Endocrine, *vol. 11, no. 3, 257–267, December 1999* 0969–711X/99/11:257–267/$12.75

Ovarian Steroid Action on Tryptophan Hydroxylase Protein and Serotonin Compared to Localization of Ovarian Steroid Receptors in Midbrain of Guinea Pigs

N. Z. Lu,[2,3] T. A. Shlaes,[1] C. Gundlah,[1] S. E. Dziennis,[2] R. E. Lyle,[2] and C. L. Bethea[1–3]

[1]Divisions of Reproductive Sciences and Neuroscience, Oregon Regional Primate Research Center, Beaverton, OR; [2]Department of Physiology/Pharmacology, Oregon Health Sciences University, Portland, OR; [3]Department of Biological Sciences, Portland State University, Portland, OR

The effect of estrogen (E) and progesterone (P) on the protein expression of the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase (TPH), and the level of serotonin in the hypothalamic terminal field was examined in guinea pigs. In addition, we questioned whether serotonin neurons of guinea pigs contain ovarian steroid receptors (estrogen receptor-α[ERα], estrogen receptor β[ERβ], progestin receptors [PRs]) that could directly mediate the actions of E or P. Western blot and densitometric analysis for TPH were used on raphe extracts from untreated-ovariectomized (OVX), OVX-E-treated (28 d), and OVX-E+P-treated (14 d E+14 d E+P) guinea pigs. The medial basal hypothalami from the same animals were extracted and subjected to high-performance liquid chromatography analysis for serotonin, dopamine, 5-hydroxyindole acetic acid, and homovanillic acid. The brains from other animals treated in an identical manner were perfusion fixed and examined for the colocalization of ERα plus serotonin and PR plus serotonin with double immunohistochemistry or for expression of ERβ mRNA with *in situ* hybridization. E and E+P treatment significantly increased TPH protein levels compared to the untreated control group ($p < 0.05$), but TPH levels were similar in the E and E+P-treated groups. By contrast, serotonin (nanogram/milligram of protein) in the hypothalamus was significantly increased by E+P treatment, but not by E alone. Neither ERα nor PR proteins were detected within serotonin neurons of the guinea pig raphe nucleus. However, ERβ mRNA was expressed in the dorsal raphe. In summary, E alone increased TPH protein expression and the addition of P had no further effect, whereas E+P increased hypothalamic serotonin and E alone had no effect. The localization of ERβ, but not ERα or PR, in the dorsal raphe nucleus suggests that E acting via ERβ within serotonin neurons increases expression of TPH, but that P acting via other neurons and transsynaptic stimulation may effect changes in TPH enzymatic activity, which in turn, would lead to an increase in serotonin synthesis.

Key Words: Serotonin; tryptophan hydroxylase; estrogen receptor-α; estrogen receptor-β; progestin receptor.

Introduction

There is a prominent serotonergic projection to the hypothalamus *(1–3)*, and serotonin plays an important role in the regulation of a variety of autonomic and behavioral functions *(3,4)*. Studies from our laboratory and others suggest that the serotonin neural system may transduce the action of ovarian steroids on pituitary hormone secretion and behavior *(5–16)*.

Ovarian steroids could affect the function of the serotonin system at multiple points. One pivotal point is at the level of tryptophan hydroxylase (TPH). TPH is the rate-limiting or committal enzyme in serotonin synthesis. Following transcription and translation, it requires phosphorylation at serine-58 for enzymatic activity *(17,18)*. We have evidence in steroid-treated primates that translation of TPH mRNA into protein proceeds linearly from transcription *(19)*. However, whether ovarian steroids affect posttranslational processing of TPH is unknown.

In guinea pigs, we previously demonstrated that serum prolactin (PRL) and the serotonin concentration in the midbrain increased with E+P treatment and not E alone *(6)*. Therefore, in this study, we questioned whether the effect of E+P on midbrain serotonin could be owing to an effect of ovarian steroids on TPH protein expression (which follows from gene expression). In addition, we questioned whether serotonin levels in the hypothalamic terminal field

Received March 26, 1999; Revised September 21, 1999; Accepted September 22, 1999.

Author to whom all correspondence and reprint requests should be addressed: Dr. Cynthia L. Bethea, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006. E-mail: betheac@ohsu.edu

reflected the serotonin content of the midbrain in steroid-treated animals.

After examination of steroid action in the serotonin system, we questioned whether the observed effects are mediated directly by nuclear estrogen (estrogen receptor-α [ERα], estrogen receptor-β [ERβ]) or progestin receptors (PRs). We previously found that serotonin neurons of macaques express PR that was induced by E treatment and maintained when P was added to the E regimen *(20,21)*. In addition, we recently reported that the dorsal raphe of monkeys contains ERβ, but little or no ERα *(22)*, suggesting that ERβ mediates the action of E in serotonin neurons. In rats, ERα and PR do not colocalize within the serotonin neurons, but instead are found in smaller interneurons containing excitatory amino acids *(23–25)*. Mice contain subpopulations of serotonin neurons with ERα *(26)*. Thus, the presence or absence of ERα, ERβ, or PR in serotonin neurons may be species specific.

In summary, we determined the manner in which E and P regulate TPH protein levels in the dorsal raphe of guinea pigs, whether serotonin levels in the hypothalamic terminal field reflect TPH protein mass and/or the serotonin content of the midbrain, and whether the guinea pig dorsal raphe contains ERα, ERβ, or PR.

Results

Assay Validation for Quantitative Analysis of TPH Enzyme

The density of the TPH enzyme signal from the Western analysis was positively correlated with the amount of protein loaded on the gel (Fig. 1A, B). The density (area under the peak in arbitrary units) of 100, 200, 300, and 400 μg of protein increased in a linear fashion as determined with the NIH Image program. Comparison of the TPH band on the films to the mol wt markers on the nitrocellulose membranes indicated that the mol wt of guinea pig TPH was approx 55–56 kDa.

Western Analysis of TPH Enzyme in Steroid-Treated Guinea Pigs

Figure 1C shows the bands on the chemilumenescent film from the Western blot analysis of the untreated, E-treated, and E+P-treated guinea pigs ($n = 5, 6$, and 5, respectively). The Ponceau-S-stained nitrocellulose membrane displayed multiple protein bands. However, the affinity-purified anti-TPH antibody specifically bound to TPH enzyme. Thus, one major band was detected. In animals with high levels of TPH protein, a minor band that migrated slower was also detected that may represent phosphorylated TPH. Only the major band was subjected to densitometric analysis. The area under the plot representing band density was used for statistical analysis of TPH protein levels.

Midbrain Raphe TPH Content

Figure 2 gives the results of the densitometric analysis of TPH protein levels. The average TPH protein level (peak area ± SEM) was 1636.40 ± 252.66 in the untreated group; 2836.67 ± 132.38 in the E-treated group, and 2515.00 ± 326.79 in the E+P-treated group. E treatment approximately doubled the TPH enzyme level in the guinea pig raphe region ($p < 0.05$) and addition of P did not change the TPH protein level compared to E only treatment. However, the level of TPH protein in the E + P treated group remained significantly higher than in the untreated group ($p < 0.05$). In summary, TPH protein levels in E treated and E+P treated groups were significantly higher than in the untreated group, but E and E+P treated groups were not different from one another.

