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Ovarian Hormones Differentially Influence Immunoreactivity for Dopamine β- Hydroxylase, Choline Acetyltransferase, and Serotonin in the Dorsolateral Prefrontal Cortex of Adult Rhesus Monkeys

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ABSTRACT

Recent studies have shown that ovariectomy reduces, and subsequent hormone replacement restores the density of axons immunoreactive for tyrosine hydroxylase in the dorsolateral prefrontal cortex of adult female rhesus monkeys. The present study indicates that three additional extrathalamic frontal lobe afferents are also sensitive to changes in the ovarian hormone environment. Specifically, the combination of hormone manipulation with qualitative and quantitative analysis of immunocytochemistry for dopamine β-hydroxylase, choline acetyltransferase, and serotonin in the primate prefrontal cortex revealed quantitative responses in both cholinergic and monoaminergic axons to changing ovarian hormone levels. However, whereas ovariectomy produced a modest net decrease in the density of fibers immunoreactive for choline acetyltransferase, this same treatment markedly increased the density of axons immunoreactive for dopamine β-hydroxylase and for serotonin. Further, the effects of ovariectomy on these afferent systems were differentially attenuated by estrogen verses estrogen plus progesterone hormone replacement. Estrogen was as effective as estrogen plus progesterone in stimulating normal prefrontal immunoreactivity for choline acetyltransferase and dopamine β-hydroxylase. The dual replacement of estrogen plus progesterone, however, was a much more potent influence than estrogen alone for serotonin immunoreactivity. Thus, ovarian hormones appear to provide stimulation that differentially affects each of four chemically identified extrathalamic prefrontal afferent systems examined to date, and may have roles in maintaining the normal balance and functional interactions between these neurotransmitter systems. J. Comp. Neurol. 409:438–451, 1999.

Indexing terms: noradrenalin; acetylcholine; association cortex; schizophrenia; neurotrophins

The prefrontal association cortices in human and nonhuman primates mediate complex processes including working memory, planning, and emotion (see Goldman-Rakic, 1987; Fuster, 1989). Sex differences and/or hormone malleability in the functional maturation (Clark and Goldman-Rakic, 1989; Overmann et al., 1996; see also Bachevalier and Hagger, 1991), lateralization (Goldberg and Podell, 1993), and the dysfunction of the frontal lobes in disorders such as schizophrenia (Seeman and Lang, 1990; Flor-Henry, 1990) suggest that gonadal hormones provide an important influence for the functional organization of the association cortices in human and/or nonhuman primates. Recent evidence in adult monkeys (Kritzer and Kohama, 1998) suggests that ovarian hormone stimulation of pre-

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frontal dopaminergic afferents, inputs which are essential for frontal lobe operations (e.g., Brozoski et al., 1979; Sawaguchi and Goldman-Rakic, 1991, 1994), may be one means by which gonadal hormones affect prefrontal function. However, the frontal lobes are also innervated by additional extrathalamic inputs including serotonergic afferents from the raphae nuclei (see Felten and Sladek, 1983), cholinergic axons projecting from the basal forebrain (e.g., Mesulam and Van Hoesen, 1976; Mesulam et al., 1983; Kitt et al., 1987), and noradrenergic afferents arising from the locus coeruleus (e.g., Porrino and Goldman-Rakic, 1982). Like dopamine inputs, these afferents terminate within the frontal lobes with a high degree of regional, laminar, and cellular selectivity (e.g., Berger et al., 1988; Lewis and Morrison, 1989; Lewis, 1991; Wilson and Molliver, 1991; Mrzljak and Goldman-Rakic, 1993; Smiley and Goldman-Rakic, 1996), and each seems to make distinctive contributions to frontal lobe function (e.g., Bartus et al., 1982; Arnsten and Goldman-Rakic, 1985; Richardson and DeLong, 1986; Araneda and Andrade, 1991; Arnsten, 1997). The present study asked whether these three chemically identified prefrontal afferents are also sensitive to changes in gonadal steroids.

Studies in rodents (e.g., Heritage et al., 1977; Sar and Stumpf, 1981; Fuxe et al., 1987; Toran-Allerand et al., 1992) and in primates (Bethea, 1993) have shown that subsets of identified cholinergic neurons of the basal forebrain, and serotonergic and noradrenergic neurons of the midbrain and brainstem contain intracellular hormone receptors. Further, hormone sensitivity for all three transmitter systems has been identified in subcortical regions e.g., neostriatum and hypothalamus, in rodents (see Vaccari, 1980; e.g., Miller, 1983; see also Bernard and Paolino, 1974). However, fewer studies have examined the hormone sensitivity of these afferents in the cerebral cortex (e.g., Luine, 1985; Battaner et al., 1987; Singh et al., 1994) and no previous investigations have focused on nonhuman primates. Accordingly, ovarian hormone stimulation of the cholinergic, serotonergic, and noradrenergic innervation of the cerebral cortex was explored in adult rhesus monkeys. Hormone effects were evaluated by qualitative and quantitative analysis of immunocytochemistry for dopamine β-hydroxylase, choline acetyltransferase, and for serotonin in the dorsolateral prefrontal cortex of intact and hormonally manipulated adult female rhesus monkeys. Comparative analyses in ovariectomized animals, ovariectomized animals supplemented with estrogen or with estrogen followed by progesterone, and in age- and sex-matched controls revealed unique quantitative responses of each of these afferent systems to hormone manipulation. These findings suggest that ovarian hormones may stimulate a group of neurotransmitters whose actions and interactions (e.g., Marchi and Raiteri, 1985; Liskowsky and Potter, 1985; Maura et al., 1992; Lynch, 1997; Rollema et al., 1997; Matsumoto et al., 1998) are important to prefrontal cortical function. Ovarian hormone dysregulation, on the other hand, could have relevance for disorders such as schizophrenia and depression, where the functional interaction between multiple transmitter systems are pivotal to disease and treatment (e.g., Halbreich and Lumley, 1993; Kapur and Remington, 1996), and where sex differences have been noted in incidence and/or clinical course (e.g., Seeman and Lang, 1990).

