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# OVARIAN HORMONE INFLUENCES ON THE DENSITY OF IMMUNOREACTIVITY FOR TYROSINE HYDROXYLASE AND SEROTONIN IN THE PRIMATE CORPUS STRIATUM

**M. F. KRITZER,[a]* A. ADLER[a] AND C. L. BETHEA[b]**

[a]*Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY 11794-5230, USA*

[b]*Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR 97006, USA*

**Abstract—The serotonergic and dopaminergic inputs to the corpus striatum in human and non-human primates participate in diverse sensorimotor, cognitive, and affective functions, are implicated in dysfunction in diseases such as Parkinson's disease and schizophrenia, and are targets for many of the drugs used to treat these disorders. Sex differences in the incidence and/or clinical course of these disorders and in the effectiveness of related dopaminergic and serotonergic drug therapies suggest that primate striatal indolamines and catecholamines are also influenced by gonadal hormones. However, while well studied in rats, relatively little is known about precisely how gonadal steroids modulate stratial dopamine and serotonin systems in primates. To begin to address this issue, the present studies explored the effects of ovarian steroids on the serotonergic and dopaminergic innervation densities of the caudate, putamen, and the nucleus accumbens in young adult rhesus monkeys. Using densitometry to quantify immunoreactivity for serotonin and for the catecholamine-synthesizing enzyme tyrosine hydroxylase, innervation densities were compared in identified, functionally specialized striatal subdomains across animals that were either ovariectomized or ovariectomized and supplemented with estradiol and/or progesterone, i.e. in a primate model of surgical menopause, with and without hormone replacement therapy. These analyses revealed clear examples of structure-, hemisphere-, and replacement regimen-specific effects of changes in circulating steroids on the densities of each afferent system examined. Further, the predominantly stimulatory effects observed occurred in striatal areas analogous to those suspected as sites of localized dopamine and/or serotonin compromise in Parkinson's disease and schizophrenia. Thus, the hormone actions identified in this study could hold relevance for some of the sex differences identified in relation to these disorders, including the findings of decreased incidence and/or symptom severity in women that have led to hypotheses of protective effects for estrogen.** 

*Corresponding author. Tel: +1-631-632-8634; fax: +1-631-632-6661.

E-mail address: mkritzer@notes.cc.sunysb.edu (M. Kritzer).

*Abbreviations:* ANOVA, analysis of variance; CB, calbindin; DA, dopamine; DAB, diaminobenzidine; E, estrogen; NSS, normal swine serum; OVX, ovariectomized; OVX-E, ovariectomized, estrogen replaced; OVX-EP, ovariectomized, estrogen and progesterone replaced; OVX-P, ovariectomized, progesterone replaced; PB, phosphate buffer; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; 5-HT, serotonin.

**Key words: estrogen, caudate, putamen, nucleus accumbens, dopamine.**

In primates, dopaminergic inputs from the ventral midbrain and serotonergic afferents from the dorsal raphe project to the corpus striatum (caudate, putamen, nucleus accumbens) and play key roles in its diverse sensorimotor, cognitive, and affective functions. These dopamine (DA) and serotonin (5-HT) systems are also implicated in the striatal dysfunction associated with Parkinson's disease and schizophrenia, and both are primary targets for some of the drug treatments used to treat symptoms of these disorders (Hornykiewicz, 1976; Dray, 1981; Marsden, 1984; Agid et al., 1987; Breier, 1995; Roth and Meltzer, 1995). However, because the striatal sectors subserving sensorimotor, cognitive, and affective functions all receive inputs from DA and 5-HT afferents, pharmacologic manipulations of these neurotransmitter systems can sometimes yield unwanted side effects as well as therapeutic benefits. For example, the use of DA D2 receptor blocking neuroleptic drugs to alleviate psychosis in schizophrenia (e.g. Farde et al., 1992), and, more rarely the use of selective 5-HT re-uptake inhibitors in treating depression (Choo, 1993; Arya, 1994) can both produce extrapyramidal motor symptoms. These complications might be minimized, however, if neurochemistry could somehow be more selectively modulated across functionally specialized striatal domains. Previous studies in rats suggest that one means for selectively altering at least the DA systems of the neostriatum may be via ovarian hormone stimulation.

Experimental manipulations of estrogen (E) levels and/or assessments of its natural flux across the estrous cycle have identified effects of circulating ovarian hormones that include a complex modulation of neostriatal DA receptor binding (e.g. Bazzett and Becker, 1994) a slowing of receptor degradation (e.g. Morissette et al., 1992) and a stimulation of amphetamine-induced fos expression (Castner and Becker, 1996) that all seem to selectively occur in the lateral and/or caudal caudate nucleus. In contrast, while sex differences and/or hormone modifiability in clinical aspects of Parkinson's disease and schizophrenia, in the efficacy of associated DA and 5-HT-based therapies, and in susceptibility to drug induced extrapyramidal side effects suggest that ovarian hormones also influence striatal indol- and catecholamines in man (Gratton, 1960; Bedard et al., 1977; Quinn and Marsden, 1986; Seeman and Lang, 1990; Dluzen et al., 1998; Lyons et al., 1998; Scott et al., 2000; Kulkarni et al., 2001), relatively little is

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known about the precise nature of this stimulation. Studies in non-human primate models, however, have begun to fill in the gaps. For example, it is known that ovariectomy and hormone replacement stimulate DA cell survival in the substantia nigra (Leranth et al., 2000) and alter gene expression in brainstem 5-HT neurons (Bethea et al., 1999). However, it is uncertain whether 5-HT and DA afferents themselves are affected by these manipulations in the primate striatum, and whether as in rats these effects occur with any degree of regional selectivity. To address these issues, the present studies measured immunoreactivity for 5-HT and for the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in the caudate, putamen, and nucleus accumbens of adult rhesus monkeys that were ovariectomized and given estradiol, progesterone (P), or both, and comparing labeling to that obtained in ovariectomized controls. By using sensitive densitometric analyses and separate analyses of areas and subareas defined in part by presumed cortical afferents, the effects of ovarian hormone manipulations modeling surgical menopause and hormone replacement therapy on TH- and 5-HT-innervation densities were gauged and compared across functional domains of the neostriatum principally engaged in sensorimotor versus cognitive versus limbic functions.

## EXPERIMENTAL PROCEDURES

### Animals

Tissue samples from 14 (5–14 yrs) female rhesus monkeys (*Macaca mulatta*) was made available for this study; the animals from which this tissue was obtained served as subjects for separate series of studies. All were originally housed at the Oregon National Primate Research Center (ONPRC, Beaverton, OR, USA) and were treated and killed in sets containing one animal from each treatment group according to procedures described below designed to optimize the utility of tissues for a series of unrelated *in situ* hybridization as well as immunocytochemical studies. These procedures are approved by the Institutional Animal Care and Use Committees of the ONPRC and of the State University of New York at Stony Brook. All efforts were made to minimize the number of animals used and their discomfort.

Animals were ovariectomized by ONPRC personnel 3–6 months prior to euthanasia. Twenty-eight days before euthanasia, they were implanted with silastic capsules in the periscapular area under ketamine anesthesia (ketamine HCl; 10 mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA, USA). Control monkeys (OVX; $n=3$) were implanted with empty capsules. The E-treated monkeys (OVX-E; $n=3$) were implanted with a 4.5-cm capsule (optical density 0.132 in.; optical density 0.183 in.; Dow Corning, Midland, MI, USA) filled with crystalline estradiol (1,3,5(10)-estratrien-3,17-β-diol; Steraloids, Wilton, NH, USA). The P-treated group (OVX-P; $n=4$) received an empty capsule, and 14 days later received a P-filled capsule (4-pregnen-3,20 dione; Steraloids). The E plus P-treated group (OVX-EP; $n=4$) received an estradiol-filled capsule, and 14 days later, a 6-cm capsule filled with P.

Serum E and P concentrations were measured by radioimmunoassay in blood samples obtained at necropsy by the ONPRC Endocrine Services Laboratory. The sensitivity of the E assay equaled 5 pg/ml; the sensitivity of the P assay equaled 0.1 ng/ml. The mean (±S.E.M.) concentration of serum E in the E and E+P-treated groups was 95.2±26.2 pg/ml. The mean (±S.E.M.) concentration of serum P in the P and E+P-treated groups was 9.59±1.1 ng/ml. These concentrations approach levels observed during the mid-luteal phase of the primate menstrual cycle (Hotchkiss and Knobil, 1994). The mean (±S.E.M.) concentrations of serum E and P in the ovariectomized controls were 5.8±0.8 pg/ml and 0.23±0.12 ng/ml, respectively.

For killing, animals were sedated with ketamine and overdosed with pentobarbital (25 mg/kg, i.v.). After deep reflexes disappeared, the chest was opened, the heart's left ventricle was cannulated, the descending aorta was clamped, and the animal was perfused with 1 l of buffered saline followed by 7 l of 4% paraformaldehyde in 3.8% borate, pH 9.5 (both solutions made with water containing 0.1% diethyl pyrocarbonate); the anterior vena cava was severed for exsanguination. Afterward, the brain was removed, blocked, and postfixed in 4% paraformaldehyde for 3 h before being transferred to 0.02 M potassium phosphate-buffered saline containing 10% glycerol, followed by 20% glycerol and 2% dimethyl sulfoxide at 4 °C for 3 days for cryoprotection. After infiltration, blocks were frozen in isopentene cooled to −55 °C, and stored at −80 °C until serial sectioning on a freezing microtome (coronal plane, thickness of 40 μm). Separate blocks from the left and right hemispheres from each animal extending from the frontal pole to the level of the anterior commissure were available for study.