Concentrations of Biogenic Amines in the Hypothalamus

Figure 3 shows the serotonin concentration (nanograms per milligram of protein ± SEM) of the hypothalamus. Serotonin levels increased significantly only in the E+P-treated group ($p < 0.05$). There was no significant difference in the hypothalamic serotonin concentration between the E-treated and the untreated groups. Hypothalamic 5-hydroxyindole acetic acid (5-HIAA), dopamine, and homovanillic acid (HVA) concentrations were not different among the untreated, E-treated, and E+P-treated groups.

Immunocytochemical Detection of ERα, PR, and Serotonin

Dense nuclear staining was detected in neurons of the periaqueductal gray (PAG) with the monoclonal antibodies (MAbs) to ERα and PR. Cytoplasmic staining of various intensities was observed in neurons of the dorsal and median raphe with the antiserum to serotonin. ERα-positive cells were detected in the PAG of animals in each group. PR-positive cells were detected in the PAG of the E-and E+P-treated groups. The number of ERα-positive cells detected with antibody 1D5 greatly exceeded the number of PR-positive cells detected with MA1-410. In the double labeling protocol, omission of the primary antibodies to the steroid receptors yielded only cytoplasmic staining for serotonin, and omission of the primary antiserum to serotonin resulted in only nuclear staining for the respective steroid receptor. The populations of ERα and PR-positive cells were completely separate from the serotonin-containing cells. The ERα- and PR-positive cells were observed in the PAG area lateral to the central canal, whereas the serotonin-containing cells were located in the dorsal and median raphe nuclei, which are ventral to the central canal (Figs. 4 and 5). The serotonin-containing cells of the dorsal and median raphe did not exhibit any nuclear staining for ERα or PR.

In situ *Hybridization for ERβ*

In situ hybridization was necessary to detect ERβ expression owing to the lack of specific antibodies at this time. As illustrated in Fig. 6, ERβ mRNA expression was detected in the dorsal raphe region of the guinea pig midbrain. The expression of ERβ overlaps with the expression of serotonin in adjacent sections, suggesting that serotonin neurons express ERβ in guinea pig.

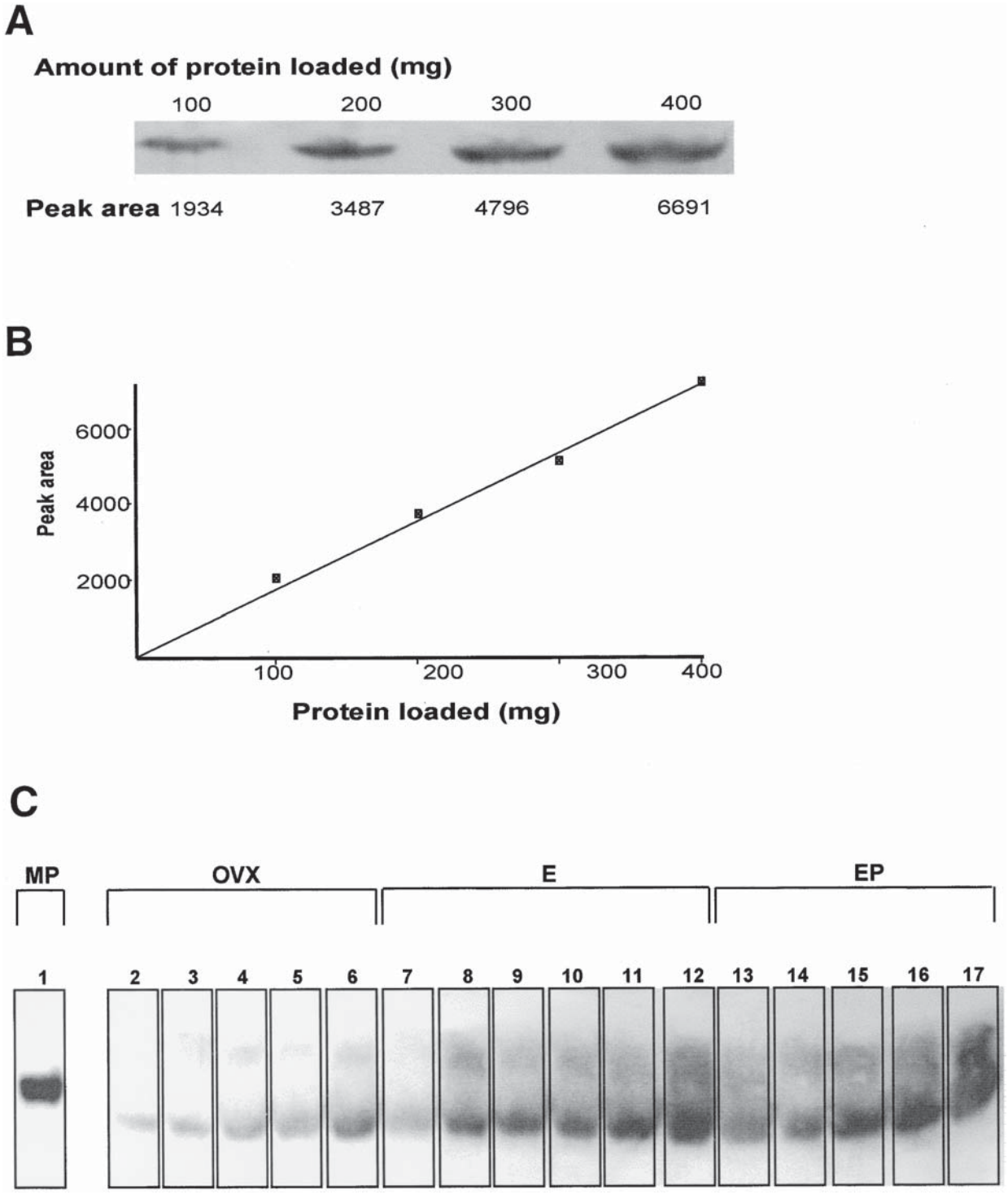

Fig. 1. Validation of quantitative analysis of TPH protein mass and the autoradiograph of the Western blot containing all the guinea pigs used in the analysis of the effect of ovarian hormones. **(A)** The chemilumenescent film representing the Western blot containing increasing aliquots of guinea pig raphe homogenate. The NIH Image Analysis software gel analysis subroutine generates a plot representing band density. The area under the curve is then calculated. This value was used for statistical analysis of TPH protein levels. **(B)** Density (peak area) of raphe protein extracts in arbitrary units plotted vs the amount of protein loaded. The quantitative analysis of TPH enzyme with Western analysis and the NIH Image Analysis software was linear in the range of 100 – 400 mg of protein. **(C)** Chemilumenescent film from the Western blot analysis of the 16 guinea pigs that were either untreated controls, E treated, or E+P treated. The Ponceau-S-stained nitrocellulose membrane displayed multiple protein bands. However, the affinity-purified anti-TPH antibody specifically bound to TPH enzyme. Thus, one major band was detected. In animals with high levels of TPH protein, a minor band that migrated slower was also detected. MP, monkey pineal gland extract, a positive control lane.