TABLE 1. Mean Pixel Densities From Digitized Camera Lucida Drawings of Dopamine-β-Hydroxylase-Immunoreactive Axons in Identified Layers (LYR) of Brodmann's Area 46[1]

	LYR				
	II	III	IV	V	VI
nml (age in yrs)					
9	1.22 ± 0.18	1.24 ± 0.17	1.15 ± 0.33	2.44 ± 0.42	2.31 ± 0.08
8	1.52 ± 0.26	1.84 ± 0.10	3.65 ± 0.06	3.24 ± 0.33	2.66 ± 0.62
17	2.13 ± 0.29	1.15 ± 0.13	2.81 ± 0.39	2.67 ± 0.27	2.75 ± 0.52
ovx (age in yrs)					
11[2]	3.69 ± 0.09	3.04 ± 0.12	4.55 ± 0.19	3.09 ± 0.08	3.99 ± 0.56
15	4.02 ± 0.11	3.87 ± 0.22	4.10 ± 0.12	3.92 ± 0.34	4.58 ± 0.10
7	3.78 ± 0.19	3.06 ± 0.22	4.22 ± 0.12	4.37 ± 0.41	4.77 ± 0.43
ovxE (age in yrs)					
12[2]	2.21 ± 0.17	1.84 ± 0.21	2.38 ± 0.03	3.11 ± 0.47	2.21 ± 0.28
13	2.12 ± 0.50	1.78 ± 0.09	2.11 ± 0.19	2.99 ± 0.38	2.10 ± 0.28
6[2]	2.33 ± 0.12	1.56 ± 0.29	2.49 ± 0.04	3.06 ± 0.19	2.50 ± 0.56
ovxE + P (age in yrs)					
7	2.00 ± 0.32	1.51 ± 0.36	2.16 ± 0.28	2.49 ± 0.09	1.83 ± 0.46
12[2]	2.12 ± 0.45	1.53 ± 0.17	2.77 ± 0.32	2.44 ± 0.19	2.02 ± 0.14
9[2]	2.51 ± 0.45	1.11 ± 0.13	2.59 ± 0.38	2.70 ± 0.22	1.96 ± 0.37

[1]Means ± standard errors from all normal (nml), ovariectomized (ovx), ovariectomized and estrogen-treated, (ovxE), and ovariectomized and estrogen plus progesterone-treated (ovxE + P) animals are shown. Animal age is on the left.
[2]Indicates animals euthanized in 1993; all others were euthanized in 1996.

MATERIALS AND METHODS

Animal subjects

Tissue from twelve adult female rhesus monkeys (*Macaca mulatta*), ages 7–17 years, was generously provided by Drs. Richard Simerly and Steven Kohama (Oregon Regional Primate Research Center, Beaverton, OR). Tissue from these animals was also used in a previous investigation of tyrosine hydroxylase (TH) immunoreactivity (Kritzer and Kohama, 1998); five animals were euthanized in 1993, and seven were euthanized in 1996 (e.g., Table 1). Three unoperated animals served as hormonally intact controls. The phase of the estrous cycle was identified in one animal (17 years old, see Table 1) as the follicular phase, a point in the cycle when circulating hormones are of intermediate level. In remaining control animals, it is only certain that at the time of euthanasia neither was in menses, when circulating hormones are lowest. The remaining nine animals had been bilaterally ovariectomized 4–7 months prior to euthanasia. Twenty-eight days prior to euthanasia, subjects were implanted with silastic capsules in the periscapular region; three ovariectomized monkeys received empty capsules (ovx) and six were implanted with capsules containing crystalline estradiol (ovxE; Sigma Chemical Corp., St. Louis, MO; of these six, three animals were implanted with capsules containing crystalline progesterone 14 days prior to euthanasia; ovxE+P; Sigma). Blood samples from implanted animals assayed for estradiol and progesterone confirmed that the implanted capsules yielded physiological levels of estrogen and progesterone (150–300 pg/ml and 4–8 pg/ml, respectively; Sprangers et al., 1990). All procedures that involved living animals had prior approval of the Oregon Regional Primate Research Center Animal Care and Use Committee (for additional detail, see Kohama and Bethea, 1995).

On the day of euthanasia, animals were deeply anesthetized with pentobarbital, and transcardially perfused with 500–1,000 ml physiological saline, followed by 4% paraformaldehyde in 3.8% borate buffer, pH 9.5. Brains were then removed, blocked, and postfixed (3 hours). Postfixed tissue was cryoprotected in a 0.02 M potassium phosphate

buffer solution, pH 7.5, containing 20% glycerol and 2% dimethyl sulfoxide, and was rapidly frozen in chilled isopentane prior to storage at -80°C. Blocks containing Brodmann's area 46 were sectioned in the coronal plane on a freezing microtome (40 μm).