### Immunocytochemistry

Tissue was immunoreacted using standard procedures. First, sections were rinsed in 0.1 M phosphate buffer (PB), pH 7.4, incubated in 1% $H_2O_2$ in PB for 30 min, and then placed in 1% sodium borohydride in PB for 30 min. Sections were then rinsed in 50 mM Tris-buffered saline (TBS), pH 7.4, placed in blocking solution [for anti-TH and anti-calbindin (CB) antibodies: TBS containing 10% normal swine serum (NSS); for anti-5-HT antibodies, TBS containing 10% NSS and 0.3% Triton X-100] for 2 h, and incubated in primary antiserum [TH (Chemicon International Inc., Temecula CA, USA), CB (Sigma Chemical Co, St. Louis, MO, USA): 1:1000, diluted in TBS containing 1% NSS, 3 days, 4 °C; 5-HT (Protos, New York, NY, USA): 1:500, diluted in TBS containing 1% NSS and 0.3% Triton X-100, 3 days, 4 °C)]. Following this incubation, sections were rinsed in TBS, placed in biotinylated secondary antibodies (Vector, Burlingame, CA, USA) overnight, 4 °C, working dilution 1:100 in TBS containing 1% NSS), rinsed in TBS, and then incubated in avidin–biotin-complexed horseradish peroxidase (Vector; 2 h, room temperature). Sections were then rinsed in Tris buffer, pH 7.6 and reacted using 0.07% 3,3′-diaminobenzidine (DAB) as chromagen and 0.002% $H_2O_2$ as catalyst. For tissues labeled with anti-TH and anti-5-HT antibodies, sections from individual animals were reacted separately at 2-min intervals to standardize reaction times (30 min) across sections. Adjacent sections reacted for CB were used to identify subdomains of the nucleus accumbens, and were reacted for similar but not exactly corresponding times across sections, animals, and animal groups. All immunoreacted sections were slide-mounted and coded for analysis so that subsequent analyses could be conducted blind to the subjects' experimental condition.

### Control experiments

The labeling procedures above were carried out on representative sections from each animal group with omission of primary antiserum or secondary antibodies. These sections were reacted in parallel with normally immunoreacted sections.

### Qualitative analyses

Representative series of sections from rostral corpus striatum to the anterior commissure were examined using a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thronwood, NY, USA), and objectives ranging between 1.25 and 63×. Analyses included assess-

ments of gross patterns of immunoreactivity and of axon morphology. Pseudocolored (25 colors) video-captured grayscaled images (Scion Image1.6) were generated to highlight density differences in immunolabeling across structures, animals, and groups.

### Quantitative analyses

Quantitative assessments of immunoreactivity were carried out on series of video-captured images of sections. For these analyses, the most posterior sections that could be matched across all animals were selected to allow assessments at levels where the caudate and putamen are most differentiated in terms of corticostriatal afferents. For each assay (immunohistochemical reaction), one section per animal, per hemisphere, per antigen was used for each antigen. Images from the reacted sections were captured at 1.25× using a SONY DXC-9000 color video camera attached to a Zeiss Axioplan microscope interfaced with a Macintosh computer equipped with frame-grabbing and image analyzing software (Scion Corporation, Frederick, MD, USA). Because of structure size, images of caudate, putamen, and accumbens were acquired separately. This enabled transmitted light levels to be adjusted by structure, hemisphere, and antigen to yield images where gray values across subjects were centered with respect to the 1–256 scale. For each data set (a given structure immunolabeled for a given antigen) camera, microscope, and ambient light settings were kept constant, and all images were collected in one session.

Acquired images were saved as grayscale PICT files (Scion Image 1.6). In these images the caudate and putamen were divided into four sectors to accommodate the expected dorsoventral gradients in innervation density for both TH and 5-HT (Lavoie et al., 1989; Lavoie and Parent, 1990) and to approximate the sensorimotor, association, and limbic, striatal sectors that are defined by corticostriatal afferents (Kunzle, 1975, 1977; Selemon and Goldman-Rakic, 1985; Haber and McFarland, 1999; Haber et al., 2000). Because the nucleus accumbens core could not be distinguished in all sections, accumbal analyses were limited to the shell that was identified using CB immunoreactivity (Fig. 1; e.g. Meredith et al., 1996; Brauer et al., 2000; Ikemoto et al., 1996).

Within each region of interest, gray values of all component pixels were tabulated (Scion Image 1.6). These gray value populations were quantitatively compared across animals and groups within an assay using decile analysis. For this procedure running sums of the pixel values over increasing grays were generated, and the gray values marking the division of the total number of pixels sampled into 10 equal portions, i.e. the gray value levels where 10, 20, 30% etc. of the pixels sampled were at or below, were tabulated on per animal, per structure, per hemisphere, and per antigen bases.

### Statistics

Statistical analyses (three-way analyses of variance [ANOVAs]) tested whether the density of TH or 5-HT innervation in the caudate, putamen or nucleus in ovariectomized monkeys (OVX-E, OVX-P or OVX-EP) differed from that in ovariectomized controls. Separate ANOVAs for caudate, putamen, and nucleus accumbens shell compared gray value deciles (above) from the individual animals and probed for main effects of hormone replacement, sector (caudate and putamen), and hemisphere, and for interactions between hormone replacement, sector, and hemisphere. Where indicated, Sheffe's post hoc comparisons were used; a $P<0.05$ level was accepted as significant.

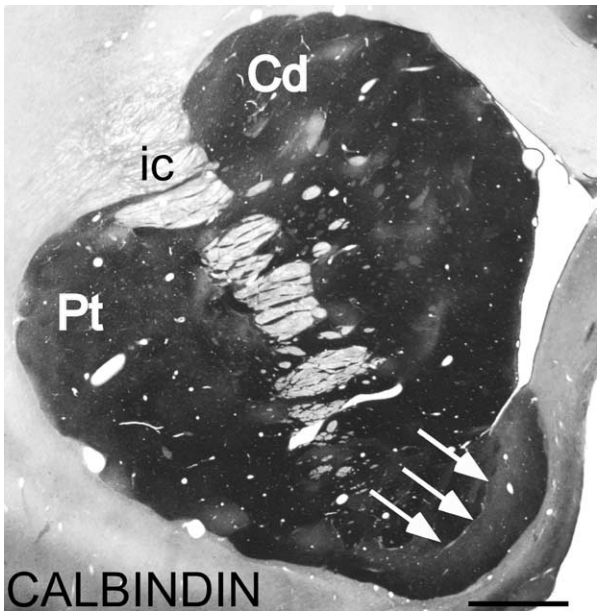


**Fig. 1.** A representative tissue section from the left hemisphere of an OVX animal immunolabeled for CB showing the light staining of the nucleus accumbens shell (arrows) used to direct quantitative analyses in adjacent sections immunoreacted for TH or for 5-HT to this region. The image shown was captured from a Zeiss Axioplan microscope using an Axiocam digital camera. Brightness and contrast were adjusted to optimize visualization of the local waxing and waning in the immunoreactivity. Scale bar=2 mm. Cd, caudate nucleus; ic, internal capsule; Pt, putamen.

## RESULTS

### Qualitative analyses of immunoreactivity

Immunoreactivity for TH and 5-HT was examined in the caudate, putamen, and nucleus accumbens in adult rhesus monkeys that were OVX or OVX-E, OVX-P, or OVX-EP. The tissue available for this study extended from the rostral poles of these structures to the level of the anterior commissure. In representative sections where primary or secondary antibodies were omitted from immunolabeling protocols, no obvious patterned staining was observed. However, in normally reacted sections, both commercially available antibodies used produced labeling in all animals that corresponded closely to patterns of catecholamine and indolamine immunoreactivity previously described in hormonally intact monkeys (Fig. 2; Lavoie et al., 1989; Lavoie and Parent, 1990). For example, TH-immunoreactivity was prominent throughout the corpus striatum, with immunoreactivity further characterized in the caudate and putamen in particular by gradients in density from more moderately stained dorsolateral to more strongly immunoreactive ventromedial zones, and by smaller, discrete patches of noticeably lighter labeling reminiscent of striatal patches or striosomes (Fig. 2A, B; Gerfen, 1992). Higher magnifications revealed that the component axonal staining was composed of dense meshwork of fine, highly varicose TH-immunoreactive processes, morphologies also anticipated in studies of intact monkeys (Fig. 2C, D; Lavoie et al., 1989). Immunoreactivity for 5-HT was also