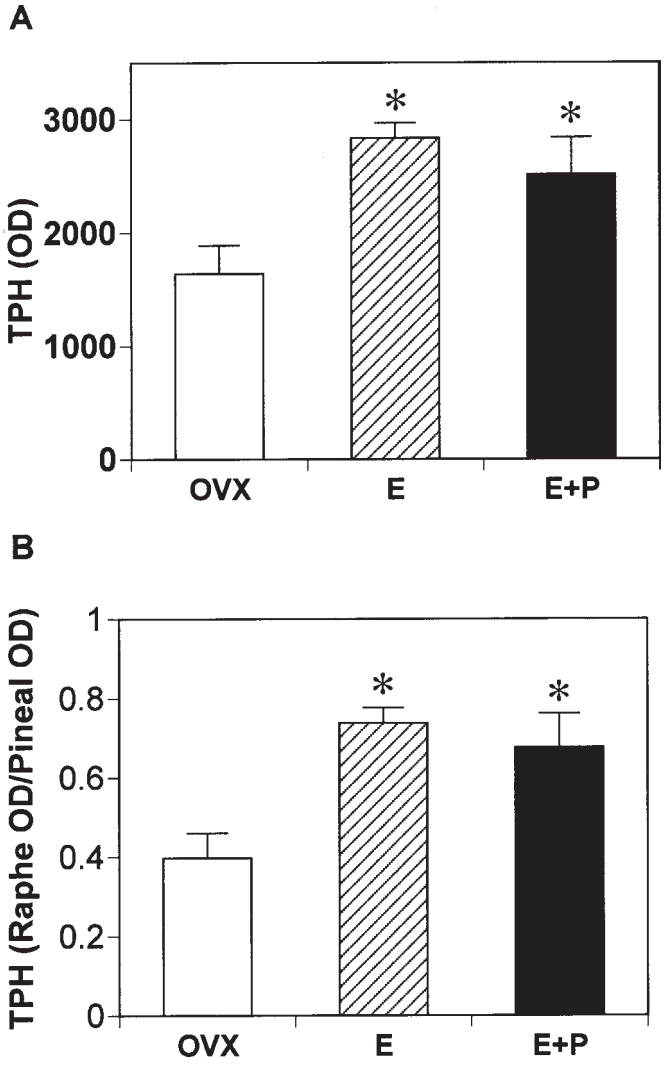

Fig. 2. (A) Results of the densitometric analysis of TPH protein signal (mean ± SEM), which is the area under the intensity curve and expressed as arbitrary units of optical density. **(B)** Density of the TPH protein signal normalized by the density of the pineal signal. E treatment approximately doubled the TPH enzyme level in the guinea pig raphe region (*$p < 0.05$) and addition of P did not change the TPH protein level or ratio compared to E-only treatment. However, the level of TPH protein in the E+P- treated group remained significantly higher than in the untreated group (*$p < 0.05$). In summary, TPH protein levels in the E- and E+P-treated groups were significantly higher than in the untreated group, but the E- and E+P-treated groups were not different from one another.

*$p < 0.05$ compared to ovx controls, Student–Newman–Keuls post-hoc pairwise comparison.

Steroid Hormone Levels

Serum E and P concentrations were determined in the trunk blood collected from the unperfused animals used for TPH Western blot and amine high-performance liquid chromatography (HPLC). Serum E levels (picograms/milliliter ± SEM) were 36.0 ± 2.65 in the untreated guinea pigs and 141.82 ± 36.72 in the E- and E+P-treated groups ($p < 0.01$). The P levels (nanograms/milliliter ± SEM) averaged 0.10 ± 0.10, 0.05 ± 0.03, and 4.13 ± 0.36 in the untreated, E-treated, and E + P treated groups, respectively. P levels in the E+P-treated group were significantly higher than in the untreated and E-treated groups ($p < 0.001$). The steroid replacement treatment achieved serum E and P levels within the physiological range reported for the guinea pig estrous cycle *(27)*.

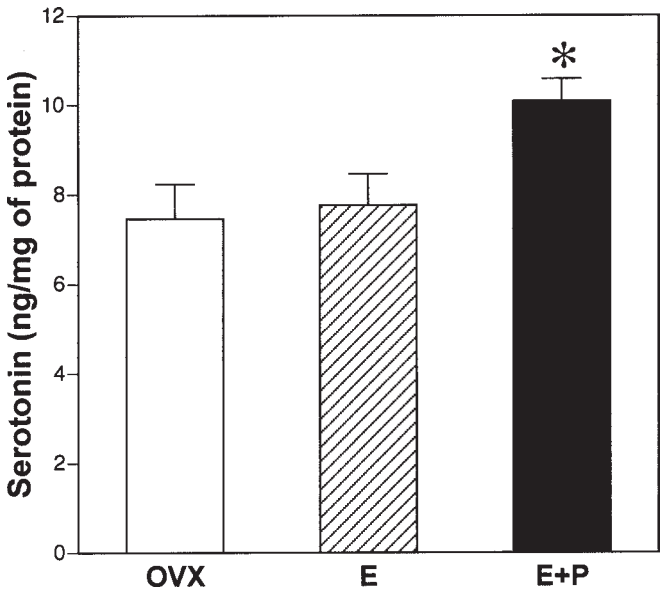

Fig. 3. The average serotonin concentration (ng/mg of protein ± SEM) in the hypothalamus of the untreated, E-treated, and E+P-treated groups, respectively. Serotonin levels increased significantly only in the E+P-treated group (*$p < 0.05$). There was no significant difference in the hypothalamic serotonin concentration between the E-treated group and the untreated group. Hypothalamic 5-HIAA, dopamine, and HVA concentrations were not different among the untreated, E-treated, and E+P-treated groups.

*$p < 0.05$ compared to ovx controls, Student–Newman–Keuls post-hoc pairwise comparison.

Vaginal Smears

Vaginal smears were taken before necropsy to verify the efficacy of steroid implants. Small leukocytes predominated in the vaginal smears of the untreated group. Large numbers of cornified squamous epithelial cells dominated the vaginal smears of the E-treated group. The infiltration of small leukocytes and the presence of round nucleated epithelial cells was observed in the vaginal smears of E+P-treated guinea pigs.