Immunocytochemistry

Tissue sections were immunoreacted according to standardized 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 minutes, and then in 1% sodium borohydride in PB for an additional 30 minutes. Sections were then rinsed in 50 mM Tris-buffered saline (TBS), pH 7.4, placed in blocking solution (TBS containing 10% normal swine serum; NSS) for 1–2 hours, and incubated in primary antiserum (diluted in TBS containing 1% NSS, 2–3 days, 4°C). Anti-serotonin (5-HT; Eugene Tech, Ridgefield Park, NJ; Chemicon International Inc. Temecula, CA) antibodies were used at working dilutions of 1:500 and 1:1,000, respectively. The anti-dopamine β-hydroxylase (DBH) antibodies (Eugene Tech International Inc.; Protos, New York, NY) were also used at working dilutions of 1:500 and 1:1,000, respectively. The anti-choline acetyltransferase (CHAT- affinity-purified) antibody (Chemicon International Inc.) was used at a working dilution of 1:500. Following incubation in the primary antibody (3 days, 4°C), sections were rinsed in TBS, placed in biotinylated secondary antibodies (Vector, Burlingame, CA, 2 hours, room temperature, working dilution 1:200), rinsed in TBS, and then incubated in avidin-biotin-complexed horseradish peroxidase (ABC, Vector, 2 hours, room temperature). Sections were then rinsed in Tris buffer, pH 7.6, and reacted by using 0.07% 3,3′-diaminobenzidine (DAB) as chromagen prior to slide-mounting and silver-based signal intensification.

Each of the two commercially available antibodies used to label serotonergic and noradrenergic fibers yielded labeling that was suitable for qualitative analysis. However, only the one anti-5-HT antibody and the one anti-DBH antibody that consistently produced a superior signal and signal-to-noise ratio in the paraformaldehyde-fixed primate cortex (Protos anti-DBH; Eugene Tech anti-5-HT) were used in quantitative studies.

Silver/gold intensification

The DAB-treated, slide-mounted sections were silver-intensified (Kitt et al., 1988) by incubating slides in 1% silver nitrate (pH 7.0, 45 minutes, 55°C). After a brief rinse in running dH_2O, sections were incubated in 0.1% gold chloride (10 minutes, room temperature). Sections were then rinsed again in running dH_20, fixed in 5% sodium thiosulfate (10 minutes, room temperature), counterstained (2% cresyl violet), dehydrated, and placed under coverslips.

Control experiments

The immunocytochemical labeling procedures outlined above were carried out on representative sections from intact and manipulated animals with the omission of primary antisera or with the omission of secondary antibodies. Control sections were silver/gold- intensified side-by-side with normally immunoreacted slides.

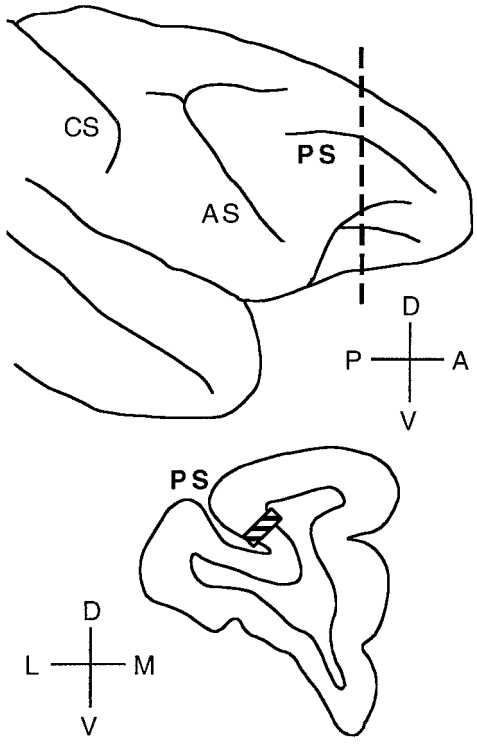

Fig. 1. Schematic diagram of the macaque frontal lobe (lateral view, above) and of a cross-section (below) indicating the level of the frontal lobe at which quantitative analyses were performed. In each section selected for quantitative study, camera lucida drawings of choline acetyltransferase-, dopamine β-hydroxylase-, or serotonin-immunoreactive fibers were obtained from cortex in the dorsal bank of the principal sulcus (PS) lying midway between its fundus and lateral lip (shaded). AS, arcuate sulcus; CS, central sulcus; A, anterior; D, dorsal; P, posterior; V, ventral; M, medial; L, lateral.

Analysis: Qualitative evaluation

Qualitative examination of the laminar distribution, the orientation, approximate density, and the morphology of immunoreactive axons was carried out at representative rostrocaudal levels within Brodmann's area 46 in all four groups of animals.

Analysis: Quantitative evaluation

For quantitative studies, a series of two to three sections was obtained from each animal for each antibody to be evaluated at the level of the midprincipal sulcus (Fig. 1). All tissue from all animals for these studies was sectioned on the same day, and immunoreacted and silver/gold-enhanced as a single group. Coded slides were counterstained with 2% cresyl violet for identification of cortical layers and were analyzed by a single observer (M.F.K.).

Camera lucida drawings were made of immunoreactive fibers under brightfield illumination using a 63× oil immersion objective. Because monoamine innervation of the primate frontal lobe is characterized by a series of smooth gradients, from rostral to caudal, medial to lateral, and from the fundus to the lip of the principal sulcus (Levitt et al., 1984), drawings were obtained from sections through the midprincipal sulcus that were matched with respect to all major axes, as well as to the fundus, lip, and bank of the principal sulcus (Fig. 1). The level selected for

analysis is characterized by emergent lateral and medial orbital sulci on the ventral surface; these landmarks were used to establish comparable anterior/posterior location of the sections to be evaluated across animals. Section thickness within all areas evaluated was also measured by roll-focusing from the top to bottom section surfaces using the calibrated fine-focus of the microscope (Zeiss Axioskop, Zeiss, Inc., Thornwood, NY) to ensure uniformity in this parameter. Other than regional selection, no attempts were made to preselect subareas for analysis.