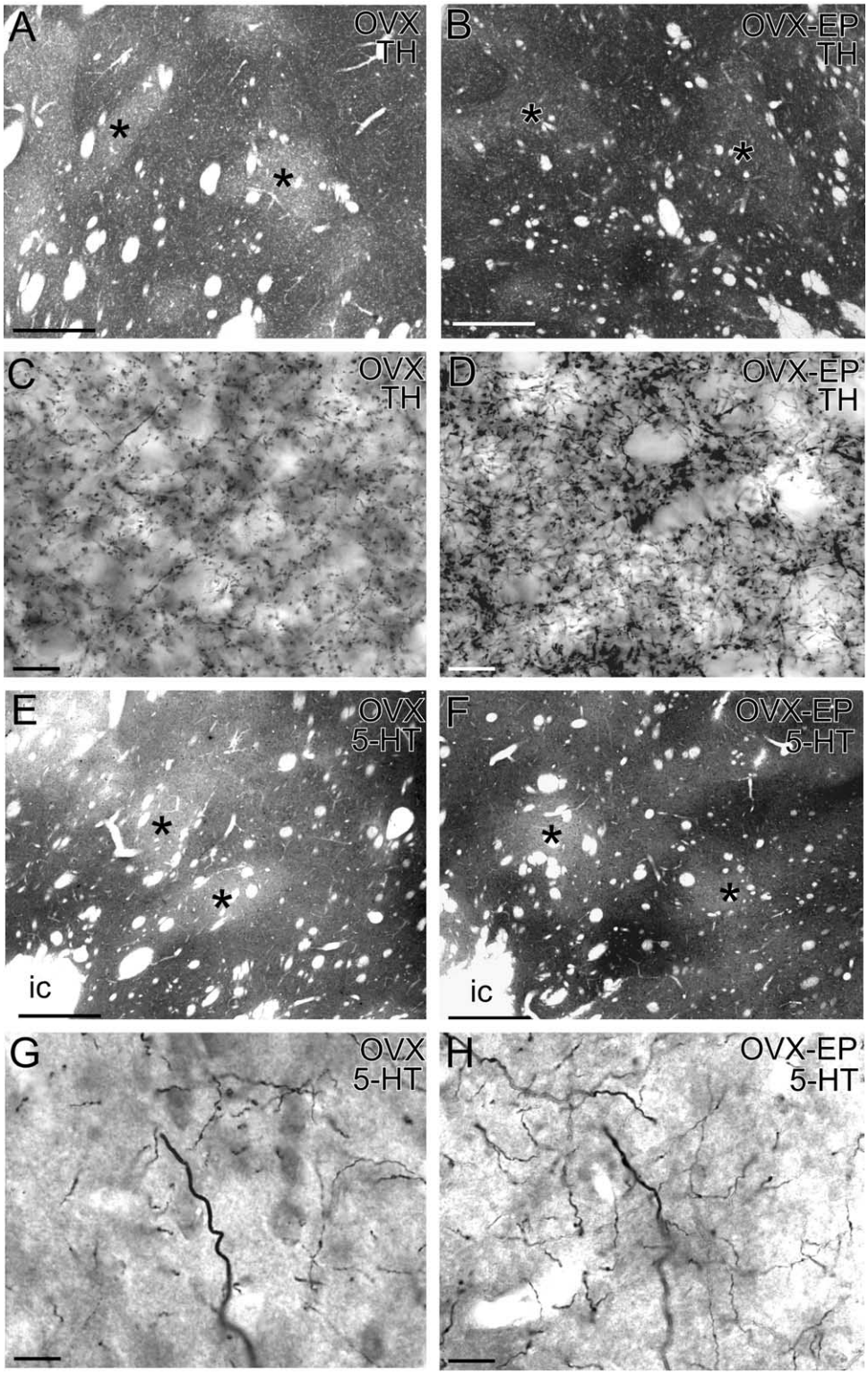


**Fig. 2.** Representative photomicrographs of sections immunoreacted for TH (panels A–D) and for 5-HT (panels E–H) in the left caudate nucleus in an OVX (panels A, C, E, G) and an OVX-EP monkey (panels B, D, F, H). Corresponding images from the OVX control and OVX-EP animals, e.g. panels A, B, were captured from a Zeiss Axioplan microscope using an Axiocam digital camera under identical conditions of illumination. Despite ovariectomy, TH and 5-HT immunoreactivity appears qualitatively normal and shows reduced labeling over presumed striosomes (asterisks, panels A, B, E, F). Labeling is less dense in the OVX control (A, E) compared with the OVX-EP (B, F) animal. Thin, varicose TH-immunoreactive axons (C, D) and populations of thick and thin 5-HT immunoreactive axons (G, H) are also present in both animal groups but are more dense in the OVX-EP (D, H) compared with the control OVX (C, G) group. Scale bars: A, B, E, F=1 mm; C, D, G, H=15 μm; ic, internal capsule.

dense (Fig. 2E, F), with component axons that tended to be thicker and somewhat smoother than the catecholaminergic axons (Fig. 2G, H). Like TH, however, the qualitative features of 5-HT immunolabeling in ovariectomized animals, including dorsoventral gradients in density and sharper punctuations with striosome-like zones of reduced labeling (Fig. 2E, F) closely matched previous descriptions in hormonally intact, adult monkeys (Lavoie and Parent, 1990).

While qualitatively normal patterns of immunoreactivity were thus preserved, in several striatal structures, immunoreactivity for TH and/or 5-HT appeared to be weaker in the control compared with the hormonally replaced animals. This diminution in labeling was readily apparent in light microscopy, and appeared to have some basis in differences in the density of individually immunoreacted axons across animal groups (see Fig. 3). To explore these differences further, patterns of innervation density were mapped onto striatal structures in digitized (video-captured under identical illumination) images of immunoreacted tissue sections using pseudocoloring to localize the waxing and waning of labeling. These analyses revealed that for TH-immunoreactivity, warmer tones representing stronger grays of more intense immunoreactivity consistently marked the dorsolateral half to three-quarters of the caudate nucleus (left and right) in the animals given ovarian steroids (OVX-E, Fig. 3B, F; OVX-P, Fig. 3C, G; OVX-EP, Fig. 3D, H) and contrasted with the cooler tones that were present in corresponding regions of the control animals (Fig. 3A, E). In the ventromedial caudate (Fig. 3) and adjacent nucleus accumbens there were no obvious group differences in innervation. However, in the putamen, highly complex differences in innervation density were present. Most striking was that these group differences were only seen in the left putamen where immunoreactivity was consistently more dense than controls in the OVX-EP animals (Fig. 3D′) throughout the structure, and more dense than controls in the OVX-P group in the ventral putamen (Fig. 3C′). In contrast, innervation densities were similar to controls in the left putamen, of the OVX-E (Fig. 3A′, 3B′) group.

Group differences in the density of 5-HT immunoreactivity in the caudate and putamen (Fig. 4) also selected mainly for structures of the left hemisphere. For the left caudate, for example, immunoreactivity in all of the steroid treated cohorts (Fig. 4B–D) was noticeably denser than in the controls (Fig. 4A). For the putamen (left only), however, immunoreactivity in the OVX-P (Fig. 4C′) and OVX-EP (Fig. 4D′) cohorts was denser than in the controls, whereas labeling in the OVX-E animals (Fig. 3B′) was not appreciably different from control (Fig. 4A′). In the nucleus accumbens, there were no discernable group differences in 5-HT immunoreactivity in either hemisphere.

### Quantitative analyses

Quantitative assessments of immunoreactivity derived from optical density measurements made from video-captured, grayscaled images of immunoreacted sections (Scion Image, 1.6) were used to evaluate group differences further. The sections used for these analyses were taken from a single matched anterior/posterior level located as close to the anterior commissure as possible. Within the sections chosen for analysis, the caudate and putamen were divided into dorsolateral to ventromedial quadrants that approximated striatal zones defined by both sensorimotor, associational, versus limbic corticostriatal inputs (e.g. Kunzle, 1975, 1977; Selemon and Goldman-Rakic, 1985; Haber and McFarland, 1999; Haber et al., 2000) and by gradients in indol- and catecholamine innervation density; the nucleus accumbens shell was defined from adjacent CB-immunoreacted sections (see Fig. 1). In each of these defined regions of interest, the expected local inhomogeneities in immunolabeling were prominent. To avoid averaging signal across these unevenly labeled zones, in each animal the gray values of all of the component pixels sampled per region were tabulated, and the gray values where 10, 20, 30% of the pixels sampled were at or below., i.e. gray value deciles (see Experimental Procedures), were determined. These population representations were used in turn in quantitative and statistical comparisons made on per region, per sector, and per hemisphere bases to assess the magnitude and robustness of group differences in striatal indolamine and catecholamine innervation densities.

### Immunoreactivity for TH in caudate and putamen

Quantitative analyses revealed that in the caudate, the group mean gray value deciles for TH-immunoreactivity in the OVX-E, OVX-P, and OVX-EP groups were 10–25% higher than corresponding values from the controls in the dorsal three sectors, but were overlapping with values from controls in the most ventral division of the caudate (Fig. 5). Subsequent ANOVAs supported the robustness of these differences in innervation density in findings of significant main effects of hormone replacement ($P<0.013$ vs. OVX-E; $P<0.021$ vs. OVX-P; $P<0.036$ vs. OVX-EP) and significant interactions between hormone replacement and caudate sector ($P<0.012$ vs. OVX-E; $P<0.007$ vs. OVX-P; $P<0.0094$ vs. OVX-EP). Although group differences were also noticeably larger on the left (Fig. 5), no significant interactions between hemisphere and hormone replacement were found. The allowed post hoc comparisons (Scheffe's, $P<0.05$) confirmed that with only one exception, decile values from the OVX-E, OVX-P, and OVX-EP groups were significantly different from the OVX control in the dorsal three fourths of both the left and the right caudate, but were not significantly different from control in the ventral aspects of this structure (Fig. 5).