Pituitary Wet Weight

Wet weights were obtained of the pituitaries from the unperfused animals (brains were used for TPH Western blot analysis and amine HPLC). The average wet wts (milligrams ± SEM) of the pituitary in the untreated, E-treated, and E+P-treated groups were 10.34 ± 1.42, 14.18 ± 0.46, and 14.48 ± 0.76, respectively. The weight of the pituitary in the E- and E+P-treated groups was significantly higher than that of the untreated group ($p < 0.05$). The ratio of the

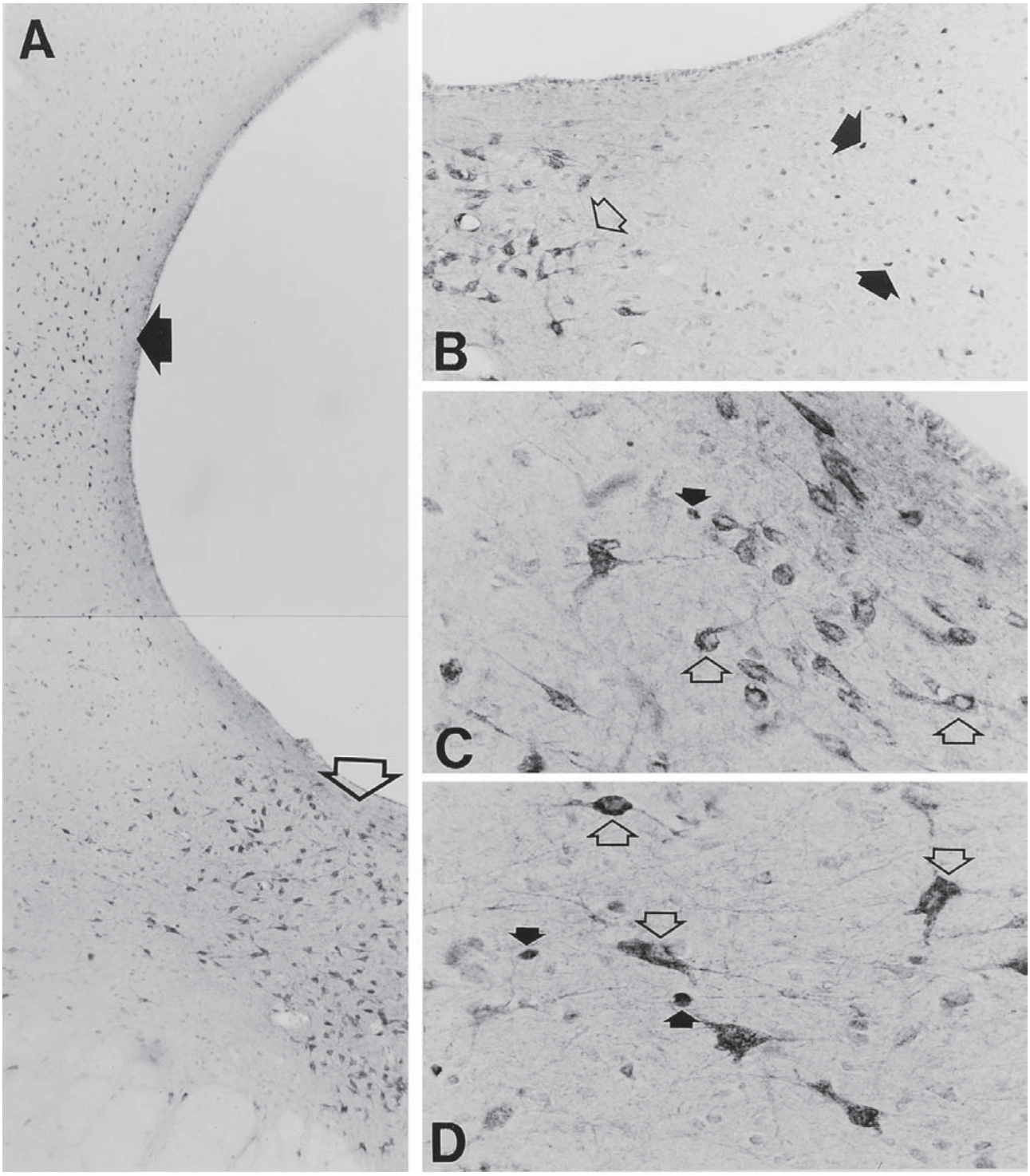

Fig. 4. Representative double immunocytochemical staining for ER and serotonin in the pontine midbrain from an untreated guinea pig. Solid arrows point to ER-positive neurons and open arrows point to serotonin-positive neurons. **(A)** Low magnification composite photomicrograph showing the central canal with the lateral population of ER-positive cells in the PAG (solid arrow) and the separate population of serotonin-positive neurons (open arrow) in the dorsal raphe nucleus (×25). **(B)** Intermediate magnification photomicrograph of the ER-positive (solid arrows) vs serotonin-positive (open arrow) neuronal populations in the midbrain (×40). There is very little overlap between the populations. **(C,D)** Higher magnification photomicrographs taken at the interface of the ER- and serotonin-positive cell populations in the midbrain (×60). In this area, ER-positive cells (solid arrows) are adjacent to serotonin-positive cells (open arrows), but the nuclei of the serotonin-containing cells are clear and do not exhibit nuclear staining for ER, which is black.