Four nonoverlapping drawings were obtained from each cortical layer, from each animal, for each antibody; individual drawings subtended widths of 100–300 μm, measured parallel to the cortical surface, heights constrained by the thickness of the layer, and depths corresponding to the thickness of the section. Drawings were then scanned as black and white drawings (Hewlett Packard 4C Scanner; Desk Scan II). These digitized images were imported into NIH Image (5.8) for further processing and quantitative evaluation. First, because it was not possible to faithfully represent the diameters of all axons in camera lucida drawings, scanned drawings were skelatinized, a process that replaces lines of varying width with ones of uniform thickness. Next, mean pixel densities (NIH Image 5.8) were obtained; because the lines representing immunoreactive fibers were previously skelatinized, this areal measure was directly proportional to total two-dimensional length of the fibers within a given drawing (see Kritzer and Kohama, 1998).

Statistical analysis

All mean pixel density values from all subjects were pooled and analyzed first using a two-way analysis of variance (ANOVA) with a repeated measures design; the contributions of cortical layer, steroid treatment, individual animals, animal age, and tissue storage time to variability in the data were tested. Where appropriate, post-hoc comparisons were carried out (Student-Newman-Keuls); a value of $P < 0.05$ was accepted as significant.

RESULTS

Specificity of immunostaining

All five commercially available antibodies labeled axons in control animals in patterns corresponding to labeling documented in previous immunocytochemical studies of primate prefrontal cortex. Thus, the anti-CHAT antiserum labeled extremely dense populations of varicose, highly arborized axons (Lewis, 1991; Mrzljak and Goldman-Rakic, 1993), the two anti-DBH antibodies labeled sparse populations of heavily beaded as well as thinner, smoother axons (Lewis and Morrison, 1989), and the two anti-5-HT antisera labeled moderately dense populations of axons that ranged from a smooth to more highly varicose morphology (Wilson and Molliver, 1991). These similarities with previous descriptions (also see below), and in the case of anti-DBH and anti-5-HT immunoreactivity, the correspondence in labeling obtained with antisera obtained from different commercial sources, indicate the specificity of immunostaining for the neurotransmitter or related synthetic enzymes of interest. Specificity in immunostaining was also supported in control experiments in which omission of primary or secondary antibodies yielded only light, unpatterned immunoreactivity.

Immunostaining in ovariectomized animals. The morphology, the distribution, and the density of axons immunoreactive for 5-HT, DBH, and CHAT were examined at representative rostrocaudal levels in Brodmann's area 46 of the dorsolateral prefrontal cortex. For all three antigens, immunolabeling in ovariectomized animals showed clear quantitative departures from labeling present in control animals. However, each of the three axon populations responded differently to ovariectomy and/or to ovariectomy followed by hormone replacement (ovariectomy paired with estrogen replacement or with progesterone treatment following estrogen priming). These differential outcomes are described separately below for the three afferent systems evaluated.

Dopamine β-hydroxylase-like immunoreactivity

Immunoreactivity for labeled axons in control animals displayed expected morphologies and layer-specific patterns of orientation and density (e.g., Lewis and Morrison, 1989). Layers I–III, for example, were innervated by sparse populations of mainly radially oriented, heavily beaded axons, with smaller numbers of thin, poorly varicose fibers (Figs 2, 3A). Immunoreactivity in layers IV–VI, on the other hand, consisted of denser aggregates of more obliquely oriented fibers. These baselines contrasted with a noticeably more dense DBH innervation present in ovariectomized animals. Although immunoreactive axons were qualitatively similar to controls, e.g., DBH-immunoreactive axons were either beaded or smooth (Fig. 3B), and displayed expected layer-specific patterns of orientations (Fig. 2), there were many more axon segments present per unit area in the cortices of ovariectomized subjects (Fig. 2). These increases were most obvious in the supragranular layers, where the normally very low densities of DBH axons provided sharpest contrast to labeling in ovariectomized animals. Axon density in ovx animals also appeared elevated relative to controls in the granular and infragranular layers, albeit less markedly so than in the supragranular laminae.

Quantitative estimates of innervation density (see Materials and Methods) in layers II–VI revealed relatively stable, layer-specific axon density measures among individual animals within a given group (Table 1). These analyses also gave further definition to the differences in DBH immunoreactivity among control and ovariectomized animals. These analyses revealed that in some layers, axon density in ovx cases was more than twice control levels. In layers II and III, for example, axon density in ovx cases were, on average, about 236% of normal. In layers IV, V, and VI, increases in innervation were more moderate, with axon density in ovx cases rising to levels that were roughly 70%, 36%, and 73% higher than controls, respectively (Fig. 4). Statistical evaluation of these data (ANOVA with repeated measures design, including data from hormonally replaced subjects, see below) revealed significant main effects of treatment [$F_{3,8} = 17.04$, $P < 0.0008$] and layer [$F_{4,12} = 13.84$, $P < 0.0001$], excluded individual animals [$P < 1.0$] as significant sources of variance in the data. There were also no main effects of animal age [$F_{1,6} = 0.461$, $P < 0.523$] or tissue storage time [$F_{1,6} = 0.036$, $P < 0.856$]. The permitted post-hoc comparisons (Student-Newman-Keuls, $P < 0.05$) confirmed that the elevated levels of DBH-immunoreactive axons in ovariectomized