Quantitative evaluation of the gray value deciles for TH-immunoreactivity in the putamen also supported the hemisphere-specific group differences in immunoreactivity suggested in visual inspection of labeled sections alone. Thus, for the left putamen, gray value deciles in the OVX-EP group were 15–20% higher than those of the OVX controls. For the OVX-P group, decile values were also about 10–15% higher than controls, but mainly in the ventral putamen (Fig. 6). Innervation was similar (deciles differing usually by less than 5%) in the OVX-E and control

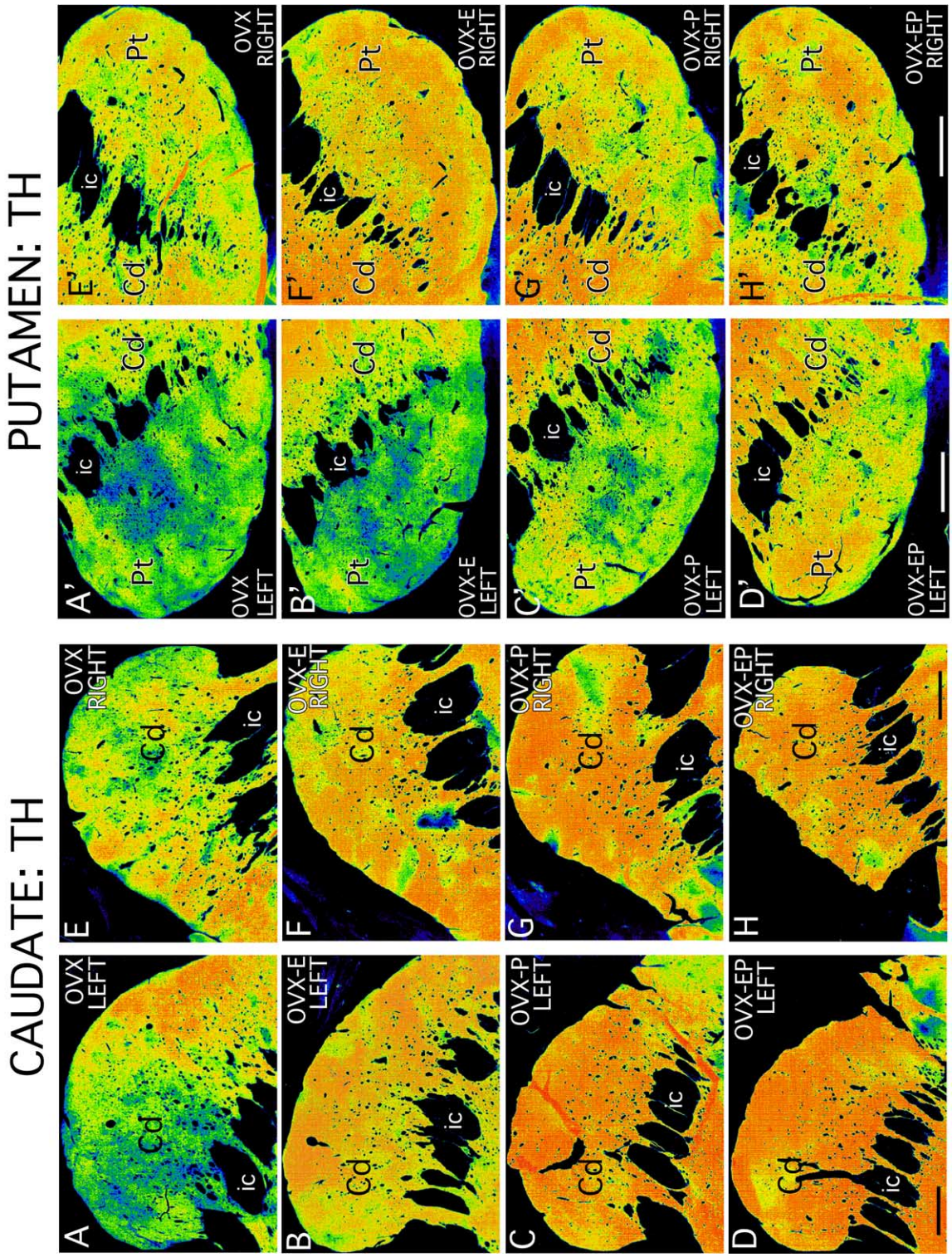


**Fig. 3.** Representative tissue sections through the precommissural left and right caudate nucleus (Cd) and putamen (Pt) of monkeys that were either OVX (panels A, E, A′, E′) or OVX-E (panels B, F, B′, F′), OVX-P (panels C, G, C′, G′) or OVX-EP (panels D, H, D′, H′). The sections shown were immunoreacted for TH and were captured, digitized and pseudocolored under identical conditions. Cooler colors (blues, greens) representing lower levels of immunoreactivity distinguish the dorsolateral caudate in the OVX control group compared with the warmer colors seen in the hormonally replaced cohorts. In the right putamen, no group differences were apparent (E′–H′), but on the left, lower levels of innervation are seen in the OVX control (A′) and OVX-E (B′) cases compared with the OVX-EP (D′) animal. Scale bars=2 mm; ic, internal capsule.

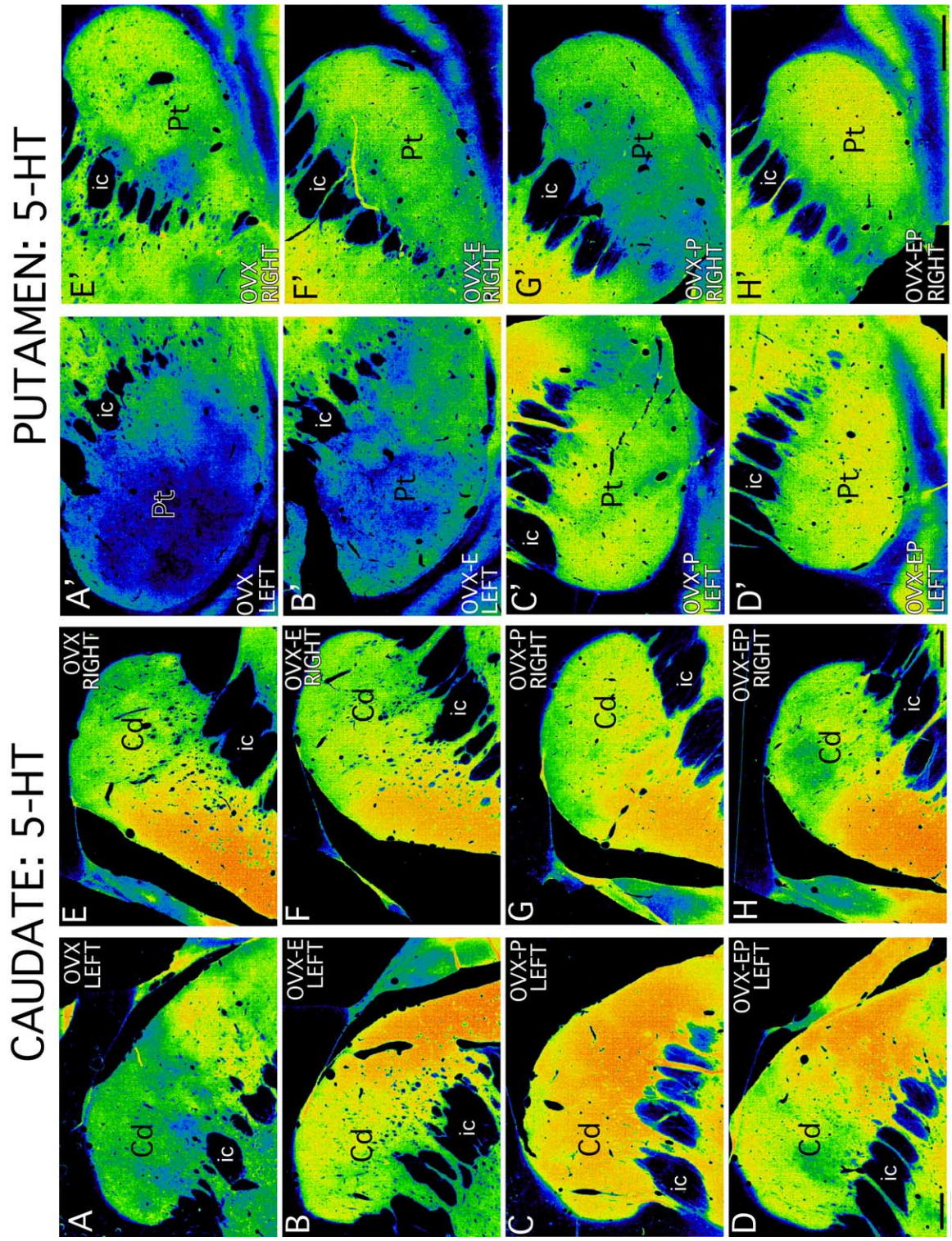


**Fig. 4.** Representative tissue sections through the precommissural left and right caudate nucleus (Cd) and putamen (Pt) of monkeys that were either OVX (panels A, E, A′, E′) or OVX-E (panels B, F, B′, F′), OVX-P (panels C, G, C′, G′) or OVX-EP (panels D, H, D′, H′). All sections were immunoreacted for 5-HT and were captured, digitized and pseudocolored under identical conditions of illumination. For the left caudate from the OVX control (A) animal, cooler colors mark lower levels in innervation density compared with the hormone-supplemented groups (B–D). For the right putamen, no group differences are discernable (E′–H′), but on the left, lower levels of innervation density are seen in the OVX control (A′) and OVX-E (B′) compared with OVX-P (C′) and OVX-EP (D′) cases. Scale bars=1 mm; ic, internal capsule.

