by steroids.

In conclusion, one month of hormone replacement downregulated 5-HT_{1A} postsynaptic receptor binding, 5-HT_{1A} autoreceptor binding, and autoreceptor mediated G protein activation and cell signaling without affecting the intrinsic property of the receptors in nonhuman primates. These data suggest that hormone replacement could be a beneficial adjunct to antidepressant treatment in postmenopausal women with mood disorders by decreasing the 5-HT_{1A} autoreceptor-specific second message pathway. It is also important to note that in this study, the rhesus macaques were treated with natural hormones, which differ in many respects from synthetic compounds commonly prescribed for hormone replacement therapy. For women at risk for breast and uterine cancer, however, hormone replacement therapy is not an option. Hence, the development of a selective estrogen receptor modulator with efficacy in the brain serotonin system but without any detrimental effects in the peripheral tissues is needed.

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