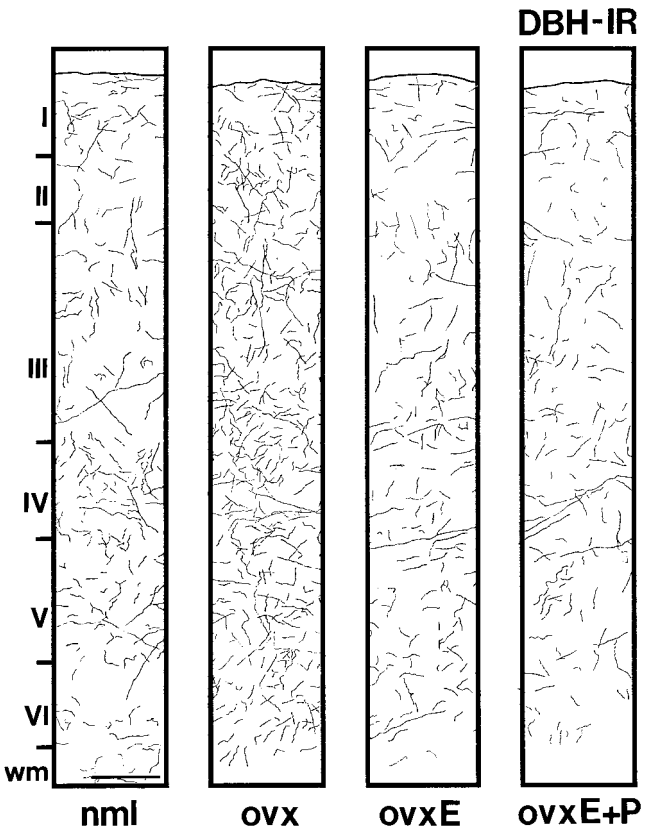

Fig. 2. Representative camera lucida drawings of dopamine-β-hydroxylase (DBH)-immunoreactive (IR) fibers from the dorsolateral prefrontal cortex of a control subject (nml), an ovariectomized (ovx) animal, an ovariectomized animal treated with estrogen (ovxE), and an ovariectomized monkey treated with estrogen followed by progesterone (ovxE+P). Cortical layers are identified by roman numerals on the left. Comparison illustrates a prominent increase in DBH axon density following ovariectomy, and qualitatively and quantitatively normal innervation in ovxE and ovxE+P animals. Despite differences in the number of processes present per unit area, the layer-specific characteristics of DBH-immunoreactive fiber orientation are preserved in all experimental groups. wm, white matter. Scale bar = 250 μm.

animals were significantly different from controls in every layer (Fig. 4).

Examination of ovariectomized animals treated with estrogen, or with estrogen followed by progesterone, indicated that both hormone replacement regimens were highly effective in attenuating ovariectomy-induced increases in cortical DBH innervation. Thus, in both groups of hormonally replaced subjects, prefrontal DBH-immunoreactive axons appeared to be morphologically intact (Figs. 2, 3C, D) and present in densities that appeared comparable to control animals. Quantitative analysis confirmed this latter point by showing that axon density was not statistically different from controls in any layer for either replacement group (Fig. 4).

Serotonin-like immunoreactivity

Similar to previous studies, hormonally intact control animals showed a moderately dense serotonergic innervation in the dorsolateral prefrontal cortex, with signature axon morphologies and orientations distinguishing particular cortical layers (e.g., Berger et al., 1988; Wilson and Molliver, 1991). Immunoreactivity in layer I, for example, consisted mainly of highly varicose axons that coursed parallel to the pial surface. In layers II and III, however, immunoreactive axons tended to be less varicose, and coursed at nearer right angles to cortical lamination (Fig. 3E). In ovariectomized animals, these qualitative features of innervation were preserved (Figs. 3F, 5). However, it was immediately evident that immunoreactive fiber density was markedly elevated relative to controls (Fig. 5). For example, the normal local waxing and waning of 5-HT axon density in layers II/III (see Wilson and Molliver, 1991) was replaced by a much more conspicuous clustering of immunoreactive fibers in ovx animals (Fig. 3). Immunoreactivity also appeared above normal in layers IV and V. In layer VI of both control and ovx animals, where axon densities were fairly low, group differences in innervation were less obvious by means of visual inspection.

Quantitative estimates of innervation showed that in layers II, III, and V, 5-HT axon density was similar among animals within a given group (Table 2). These studies further showed that axon density was, on average, two to more than three times greater in ovx animals than in controls. In layers IV and VI, densities were also above normal, but less strikingly so; on average, axon density was 193% and 164% of normal in these two layers, respectively (Fig. 6). An initial ANOVA with repeated measures design (that included hormonally replaced subjects) revealed significant main effects of treatment [$F_{3,8} = 10.80$, $P < 0.0035$] and layer [$F_{4,12} = 41.52$, $P < 0.0001$]. A significant layer-by-treatment interaction [$F_{12,312} = 5.99$, $P < 0.0001$] was also revealed that may reflect the considerable differences in the magnitude of the effects of ovariectomy in layers II, III, and V, verses layers IV and VI. Individual animals, however, were excluded as significant sources of variance in the data [$P > 1.0$], and no main effects of animal age [$F_{1,6} = 0.78$, $P < 0.411$] or tissue storage time [$F_{1,6} = 1.08$, $P < 0.354$] were identified. Subsequent post-hoc comparisons (Student-Newman-Keuls, $P < 0.05$) revealed that with the exception of layer VI, the elevated density values of 5-HT innervation in ovariectomized animals were significantly different from controls in all layers (Fig. 6).