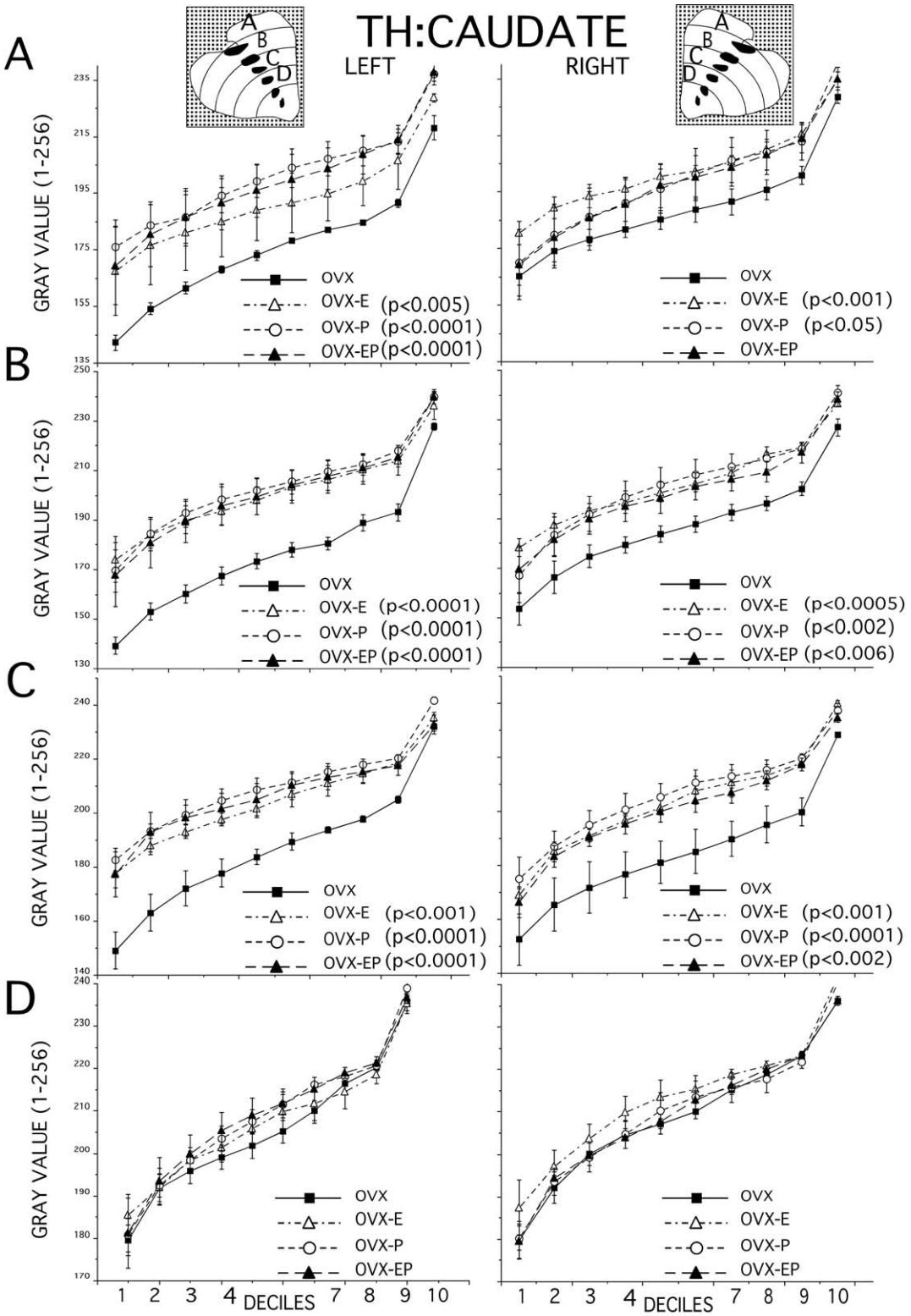


**Fig. 5.** Graphs plotting the mean gray value deciles of TH immunoreactive density measured in captured images of the caudate (left and right) of monkeys that were OVX or OVX-E, OVX-P or OVX-EP; separate plots are shown for group mean decile values measured in dorsolateral to ventromedial caudate sectors (insets show approximate locations), and error bars represent S.E.M. The generally weaker TH labeling in dorsolateral caudate of OVX control compared with the OVX-E, OVX-P and OVX-EP groups is illustrated in the downward shifts of OVX control plots (A–C), while the relative insensitivity of innervation in the ventral caudate produced overlapping graphs for all four animal groups (D). The significance levels of statistically identified group differences are shown in the graph keys.

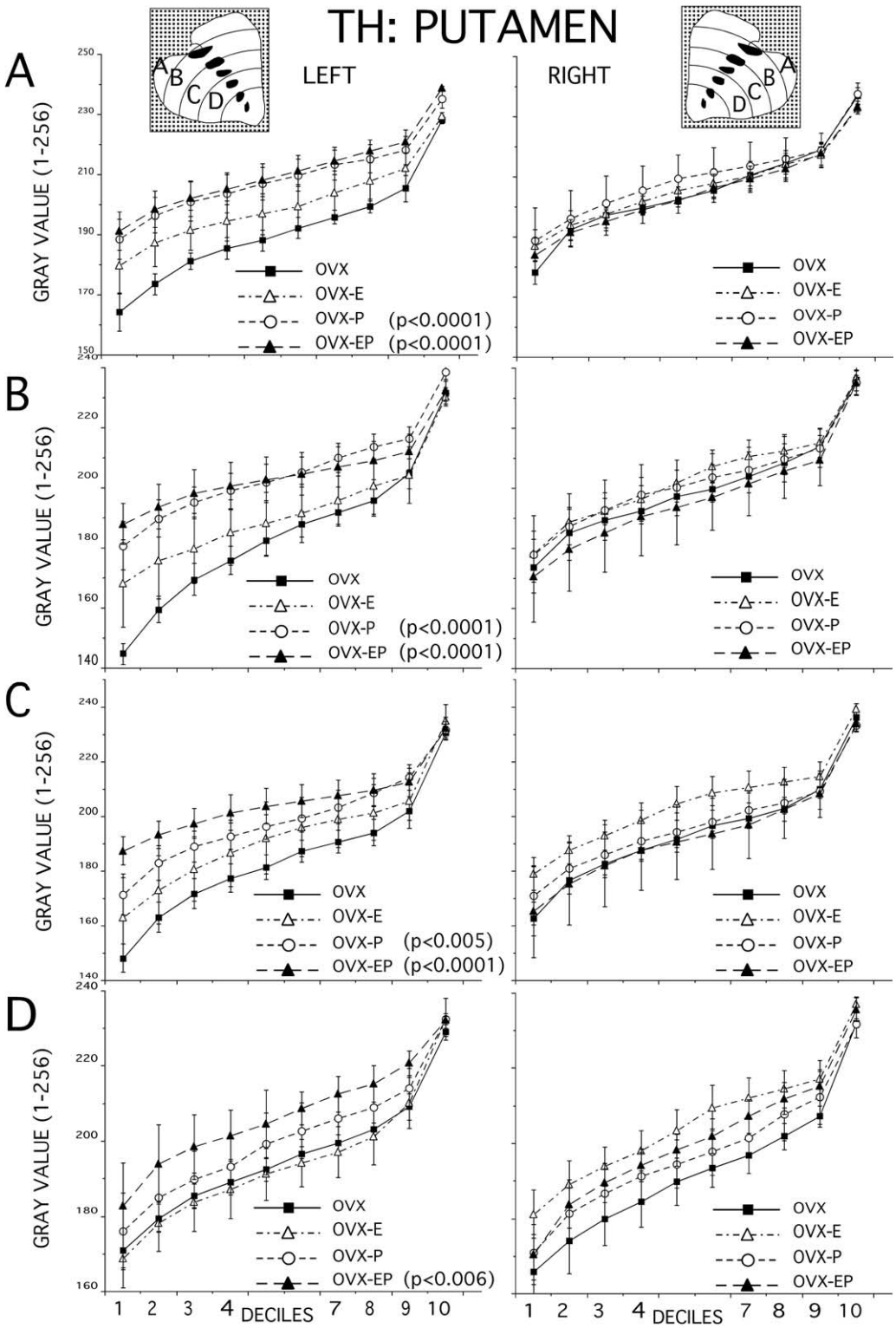


**Fig. 6.** Graphs plotting the group mean gray value deciles of TH immunoreactive density measured in captured images of the putamen (left and right) of monkeys that were OVX or OVX-E, OVX-P or OVX-EP; separate plots are shown for deciles measured in dorsolateral to ventromedial putamen sectors (see insets), and error bars represent S.E.M. The relative insensitivity of innervation in the right putamen is indicated in the overlapping graphs from all animal groups (A–D, right). For the left putamen, a decreased density of TH immunoreactivity in the OVX control and OVX-E groups compared with the OVX-EP cohort is observed from dorsal to ventral quadrants. The OVX-P group has gray value deciles that are similar to the OVX-EP cohort dorsally, but in more ventral sectors, the density of this group begins to wane relative to the OVX-EP group. The significance levels of statistically identified group differences are shown in the graph keys.