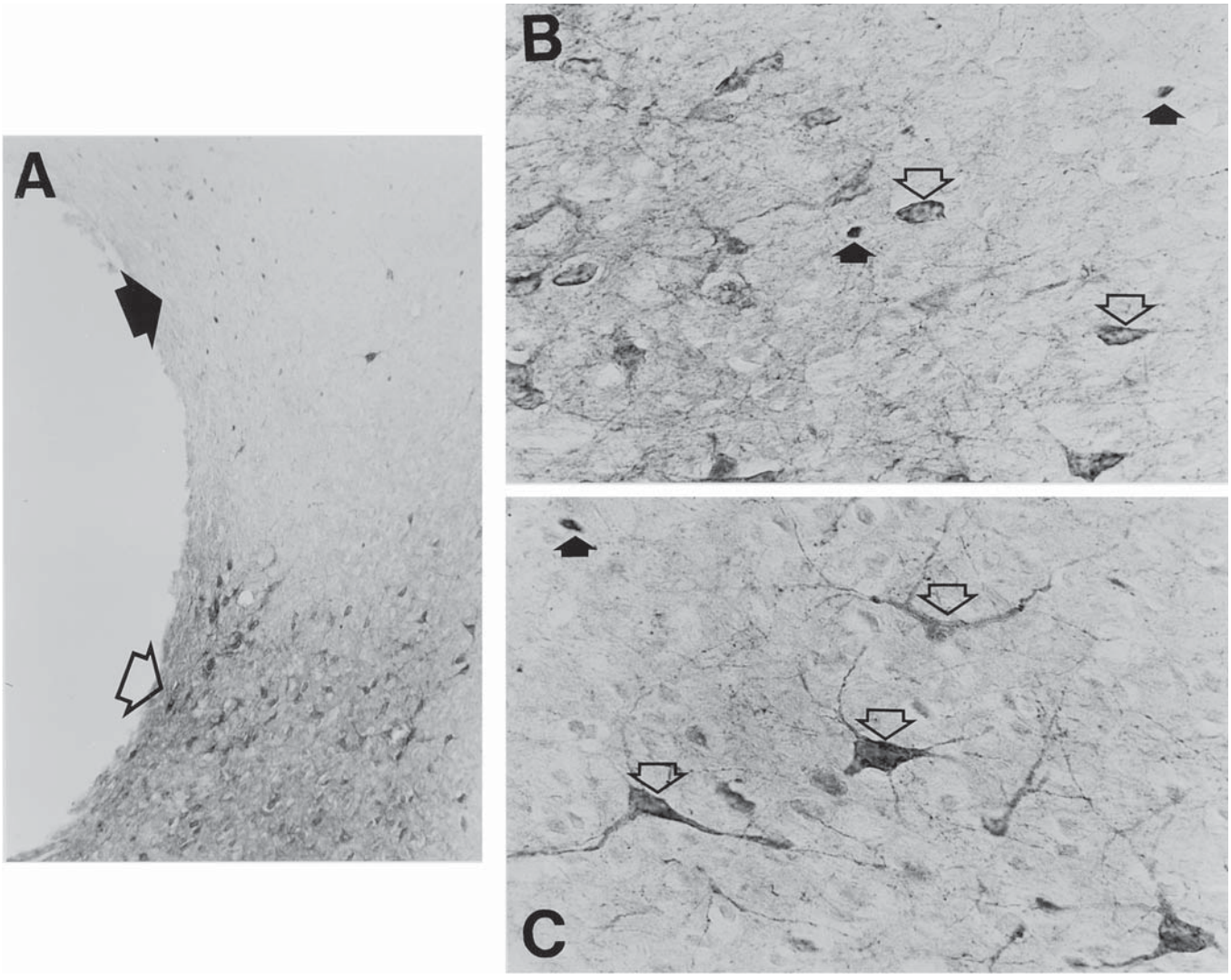

Fig. 5. Representative double immunocytochemical staining for PR and serotonin in the pontine midbrain of an E-treated guinea pig. Solid arrows point to PR-positive neurons and open arrows point to serotonin-positive neurons. **(A)** Low magnification photomicrograph showing the central canal with the lateral population of PR-positive cells in the PAG (solid arrow) and the separate population of serotonin-positive neurons (open arrow) in the dorsal raphe nucleus (×25). For this photomicrograph, the stage was angled to obtain both populations in the same field. The open arrow points toward the actual ventral surface of the brain. **(B,C)** Higher magnification photomicrographs taken at the interface of the PR-positive population and the serotonin-positive population in the midbrain (×60). In this area, PR-positive cells (solid arrows) are adjacent to serotonin-positive cells (open arrows), but the nuclei of the serotonin-containing cells are clear and do not exhibit nuclear staining for PR.

pituitary wet wt to the body weight equaled 0.019 ± 0.0014, 0.027 ± 0.0014, and 0.025 ± 0.0012 in the untreated, E-treated and E+P-treated groups, respectively. The ratio was significantly higher in the E- and E+P-treated groups than in the untreated group ($p < 0.05$). However, the E- and E+P-treated groups were not different.

Wet Weight and Protein Content of Brain Regions

The wet wt and protein content of the dissected raphe and hypothalamic blocks were consistent, indicating that the dissection was reproducible. The wet wts (milligrams) of the raphe were 105.32 ± 5.73, 113.93 ± 8.58, and 102.48 ± 3.26 (not different), and the protein contents (milligrams) were 3.05 ± 0.26, 3.71 ± 0.36, and 3.22 ± 0.27 (not different) in the untreated, E-treated and E+P-treated groups, respectively. The wet wts (milligrams) of the hypothalamic block were 164.88 ± 20.42, 166.25 ± 14.5, and 172.4 ± 7.89 (not different), and the protein contents (milligrams) were 2.86 ± 0.33, 3.47 ± 0.24, and 3.06 ± 0.46 (not different) in the untreated, E-treated, and E+P-treated groups, respectively.

Discussion

The role of serotonin in behavior, mood disorders, and pituitary hormone secretion is undisputed *(28,29). S*erotonin neurotransmission can be regulated at many points. Within the serotonin neuron, complex mechanisms govern precursor uptake, serotonin synthesis, release, reuptake,

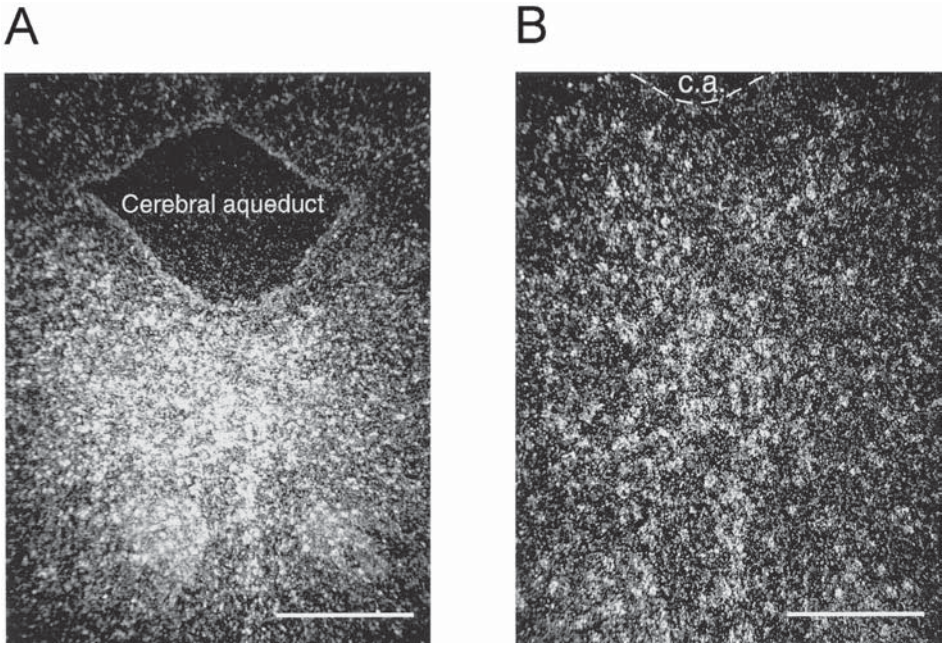

Fig. 6. Dark-field photomicrographs of ERβ mRNA expression (silver grains) in the guinea pig dorsal raphe. **(A)** Low-power magnification bar = 500 μm; **(B)** high-power magnification bar = 250 μm. (c.a., cerebral aqueduct.)

degradation, and autoreceptor inhibition of firing. Each of these mechanisms involves highly specific proteins that, minimally, are transcribed as RNA, translated into amino acid sequences, and then frequently subjected to further posttranslational trafficking and modifications such as phosphorylation.

We found that guinea pigs show little increase in serum prolactin (PRL) or the level of serotonin in the dorsal raphe after 28 d of E treatment. However, addition of P to the E regimen caused significant elevation in both PRL and serotonin *(6)*. Therefore, we questioned whether the raphe serotonin levels were owing to an increase in the mass of the rate-limiting enzyme, TPH, and whether serotonin in the hypothalamic terminal field is regulated in the same manner as serotonin in the dorsal raphe.

This study found that hypothalamic serotonin concentrations increased with E+P but not E alone, as previously reported for dorsal raphe serotonin and serum PRL. However, TPH protein mass increased in response to E, and the addition of P had no further effect. Thus, there is an interesting discrepancy between the steroid regulation of protein expression for the rate-limiting enzyme in serotonin synthesis and the level of serotonin in the dorsal raphe nucleus and in the hypothalamic terminal field. E increases expression of the committal enzyme with little subsequent effect on serotonin concentrations, and then P increases serotonin levels.