As in ovariectomized subjects, 5-HT-immunoreactive axon morphology and orientation appeared to be intact in ovxE and ovxE+P animals (see Figs. 3G,H, 5). Axon densities, however, corresponded to values that were intermediate between ovx and control densities. In ovxE animals, 5-HT density was slightly lower than ovx, but remained well above control levels (between 233% of normal in layer V, and 169% of normal in layer IV, see Fig. 6). In ovxE+P animals, axon density was lower than in ovxE animals. However, axon density consistently remained at least marginally greater than values in controls in these dually replaced animals. Statistical evaluation (ANOVA followed by Student-Newman-Keuls, $P < 0.05$) revealed that ovxE was significantly different from controls in all layers. Axon densities in ovxE+P cases, on the other hand, although also higher than normal, were only significantly different from controls in layer II (Fig. 6).

Choline acetyltransferase-like immunoreactivity

As anticipated in previous studies, CHAT innervation in the prefrontal cortex of control animals was extremely

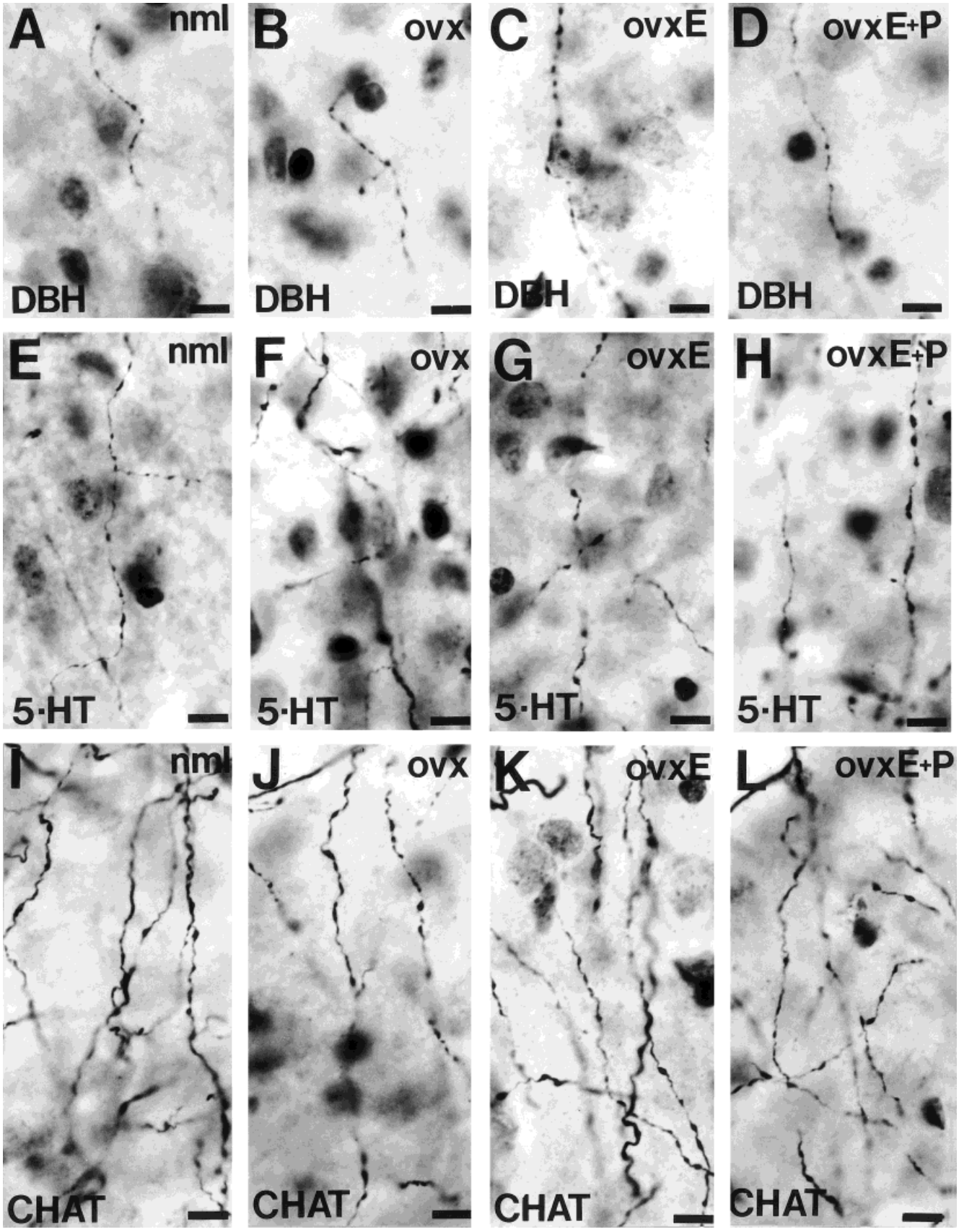

Fig. 3. Representative high power photomicrographs of layer III in the dorsolateral prefrontal cortex showing that axons labeled with immunocytochemical markers for noradrenalin (**A–D**), serotonin (**E–H**), and acetylcholine (**I–L**) are qualitatively similar in intact animals (nml), ovariectomized (ovx) monkeys, ovariectomized animals treated with estrogen (ovxE), and ovariectomized monkeys treated with estrogen and progesterone (ovxE+P). Antibodies recognizing and dopamine β-hydroxylase (DBH: A–D), serotonin (5-HT: E–H), and choline acetyltransferase (CHAT: I–L) label populations of axons in ovariectomized animals that are morphologically indistinguishable from those present in hormonally intact control subjects and are also oriented at similar angles (A, E, I). Even at this high power, some appreciation for the quantitative differences in axon innervation among animal groups can also be discerned. Scale bars = 10 μm.