OVX groups, however, in the left putamen, and was similar across all animal groups on the right (Fig. 6). In initial analyses where decile values from both hemispheres were evaluated together, ANOVAs did not identify significant main effects of hormone replacement or significant interactions between hormone replacement and putamen sector. However, significant interactions were identified between hormone replacement and hemisphere for the OVX-P ($P<0.033$) and OVX-EP groups ($P<0.0069$). When these data were separated by hemisphere, significant main effects of hormone replacement also emerged for both treatment groups on the left, but not the right ($P<0.0073$ vs. OVX-P; $P<0.0045$ vs. OVX-EP). Allowed post hoc comparisons revealed that for the left putamen, with few exceptions the OVX-P and OVX-EP deciles were significantly different from controls (Fig. 6).

### Immunoreactivity for 5-HT in the caudate and putamen

Quantitative analyses of pixel gray value deciles measured for 5-HT immunoreactivity in the dorsal striatum revealed significant group differences in immunolabeling that were seen only in the left hemisphere. For the left caudate, gray value deciles in the OVX-E, OVX-P, and OVX-EP groups were all 15–20% higher than corresponding values from the controls dorsally, and some 10–15% higher than controls in the more ventral caudate (Fig. 7). Although ANOVAs did not reveal significant main effects of hormone replacement, they did identify significant interactions for all three treatment groups between hormone replacement and hemisphere ($P<0.008$ vs. OVX-E; $P<0.030$ vs. OVX-P; $P<0.032$ vs. OVX-EP), and for each of these groups as well, significant main effects of hormone replacement emerged for the left but not the right caudate when the data were evaluated by hemisphere ($P<0.033$ vs. OVX-E; $P<0.044$ vs. OVX-P; $P<0.0032$ vs. OVX-EP). Permitted post hoc comparisons further revealed that with the single exception of the OVX-EP group in ventral-most sector, gray value deciles from the left caudate of the hormonally replaced cohorts were all significantly different from those of the OVX controls (Fig. 7).

In the left putamen, OVX-EP deciles were also about 15–20% higher than the corresponding values in the OVX control group (Fig. 8). The deciles in the OVX-P group were also about 15% higher than controls in the dorsal putamen, but were only about 10% higher than controls more ventrally. For the OVX-E group there was very little separation (10% or less) between the gray value deciles of this group and the controls (Fig. 8). Perhaps as a consequence, the only effects identified in the putamen as statistically significant were interactions between hormone replacement and hemisphere for the OVX-EP group ($P<0.081$), with significant main effects of hormone replacement identified for this group in the left ($P<0.0023$) but not the right hemisphere only after hemispheric data were evaluated separately. Post hoc comparisons, thus limited to OVX controls vs. OVX-EP groups, revealed that all gray value deciles for the left putamen in the OVX-EP group were significantly different from those of the OVX controls (Fig. 8).

### Immunoreactivity for TH and 5-HT in the nucleus accumbens

Quantitative analyses of pixel gray value deciles in the nucleus accumbens (shell) revealed no group differences in either TH or 5-HT immunoreactivity. Rather, gray value deciles for both antigens were overlapping for all of the animal groups in both hemispheres (Fig. 9). Subsequent ANOVAs likewise identified no significant main effects of hormone replacement, and no significant interactions between hormone replacement and hemisphere.

## DISCUSSION

Previous studies in rats have identified ovarian hormone effects on 5-HT- and/or DA-mediated behaviors, levels, release, metabolism, and receptor binding in the dorsal and ventral striatum (Shimizu and Bray, 1993; see Becker, 1999). In those studies where endpoints were examined on a regional basis, clear examples of anatomical selectivity in stimulation were also found. For example, ovarian hormone modulation of striatal DA D2 receptor binding, (Roy et al., 1990; Bazzett and Becker, 1994), retardation of D1 and D2 receptor degradation (Morissette et al., 1992), and stimulation of amphetamine-induced fos expression (Castner and Becker, 1996) were observed especially in lateral and/or caudal compared with more medial neostriatal subdivisions. The sex differences marking the incidence, severity, and response to drug treatments in striatal dysfunctions such as Parkinson's disease and schizophrenia have been taken as evidence that ovarian hormones also influence the catecholamine and indolamine systems of the corpus striatum in man (e.g. Seeman and Lang, 1990; Lyons et al., 1998). Although it is uncertain how they relate to effects of hormonal flux over the menstrual cycle, findings from this study of ovariectomy and hormone replacement in young adult female rhesus monkeys provide experimental evidence in support of this influence, including the suggestion of regionally specific stimulation of 5-HT and DA that differentially affects innervation in the dorsal versus ventral striatum, the caudate versus the putamen, and neostriatal structures of the left versus the right hemisphere.

### Technical considerations

The conclusions of this study are based on quantitative evaluation of anatomically matched sections taken from ovariectomized control and hormonally replaced rhesus monkeys that were immunolabeled in parallel for TH or for 5-HT. In addition to the documented specificity of the commercial antisera used, patent labeling of catecholamine and indolamine afferents was supported by findings that both antibodies produced staining that was qualitatively similar to previous descriptions of serotonergic and dopaminergic innervation in the corpus striatum of monkeys from a level of overall patterning to axonal staining (Lavoie et al., 1989; Lavoie and Parent, 1990), and that neither

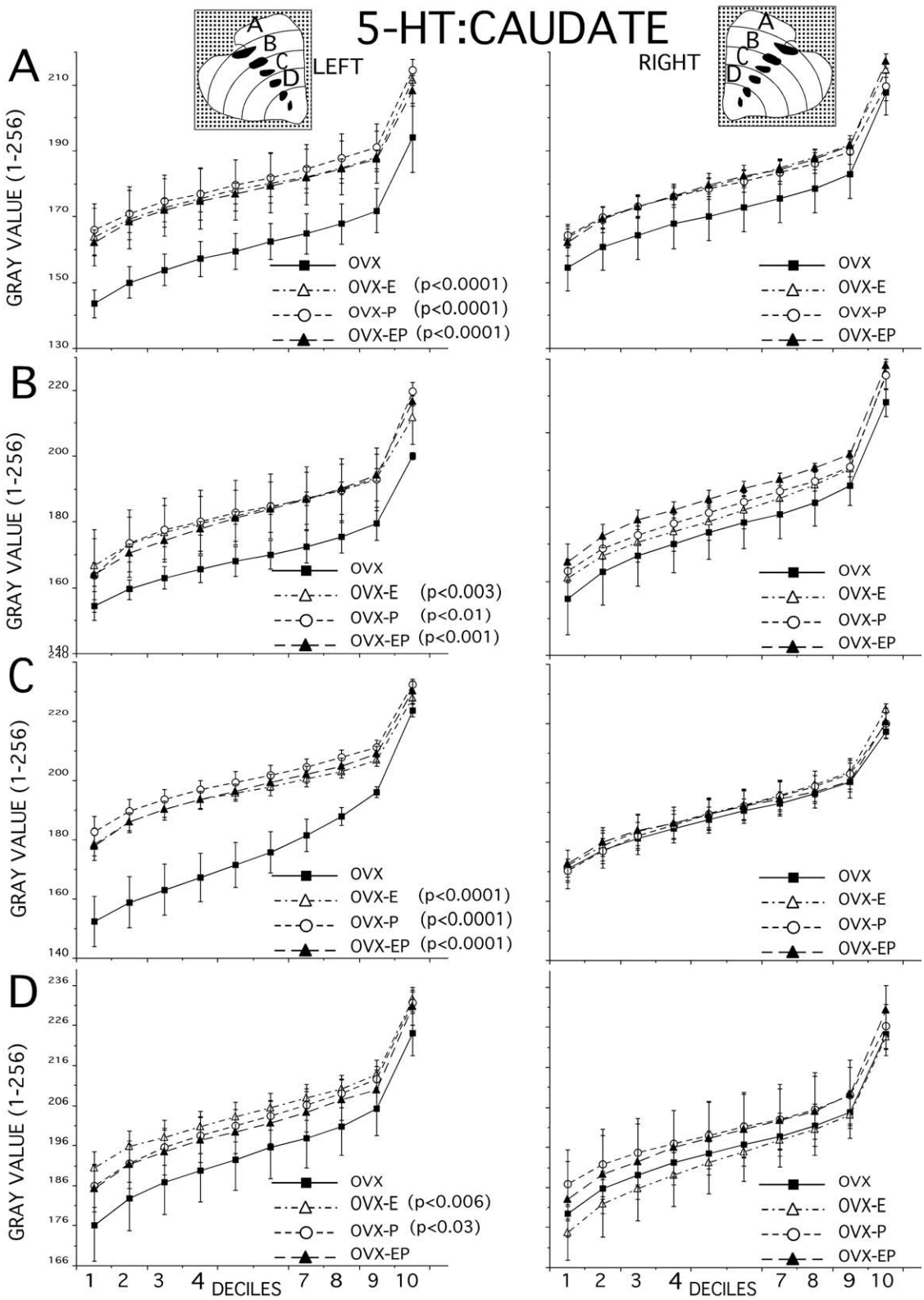


**Fig. 7.** Graphs plotting group mean gray value deciles of 5-HT immunoreactive density measured in captured images of the caudate nucleus (left and right) of monkeys that were OVX or OVX-E, OVX-P or OVX-EP; separate plots are shown for deciles measured in dorsolateral to ventromedial caudate sectors (see insets), and error bars represent S.E.M. The relative insensitivity of the indolamine innervation of the right caudate to ovariectomy and hormone replacement is indicated in the corresponding, overlapping graphs from the four animal groups (A–D, right). For the left caudate, a significantly decreased density of 5-HT immunoreactivity in the OVX control compared with OVX-E, OVX-P and OVX-EP cohorts is observed in all sectors.