The examination of the nuclear steroid receptors may provide some insight. The serotonin neurons in the dorsal or median raphe of guinea pigs did not exhibit nuclear ERα or PR in the presence or absence of the ovarian hormones. Moreover, as previously reported *(30)*, the midbrain populations of ERα and PR-positive cells are located in the lateral aspects of the PAG whereas the serotonin neurons are located ventral to the central canal in the raphe nuclei. However, ERβ mRNA has now been observed in the dorsal raphe of rats *(31)*, monkeys *(22)*, and guinea pigs (herein). If the ERβ mRNA observed in the dorsal raphe is expressed as protein in serotonin neurons, then E acting via ERβ may increase gene expression for TPH, which is reflected in protein mass. The absence of nuclear PR in serotonin neurons is consistent with the lack of a further effect of P on TPH protein mass. However, P then increases serotonin concentrations in both the dorsal raphe and hypothalamic terminal field in some manner that does not involve a direct action via a nuclear receptor in serotonin neurons.

A recent report by Alves et al. *(25)* indicated that PR-positive neurons in the rat raphe region contain excitatory amino acids (EAAs). This leads to the speculation that in rodents, P acting via PR in other neurons (perhaps EAAs) increases transsynaptic stimulation of serotonin neurons and thereby increases serotonin synthesis. Along this line of reasoning, the stimulation of membrane receptors could evoke a cascade of intracellular events that culminates in phosphorylation and activation of TPH, which in turn, would increase synthesis of serotonin. The PR in the adjacent PAG of guinea pigs could potentially function in this role. Anatomical tracing and recording studies support the existence of projections from EAA neurons in the PAG to the raphe nucleus, but the projection to the raphe magnus has been studied most intensely because of its role in pain *(32,33)*.

Alternative hypotheses are also viable at this point. An effect of P on degradation of serotonin via expression of monamine oxidases *(34)* or an effect of P on membrane receptors *(35)* leading to changes in reuptake can also be envisioned. As with most complex biological systems, a combination of effects may be enacted.

This scenario differs somewhat from the primate in which PR resides within the serotonin neuron. We have recently reported that serotonin neurons of monkeys express ERβ mRNA *(22)*, and that TPH mRNA in monkey dorsal raphe is increased by E and P has no further effect despite the presence of nuclear PR *(20,36)*. Therefore, we do not know the mechanism(s) by which P increases serotonin—whether the cognate nuclear receptor resides within the serotonin neuron (as in primate) or whether it resides in adjacent or PAG EAA neurons (rodents).

In summary, there is a discrepancy between the expression of TPH protein levels and the level of the final transmitter product, serotonin, in the dorsal raphe and the hypothalamic terminal field of guinea pigs treated with E or E+P. E alone increased TPH protein expression and the addition of P had no further effect, whereas E+P increased raphe and hypothalamic serotonin and E alone had no effect. Therefore, we speculate that E acting via ERβ increases transcription and translation of TPH, but that P acting via interneuronal stimulation in guinea pigs increases the enzymatic activity of TPH via phosphorylation. This does not rule out the potential for P to affect other mechanisms of serotonin neurotransmission such as degradation, release, or reuptake. However, the presence of ERβ mRNA in the dorsal raphe and the presence of ERα and PR in adjacent neurons of the PAG support this new working hypothesis.

Materials and Methods

The experiments were approved by the Oregon Regional Primate Research Center Animal Care and Use Committee. Unless otherwise stated, reagents were obtained from Sigma, St. Louis, MO.

Animals and Experimental Groups

For TPH protein and serotonin analysis, 16 adult ovariectomized (OVX) guinea pigs were purchased from Charles River Laboratories (Wilmington, MA). For immunocytochemical study, nine adult OVX guinea pigs were purchased. For *in situ* hybridization study, four adult OVX guinea pigs were purchased. Guinea pigs were housed in individual cages in a temperature- and light-controlled (12 h light/12 h dark) room. They were fed Purina guinea pig chow, and water was available at all times. Green vegetables were supplied every other day.

Anesthetic

The guinea pigs were anesthetized for all invasive procedures with a mixture of 50 mg/kg of Ketamine HCl (Mallinckrodt, Mundelein, IL), 1 mg/kg of Acepromazine Maleate (Burns Veterinary Supply, Rockville Center, NY), and 2 mg/kg of Rompun (Miles, Shawnee Mission, KS).

Surgery and Treatments

Two weeks following ovariectomy, the guinea pigs were weighed and anesthetized. Silastic capsules (0.5 cm long; i.d. 0.062 in., o.d. 0.125 in; Dow Corning, Midland, MI) that were either empty (n = 8) or filled with crystalline estradiol 17-β (n = 17), (Steroloids, Wilton, NH) were inserted subcutaneously in the periscapular area. Fourteen days later, half of the E-treated guinea pigs were sedated for insertion of Silastic capsules (1.5 cm long) filled with crystalline progesterone (Steroloids). Fourteen days later, the animals were killed and the brains were processed for either TPH Western blot and HPLC (not perfused, n = 16) or double immunocytochemistry (perfused, n = 9) or *in situ* hybridization (perfused, n = 4 OVX untreated). Vaginal smears were obtained prior to decapitation. All animals were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Medical Association.

Tissue Harvest for TPH Protein and Serotonin Assays

After deep anesthesia, the guinea pigs were decapitated in sets containing an untreated, an E-treated, and an E+P-treated animal. Trunk blood was collected for determination of serum E and P levels by radioimmunoassay (RIA). The brains were immediately dissected. The hypothalamus and midbrain were weighed. The raphe block was immediately homogenized for Western blot assay of TPH protein levels. The hypothalamic block was frozen in liquid nitrogen for later microdissection and HPLC measurement of biogenic amines. The wet wt of the pituitary was obtained following removal of the brain from the skull.

Midbrain Dissection for TPH Protein Assay

The freshly dissected pontine midbrain section (approx 3 mm thick) displayed the rounded central canal on its anterior surface and the wing-shaped canal on its caudal surface. This section was microdissected immediately at necropsy, and a small square piece of tissue was harvested that extended from the top of the central gray to the decussation of the cerebellar peduncles. The piece of tissue was the width of the central gray and contained the major portion of the dorsal raphe. Each piece of tissue was immediately homogenized in 250 μL of 50 m*M* Tris (pH 7.5) (ICN, Aurora, OH) and 20 m*M* 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. The pellet was dissolved in 0.5 *N* NaOH by boiling for 15 min. Protein assays were performed on both the supernatant and the dissolved pellet with the Bio-Rad (BioRad, Hercules, CA) protein determination reagent according to the method of Bradford *(37)*.

Hypothalamic Dissection for Serotonin Assay

Hypothalamic blocks were obtained from each animal that donated a raphe for TPH assay. The hypothalamic block was bordered by the optic chiasm, mamillary bodies, and lateral sulci. The block was wrapped in foil and dropped in liquid nitrogen at necropsy, then stored at –80°C until HPLC analysis. Immediately prior to analysis, each hypothalamic block was slightly thawed and the rostral one-third of the block was dissected and discarded. The medial part, containing the dorsal medial hypothalamus (DMH) and the mediobasal hypothalamus (MBH), was trimmed on the sides, vertical to the innermost border of the optic tract and horizontal to the caudal tip of the third ventricle. The DMH was discarded. The remaining MBH contained the ventromedial, ventrolateral, and arcuate nuclei.