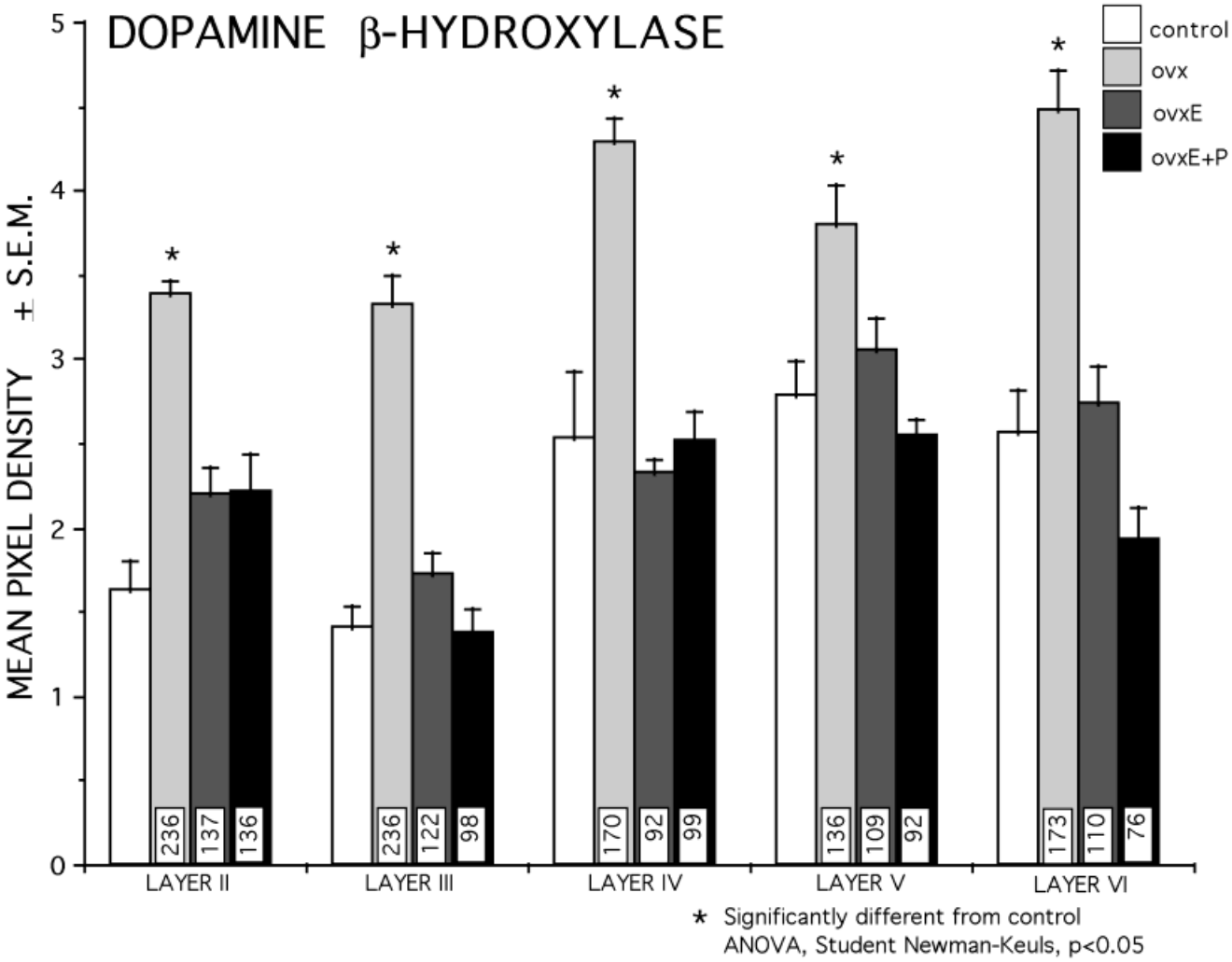

Fig. 4. Bar graphs of group mean pixel densities measured from digitized camera lucida drawings of dopamine-β-hydroxylase (DBH)-immunoreactive axons in identified cortical layers of controls (nml), ovariectomized animals (ovx), ovariectomized animals treated with estrogen (ovxE), and ovariectomized animals treated with estrogen plus progesterone (ovxE+P). In each of the major cellular layers, fiber density in ovariectomized animals (ovx) is higher than normal, but is not statistically different from normal in ovxE and ovxE+P cases. Mean density values expressed as percent of normal appear in the white boxes at the base of graph bars. These graphs summarize the stimulatory effects of ovariectomy on DBH innervation density, and indicate a sensitivity of cortical DBH innervation to both estrogen and estrogen plus progesterone treatment.

dense, especially in layers I and II. In addition, individual layers were invested with immunoreactive axons with morphologies and trajectories that were similar to descriptions in previous studies in primate prefrontal cortex (e.g., Lewis, 1991; Mrzljak and Goldman-Rakic, 1993, see Figs. 3I, 7). Normal appearing and coursing CHAT-immunopositive axons were also present in ovx animals. However, the density of these axons appeared to be somewhat lower than normal; whereas in control animals CHAT innervation provided a fairly uniform cortical coverage, in ovx animals, immunoreactive axons tended to form looser meshworks that left noticeably wider immunonegative trabeculae (Fig. 7). Quantitative analysis of fiber density indicated similar axon densities for all animals within a given group (Table 3) and confirmed that axon innervation was slightly but consistently lower than normal in ovariectomized monkeys. Thus, in ovx animals, average axon density values ranged from 76% of normal in layer V to 88% of normal in layer II (Fig. 8). Statistical evaluation of these data uncovered a significant main effect of layer [$F_{4,12} = 41.40$, $P < 0.001$] ruled out individual animals [$P > 1.0$], animal age [$F_{1,6} = 2.74$, $P < 0.149$], and tissue storage time [$F_{1,6} = 0.42$, $P < 0.523$] as significant sources of variance in the data. A main effect of treatment, however, failed to reach significance [$F_{3,8} = 2.27$, $P < 0.1573$] and subsequent post-hoc comparisons (Student-Newman-Keuls, $P < 0.05$) revealed that the trend for reduced CHAT innervation in ovx animals was only significant in layer II (Fig. 8).