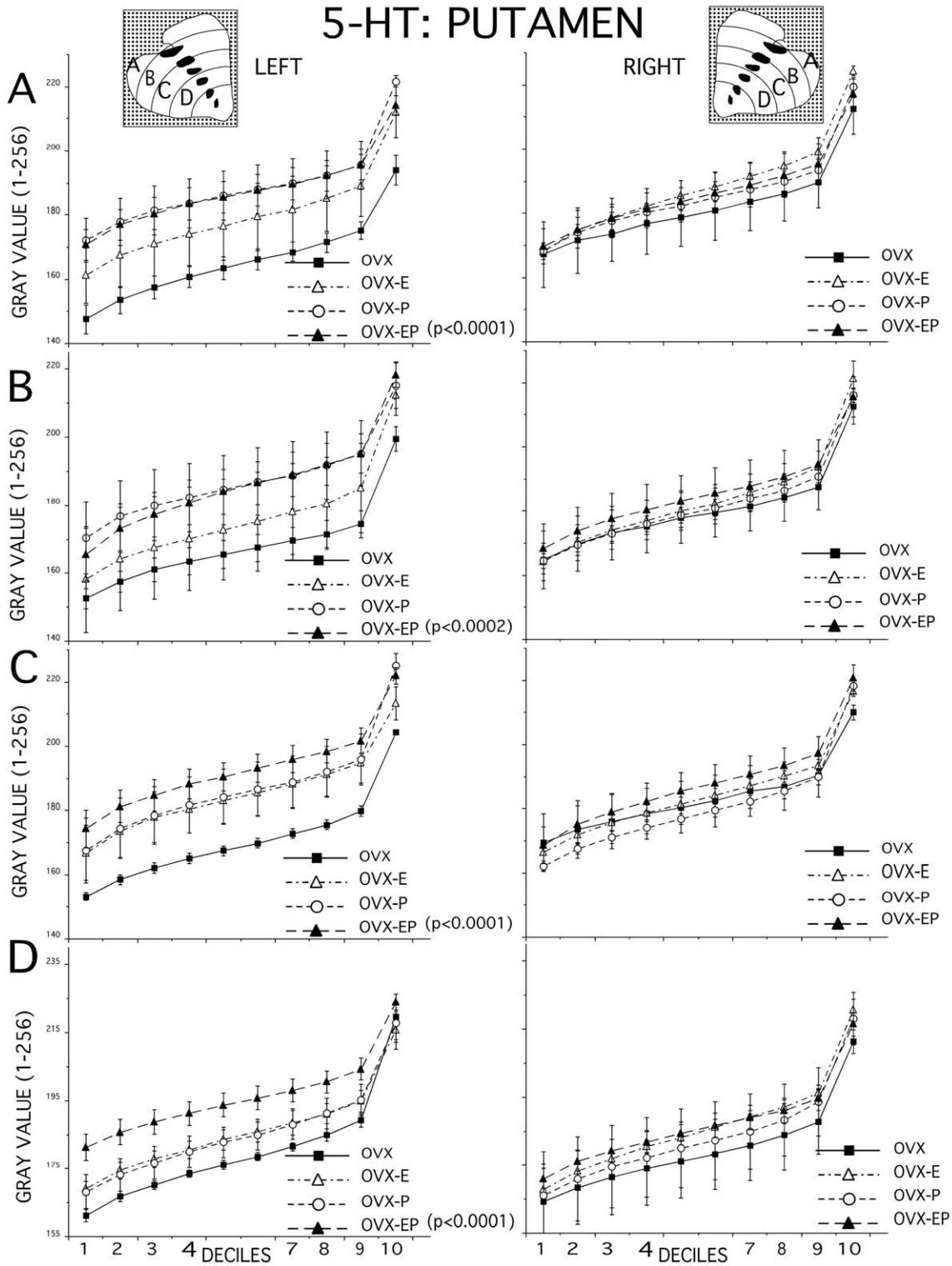


**Fig. 8.** Graphs plotting the group mean gray value deciles of 5-HT immunoreactive density measured in captured images of the putamen (left and right) of monkeys that were OVX or OVX-E, OVX-P or OVX-EP; separate plots are shown for measurements obtained in dorsolateral to ventromedial putamen sectors (see insets), and error bars represent S.E.M. The relative insensitivity of the idolamine innervation of the right putamen to ovariectomy and hormone replacement is indicated in the overlapping group decile graphs (A–D, right). For the left putamen, a decreased density of TH immunoreactivity in OVX control animals and to a lesser extent, the OVX-E group compared with the OVX-EP cohort is apparent in dorsal and ventral quadrants (A–D, left). Deciles for the OVX-P and OVX-EP groups overlap dorsally (A, B, left). In the ventral putamen (C, D), the deciles of the OVX-P group align with those of the OVX-E cohort; these fall midway between the low values of the OVX control monkeys and the higher densities of the OVX-EP animals.

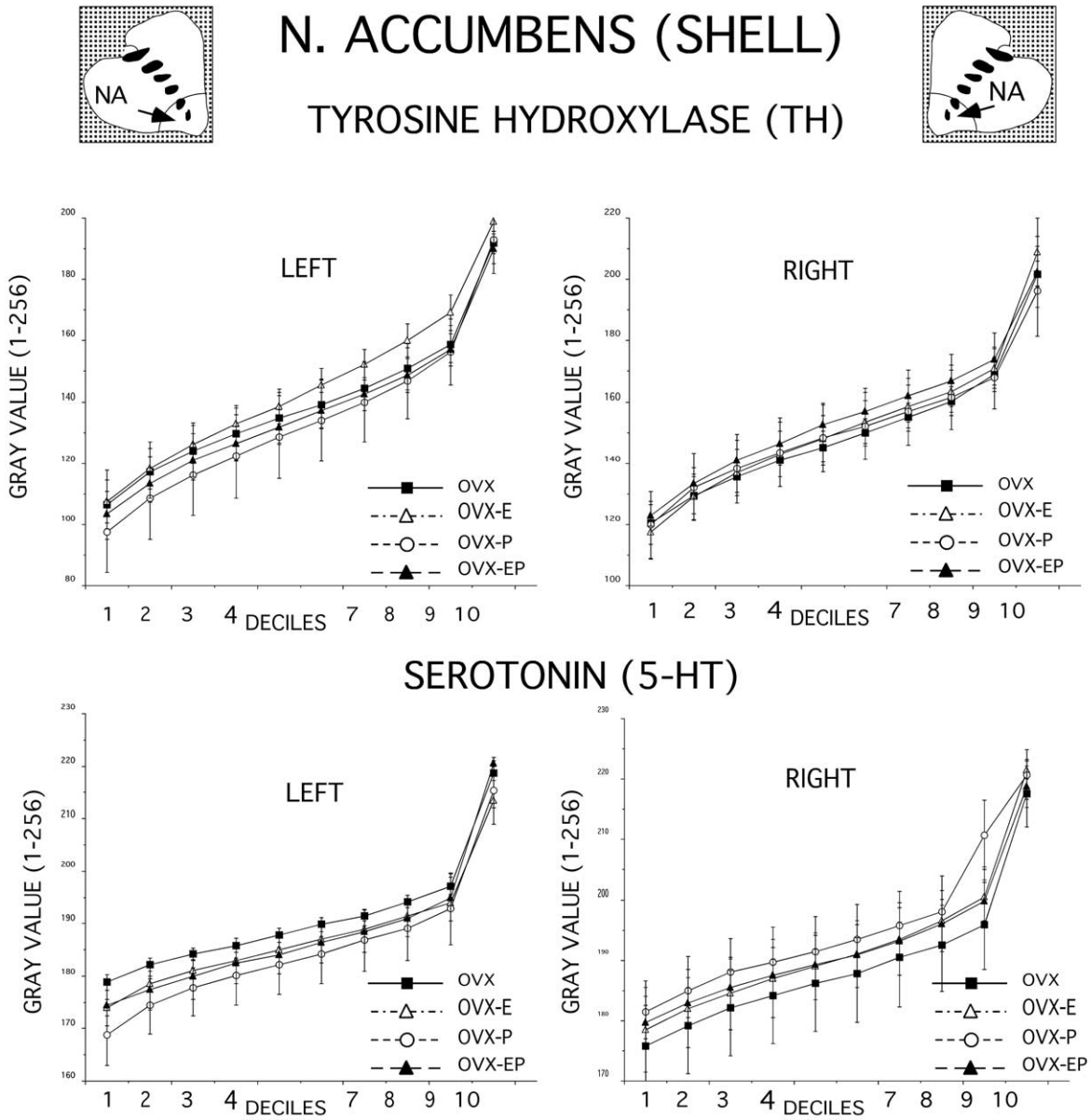


**Fig. 9.** Graphs plotting the group mean gray value deciles of TH and 5-HT immunoreactivity measured captured images of the nucleus accumbens shell (see insets, left and right) of monkeys that were OVX or OVX-E, OVX-P or OVX-EP; error bars represent S.E.M. The relative insensitivity of the catecholamine and indolamine innervations of the accumbal shell to ovariectomy and hormone replacement is indicated in the overlapping group decile graphs.

produced patterned staining in control experiments in which primary or secondary antibodies were omitted from labeling procedures. The low levels of noradrenalin present in the primate striatum (e.g. Ikemoto et al., 1996) also gave further selectivity in this study to the anti-TH antibody for identifying DA afferents.