Each MBH was homogenized in 1 mL of mobile phase containing 1 pg/μL of dihydroxybenzylamine internal standard. The sample was centrifuged at 10,000*g* for 2 min, and the supernatant was removed and filtered through Microcon-10 microconcentrators (Amicon, Beverly, MA) for amine HPLC. The pellet was resuspended in 1 mL of 0.5 *N* NaOH to determine protein levels. Previous characterization has found that 95–98% of the total protein is precipitated by the mobile phase *(6)*.

Brain Harvest for Immunocytochemistry and In Situ Hybridization

After deep anesthesia, the head of each animal was perfused with 200 mL of saline and 800 mL of 4% paraformaldehyde via intracardiac puncture. The brain of each animal was removed from the cranium and the pontine midbrain block was obtained. Each block was submerged in 20% sucrose at 4°C until complete penetration. For immunocytochemistry, the blocks were frozen in OCT cryoprotectant (O.C.T. Compound, Miles Laboratories Inc., Elkhart, IN) and stored at –80°C. The midbrain blocks were sectioned at 25μm on a Hacker-Bright cryostat, thaw mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA), and then stored at –80°C until immunocytochemical staining was performed for ovarian steroid receptors and serotonin. For *in situ* hybridization, only untreated OVX animals were used. The blocks were frozen in isopentane at –60°C, stored at –80°C, and then sectioned at 16μm on a Zeiss Microm MH400 microtome and mounted on Superfrost Plus slides. Tissue sections were vacuum-dried overnight and stored desiccated at –80°C until processing for *in situ* hybridization.

Midbrain TPH Western Analysis

Raphe extracts containing 300 μg of protein were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel. A monkey pineal extract containing 129 μg of protein was used as a positive control. Western blotting was performed according to the modified procedures of Dumas et al. *(38)* with blotting buffer containing 25 m*M* Tris base (ICN) and 192 m*M* glycine. The nitrocellulose membrane (Schleicher & Schuel, Keene, NH) was blocked with 5% aqueous Carnation nonfat dry milk (Nestle, Glendale, CA) for 45 min. Affinity purified sheep anti-TPH (Chemicon, Temecula, CA) was used at a dilution of 1/500 in buffer containing 50 m*M* Tris and 150 m*M* NaCl (pH 7.5). Rabbit antisheep antibody conjugated with horseradish peroxidase (Chemicon) was used as second antibody at a dilution of 1/7000. TPH signal was detected by exposing the blot to chemilumenescent film after developing with ECL detection reagents (Amersham, Buckinghamshire, England). Scientific imaging film (Eastman Kodak, Rochester, NY) was used for development of signal.

Assay Validation

The quantitative analysis for TPH protein was confirmed by analyzing increasing volumes of a pool of guinea pig raphe extracts. Samples containing increasing amounts of protein (100–400 μg) were electrophoresed and blotted as described above.

Densitometric Analysis of Western Blotting Results

The NIH Image Analysis software program was used for quantitative analysis of the TPH band. The TPH band image 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.

HPLC for Monoamines

The content of serotonin, 5-HIAA, dopamine, and HVA in the hypothalamic block was determined by HPLC with electrochemical detection (Waters, Milford, MA). A Keystone (Keystone, Bellefonte, PA) catecholamine column (ODS Hypersil, 100 × 4.4 mm, 3-mm particle size) was used with mobile phase consisting of 101 m*M* sodium acetate trihydrate, 67 m*M* citric acid monohydrate, and 4% methanol. The eluent was passed through an electrochemical detector (Waters 460) set at +0.60 V and 0.2 nA. The average retention times for dopamine, 5-HIAA, HVA, and serotonin were 7.0, 9.7, 15.1, and 22.0 min, respectively. The sensitivity of detection was 10 pg for dopamine, 5-HIAA, and serotonin and 50 pg for HVA. The concentration of the calibration standard for biogenic amines was 5 pg/mL. Standard preparations were analyzed at increasing injection volumes (2, 5, 10, 50, 100 μL), and the measurement of dopamine, 5-HIAA, and serotonin was linear between 10 and 500 pg, and linear between 50 and 500 pg for HVA. The concentrations of monoamines were quantitated by comparing the area under the peak of the unknown sample with the linear increase of a standard. The unknown samples were assayed at 5 μL for dopamine, 5-HIAA, and serotonin and at 100 μL for HVA, which fell within the linear range for each amine. Amine

levels were normalized to nanograms per milligram of protein.

Double Immunocytochemical Assay for ER+ Serotonin or PR+Serotonin

Localization of ERα and PR in serotonin neurons was sought using double label immunocytochemistry. Sections (25 μm) of the pontine midbrain were obtained from untreated, E-treated, and E+P-treated guinea pigs, thaw mounted, and stored at –80°C until the day of assay.

For assay, the slides were washed in precooled (4°C) 85% ethanol followed by 10 m*M* phosphate-buffered saline (PBS) (pH 7.3). The sections were blocked with normal horse serum (Elite PK-6102 Mouse Vectastain ABC Kit, Vector, Burlingame, CA) and then incubated at 4°C overnight with mouse anti-PR MAb MA1-410 (1:50) (Affinity BioReagents, Golden, CO) or with mouse anti-ER MAb 1D5 (1:25) (NeoMarkers, Fremont, CA).

The next day, the sections were washed in 10 m*M* PBS, reblocked with normal horse serum, drained, incubated with biotinylated horse antimouse second antibody, and then washed in PBS followed by 0.2 m*M* sodium acetate (pH 6.5). Sections were then placed in 200 mL of 0.2 m*M* sodium acetate containing 140 mg of diaminobenzidine tetrahydrochloride (DAB) (Wako Chemical, Richmond, VA), 40 μL of 30% hydrogen peroxide, and 0.5 g of nickel ammonium sulfate for 5–10 min. After a rinse in 0.2 m*M* sodium acetate to stop the reaction, the sections were placed in 4% paraformaldehyde (pH 7.4) for 60 min to inactivate any residual peroxidase. This was followed by rinsing in 10 m*M* PBS and blocking in normal goat serum and 3% bovine serum albumin. The blocking solutions were drained and the sections were incubated at 4°C overnight with rabbit antiserotonin polyclonal antiserum (1:5000) (Incstar, Stillwater, MN).

The next day, the slides were washed in PBS, blocked again with normal goat serum and incubated with goat antirabbit biotinylated second antibody. After rinses in 10m*M* PBS and 0.05 m*M* Tris (pH 7.6), the slides were placed in 200 mL of 0.05 m*M* Tris containing 140 mg of DAB and 40 μL of 30% hydrogen peroxide for 5–10 min. The reaction was stopped in 0.05 m*M* Tris, and the sections were dehydrated in a graded series of ethanols, infiltrated with xylene, and cover-slipped.

In situ *Hybridization for ER*β

Sections were treated with 4% paraformaldehyde for 15 min, rinsed in TE (0.1 *M* Tris, 0.05 *M* EDTA, in diethyl pyrocarbonate-treated water, pH 8.0) twice for 5 min each, and permeabilized at 37°C with proteinase K (10 μg/mL) in TE for 20 min. Then, sections were acetylated with 0.2% acetic anhydride in 0.1 *M* triethanolamine for 10 min and rinsed in 2X saline sodium citrate (SSC), 0.3 *M* NaCl, 0.03 *M* sodium citrate) twice for 2 min each. Dehydration through increasing concentrations of ethanol was followed by 2 h of drying under vacuum. ^{33}P-labeled riboprobes for ERβ mRNA were generated from monkey-specific clones of 5' and 3' plus UT regions according to Gundlah et al. *(22)*. The concentration of the probe was 5×10^4 cpm/μL and 100 μL was applied to each section. Hybridization was conducted at 50°C overnight. Following hybridization, the sections were washed with 4X SSC, treated with ribonuclease A (25 mg/mL) at 37°C for 30 min, and washed in 0.1X SSC at 60°C for 30 min. Slides were then dipped in Kodak NTB-2 (Eastman Kodak, Rochester, NY) emulsion, incubated at 4°C in the dark for 2 wk, and then developed. The distribution of ERβ mRNA in guinea pig pontine sections was visualized using dark-field optics on a Leica DMLS microscope and photographed with a Spot 2 digital camera.

Estrogen and Progesterone RIAs

Serum concentrations of estradiol-17β and progesterone were determined with RIA in the P30 Hormone Assay Core as described by Resko et al. *(39,40)*.

Statistical Analysis

Midbrain TPH protein levels, hypothalamic monoamine concentrations, pituitary wet wt, serum E and P concentrations, hypothalamic and raphe wet wts, and protein contents were compared using analysis of variance followed by the Student-Newman-Keuls pairwise post-hoc comparison. Data analysis was conducted using the Instat Statistic Program (GraphPad, San Diego, CA) on a Macintosh computer. A confidence level of $p < 0.05$ was considered significant.

Acknowledgments

We thank Dr. David Hess, Director of the Hormone Assay Core of the P30 Population Center grant for assistance with the steroid hormone assays. This work was supported by NIH grant HD17269, P30 Population Center Grant HD18185, and RR00163 for operation of the Oregon Regional Primate Research Center.

References

1. Azmitia, E. C. and Segal, M. (1978). *J. Comp. Neurol.* **179,** 641–668.
2. Sawchenko, P. E., Swanson, L. W., Steinbusch, H. W. M., and Verhofstad, A. A. J. (1983). *Brain Res.* **277,** 355–360.
3. Jacobs, B. L. and Azmitia, E. C. (1992). *Physiol. Rev.* **72,** 165–231.
4. Van de Kar, L. (1991). *Annu. Rev. Pharmacol. Toxicol.* **31,** 289–320.
5. Pecins–Thompson, M. and Bethea, C. L. (1997). *Neuroendocrinology* **65,** 335–343.
6. Bethea, C. L., Hess, D. L., Widmann, A. A., and Henningfield, J. M. (1995). *Neuroendocrinology* **61,** 695–703.
7. Mathiasen, J. R., Tomogane, H., and Voogt, J. L. (1992). *Endocrinology* **131,** 2527–2532.
8. Mistry, A. M., Vidal, G., and Voogt, J. L. (1991). *Brain Res.* **550,** 239–246.
9. Mistry, A. M. and Voogt, J. L. (1990). *Life Sci.* **47,** 693–701.
10. Maswood, S., Andrade, M., Caldarola–Pastuszka, M., and Uphouse, L. (1996). *Neuropharmacology* **35,** 497–501.
11. Jackson, A. and Uphouse, L. (1996). *Horm. Behav.* **30,** 145–152.

12. Eriksson, E., Hedberg, M. A., Andersch, B., and Sundblad, C. (1995). *Neuropsychopharmacology* **12,** 167–176.
13. Halbreich, U. and Tworek, H. (1993). *Int. J. Psychiatry Med.* **23,** 1–27.
14. Halbreich, U., Rojansky, N., Palter, S., Tworek, H., Hissin, P., and Wang, K. (1995). *Biol. Psychiatry* **37,** 434–441.
15. Mortola, J. F. (1994). *Drug Safety* **10,** 160–169.
16. Steiner, M., Steinber, S., Stewart, D., Carter, D., Berger, C., Reid, R., Grover, D., and Streiner, D. (1995). *N. Engl. J. Med.* **332,** 1529–1534.
17. Kuhn, D. M., Arthur, R. Jr., and States, J. C. (1997). *J. Neurochem.* **68,** 2220–2223.
18. Johansen, P. A., Jennings, I., Cotton, R. G., and Kuhn, D. M. (1996). *J. Neurochem.* **66,** 817–823.
19. Bethea, C. L., Mirkes, S. J., Shively, C. A., Adams, M. R., and Williams, J. K. (2000). *Biol. Psychiatry***,** in press.
20. Bethea, C. L. (1994). *Neuroendocrinology* **60,** 50–61.
21. Bethea, C. L. (1993). *Neuroendocrinology* **57,** 1–6.
22. Gundlah, C., Kohama, S. G., Mirkes, S. J., Garyfallou, V. T., Urbanski, H. F., and Bethea, C. L. (1999). *Endocr. Soc.* **81,** 234 (abstract P1–469).
23. Alves, S. E., Weiland, N. G., Hayashi, S., and McEwen, B. S. (1996). *Soc. Neurosci.* **22,** 618 (abstract 246.17).
24. Alves, S. E., Weiland, N. G., Hastings, N. B., Tanapat, P., and McEwen, B. S. (1997). *Soc. Neurosci.* **23,** 1222 (abstract 484).
25. Alves, S. E., Weiland, N. G., Lopez, V., and McEwen, B. S. (1998). *Soc. Neurosci.* **24,** 1101 (abstract 436).
26. Kennetat, G. A., Chaouloff, F., Marcou, M., and Curzon, G. (1988). *Brain Res.* **382,** 416–421.
27. Croix, D. and Franchimont, P. (1975). *Neuroendocrinology* **19,** 1–11.
28. Van de Kar, L. D. (1989). *Neurosci. Biobehav. Rev.* **13,** 237–246.
29. Owens, M. J. and Nemeroff, C. B. (1994). *Clin. Chem.* **40,** 288–295.
30. Turcotte, J. C. and Blaustein, J. D. (1993). *J. Comp. Neurol.* **328,** 76–87.
31. Shughrue, P. J., Lane, M. V., and Merchenthaler, I. (1997). *J. Comp. Neurol.* **388,** 507–525.
32. Beitz, A. J. (1990). In: *The Human Nervous System.* Paxinos, G. (ed.). Academic: San Diego.
33. Beitz, A. J. (1990). *J. Histochem. Cytochem.* **38,** 1755–1765.
34. Richards, J. G., Saura, J., Ulrich, J., and Da Prada, M. (1992). *Psychopharmacology* **106,** S21–S23.
35. Majewska, M. D. (1992). *Prog. Neurobiol.* **38**, 379–395.
36. Pecins–Thompson, M., Brown, N. A., Kohama, S. G., and Bethea, C. L. (1996). *J. Neurosci.* **16,** 7021–7029.
37. Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–254.
38. Dumas, S., Darmon, M. C., Delort, J., and Mallet, J. (1989). *J. Neurosci.* **24,** 537–547.
39. Resko, J. A., Norman, R. L., Niswender, G. D., and Spies, H. G. (1974). *Endocrinology* **94,** 128–135.
40. Resko, J. A., Ploem, J. G., and Stadelman, H. L. (1975). *Endocrinology* **97,** 425–430.