The links between changes in circulating ovarian hormone levels and changes in the endpoint of afferent innervation measured in this study was established by organizing experiments around the classical framework of comparing ovariectomized control females to hormonally replaced, ovariectomized cohorts, and by applying a block design to standardized methods of preparing, preserving, and labeling the tissue. The specific problem of quantitatively evaluating the mottled, uneven DA and 5-HT innervations that are characteristics of the primate striatum was addressed by modifying established means of densitometric evaluation of immunocytochemically (DAB) labeled brain antigens (e.g. Peretti-Renucci et al., 1991; Huang et al., 1996); whereas average density measurements are usually used, in this study the collection and comparison of data were made on pixel-by-pixel, population bases. As discussed below, the differentiated ovarian hormone actions that emerged from these studies among anatomically discrete, functionally specialized striatal structures and subdomains suggest a spectrum of actions on 5-HT and

DA systems, including those which could relate to hypotheses of protective effects for circulating Es that observations such as decreased incidence and symptom severity in female patients with Parkinson's disease and with schizophrenia have helped to formulate (Seeman and Lang, 1990; Tanner, 1995; Lyons et al., 1998).

### Stimulation of striatal catecholamines

The caudate and putamen in monkeys are divided into functionally specialized zones defined in part by both gradients in DA innervation and by discrete terminal territories of corticostriatal afferents arising from sensorimotor versus associational versus limbic areas of the cerebral cortex (Kunzle, 1975, 1977; Selemon and Goldman-Rakic, 1985; Haber and McFarland, 1999; Haber et al., 2000). Although these dorsolateral to ventromedial associational to sensorimotor to limbic corticostriatal subdivisions are most elaborated in the postcommissural striatum, each has at least some discrete representation in precommissural striatal levels, including those analyzed here (Selemon and Goldman-Rakic, 1985; McGuire et al., 1991). Recent evidence indicates that these different striatal domains are also marked by physiological differences in their DA inputs, including parameters of DA uptake and release (Cragg et al., 2000, 2002). Findings from this study now suggest that the DA innervations of these compartments are also differentially sensitive to ovarian hormones. In the caudate, for example, TH immunoreactivity in each of the hormonally replaced groups was significantly denser than controls in dorsolateral, i.e. sensorimotor/associational regions, but was similar to controls ventrally and in the adjacent nucleus accumbens, i.e. in striatal zones where limbic cortical territories terminate (Selemon and Goldman-Rakic, 1985; Haber et al., 2000). In the putamen, very different patterns of hormone sensitivity were observed that included an exclusive localization of effects to the left hemisphere, and a striking insensitivity to E administered on its own.

Laterality aside, a feature that is common to all of the striatal areas where TH-innervation was responsive to circulating steroids is that they receive DA inputs primarily from ventral tier mesencephalic cells, i.e. DA neurons of the substantia nigra, pars compacta (Smith and Parent, 1986; Lynd-Balta and Haber, 1994). This fits well with recent findings indicating that subsets of these nigral neurons contain E receptor $\beta$ mRNA in pigtail macaques (Gundlah et al., 2000), and that in African Green monkeys, roughly 30% of these cells depend on circulating E for survival (Leranth et al., 2000). The selective ovarian hormone sensitivity of presumed ventral tier DA cell terminals could also be important for understanding the observed protective effects of E that have been described in Parkinson's disease (Tanner, 1995; Lyons et al., 1998), as in this disorder these ventral tier nigrostriatal DA neurons and their projections are those most vulnerable to degeneration (Kish et al., 1988). However, the present results suggest a further functional division among ventral tier DA cells. Specifically, while the presumed DA afferents to the caudate responded to E as well as E in combination with P, afferents in the putamen were insensitive to E alone. This putamen-specific requirement for P in stimulating DA afferents in chronically manipulated animals may explain why E alone was also found to be effective in attenuating ovariectomy-induced decreases in nigral TH-immunostaining and cell number only after 10 but not after 30 days of hormone deprivation (Leranth et al., 2000). These findings may also hold promise for means of separately modulating catecholamines in the caudate, as well as suggest a particular clinical benefit for combined E plus P therapies as adjuncts in treating striatal DA deficiencies in disorders like Parkinson's disease and in hormone replacement therapies following surgical menopause.

### Stimulation of 5-HT and DA/5-HT interactions

Both the present and previous studies in primates have identified potentiating effects for ovarian hormones over nigral DA systems and/or their terminal fields. However, evidence from experimental animals and humans suggests that at least functionally, E stimulation of midbrain DA systems is bidirectional. In rats, for example, E has been shown to potentiate behavioral actions of both DA receptor agonists (e.g. Chiodo et al., 1981) and antagonists (Chiodo et al., 1979), and increase firing rates in some nigrostriatal DA neurons while inhibiting activity in others (Chiodo and Caggiula, 1983). Similarly, in humans E seems able to ameliorate both the DA depletion in Parkinson's disease (Lyons et al., 1998) and exacerbate extrapyramidal side effects caused by DA receptor-blocking neuroleptics (Gratton, 1960). As discussed below, the effects identified in this study on 5-HT, a neurotransmitter that at least in part functionally inhibits mesostriatal DA systems at levels of their terminal fields (see Kapur and Remington, 1996), may represent means whereby the net effects of ovarian hormone stimulation over striatal DA tone could include functional inhibition as well as facilitation.

Hormone replacement in ovariectomized animals influenced 5-HT innervation in the putamen in essentially the same, complex manner that it affected TH-immunoreactivity, i.e. innervation was increased relative to controls in the left but not the right putamen in OVX-EP animals, with estradiol and P replacements alone showing little to no indolamine stimulation. However, in the caudate nuclei there were discrete areas where ovarian hormones seemed to differentially affect indolamine and catecholamine afferents. One example of this was the right caudate where TH but not 5-HT afferents were responsive to ovarian hormone stimulation (below). A second focus of disparate stimulation also occurred in the ventral, limbic caudate on the left side, where ovarian hormones stimulated 5-HT afferents but had no corresponding effects on DA innervation. Although there are findings especially in rats to the contrary (e.g. Benloucif et al., 1993; Benloucif and Galloway, 1991) there is a significant literature illustrating 5-HT's ability via interactions with 5-HT2 receptors to inhibit striatal DA release in several species including primates (Dewey et al., 1995; for review, see Kapur and Remington, 1996). Such a scenario could yield a discrete zone in which ovarian hormone stimulation indirectly produces a region of functionally depressed DA innervation. As DA over-activity in corresponding regions of the human

neostriatum may be related, at least in part, to the positive symptoms of schizophrenia (see Davis et al., 1991), this potentially unique sphere of negative hormone influence could be important for the protective, neuroleptic-like effects of circulating E that have been reported in relation to these symptoms (Seeman and Lang, 1990). However, in view of the disparate findings that currently make up the literature describing 5-HT modulation of striatal DA, the alternative possibility that these same territories are sites of selective sparing from the deleterious effects of ovariectomy on striatal DA systems must also be considered.

### Lateralized stimulation of neostriatal DA and 5-HT

In the human neostriatum, laterality in DA systems is suggested in findings of distinct sidedness in dyskinesias induced by long-term neuroleptic use (Waziri, 1980), as well as in measurements of neurotransmitter levels in left- and right-hand structures of postmortem human brain (Glick et al., 1982). These findings complement data obtained in rats where functional and biochemical asymmetries in nigrostriatal DA systems have been well characterized (see Glick et al., 1977). In these animal models as well, analyses of spontaneous and amphetamine-, cocaine-, or electrically stimulated nigrostriatal motor functions, e.g. stereotypy, circling/rotational behavior, suggest that ovarian hormones make important contributions to laterality (e.g. Robinson et al., 1981; Glick et al., 1983; Hyde and Jerussi, 1983; Camp et al., 1986). Electrically stimulated rotational behavior, for example, is decreased by ovariectomy in females, but is not affected by gonadectomy in males (Robinson et al., 1981). The present studies suggest that ovarian hormones may also shape asymmetries in the neostriatal catecholamine systems of man. For example, consistent with the left- over right-sided postmortem neurotransmitter measurements and with the prevalence of right-sided, neuroleptic-induced dyskinesias in man (Waziri, 1980; Glick et al., 1982), ovarian hormone stimulation of TH-immunoreactivity in this study in monkeys was either greatest in or exclusive to the left caudate/putamen. Although underlying mechanisms are unknown, these lateralized effects add to a list in which cortical and subcortical neurochemistry, structure and/or function in the left hemisphere have been found to be selectively or especially hormone sensitive (Diamond, 1991; Geschwind and Behan, 1982; Sandhu et al., 1986). The findings of asymmetric ovarian hormone stimulation in this study may also be relevant for the sex differences observed in schizophrenia, as pathophysiology in this disorder not only impacts the striatal catechol- and indolamines, but may also involve an especial vulnerability of the left hemisphere (e.g. Gur, 1978).

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