In view of the relatively modest quantitative differences in axon density separating animal groups, it was important to more rigorously examine the possibility of group-specific bias in the axon measures themselves. Thus, although axon orientation was not appreciably different in

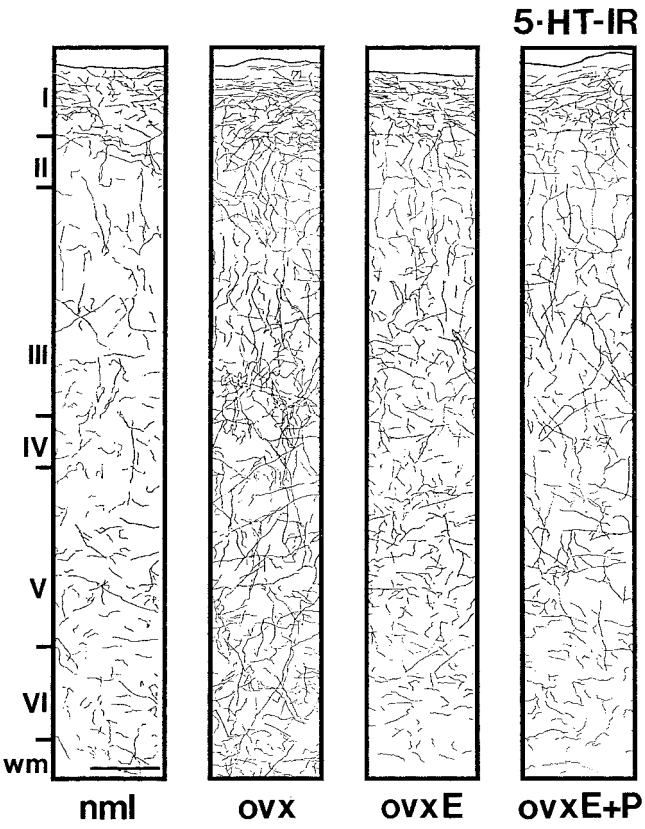

Fig. 5. Representative camera lucida drawings of serotonin (5-HT)- immunoreactive (IR) fibers from the dorsolateral prefrontal cortex of a control subject (nml), an ovariectomized (ovx) animal, an ovariectomized animal treated with estrogen (ovxE), and an ovariectomized monkey treated with estrogen followed by progesterone (ovxE+P). Cortical layers are identified by roman numerals on the left. In every group, qualitatively normal fiber orientation is observed. Quantitatively, however, there is a striking increase in 5-HT axon density following ovariectomy. In all layers, axon density is moderately reduced in ovxE compared to ovx cases, and is lower still in ovxE+P animals. Although this latter treatment rendered axon densities that were on average higher than normal, these densities were not significantly different from controls in most layers. wm, white matter. Scale bar = 250 μm.

ovx and control groups, because the two-dimensional camera lucida drawings are sensitive to fiber orientation (see Discussion, Kritzer and Kohama, 1998), quantitative estimates of fiber orientation were also carried out in two representative layers (layers III and V). This was accomplished by measuring the two-dimensional lengths of 100 axon segments residing within single focal planes (see Kritzer and Kohama, 1998). Although the sampled fiber populations were not statistically invariant (Kolmogorov-Smirnov nonparametric comparison), there was no evidence of a greater percentage of fibers oriented steeply with respect to the plane of section in ovx compared to control animals (Fig. 9), a scenario which could have resulted in underestimation of fiber content in the ovx animals (see Discussion, Kritzer and Kohama, 1998).

Investigation of ovxE and ovxE+P animals revealed no obvious differences in the cholinergic innervation of either of these two groups compared to controls. Thus, in both ovxE and ovxE+P animals, the prefrontal cortices were

TABLE 2. Mean Pixel Densities From Digitized Camera Lucida Drawings of Serotonin-Immunoreactive Axons in Identified Layers (LYR) of Brodmann's Area 46[1]

	LYR				
	II	III	IV	V	VI
nml (age in yrs)					
9	3.91 ± 0.20	3.10 ± 0.32	4.26 ± 0.52	3.28 ± 0.71	2.16 ± 0.28
8	3.87 ± 0.34	4.15 ± 0.59	5.99 ± 0.81	3.49 ± 0.27	2.29 ± 0.29
17	2.93 ± 0.31	4.88 ± 0.95	3.95 ± 0.43	3.38 ± 0.21	2.98 ± 0.22
ovx (age in yrs)					
11[2]	9.90 ± 0.56	9.13 ± 0.31	8.97 ± 0.32	8.93 ± 0.72	4.25 ± 0.79
15	11.61 ± 1.01	9.73 ± 0.52	10.10 ± 0.26	9.06 ± 0.70	4.14 ± 0.72
7	11.90 ± 0.51	10.26 ± 0.55	8.35 ± 0.75	8.22 ± 0.86	3.75 ± 0.94
ovxE (age in yrs)					
12[2]	7.74 ± 0.68	6.81 ± 0.69	7.87 ± 0.92	8.02 ± 1.06	4.91 ± 0.80
13	8.26 ± 1.30	7.39 ± 0.74	8.97 ± 1.28	8.25 ± 1.26	5.44 ± 0.59
6[2]	6.88 ± 1.11	7.38 ± 0.75	7.19 ± 1.07	7.29 ± 1.30	4.92 ± 0.26
ovxE + P (age in yrs)					
7	6.36 ± 0.49	5.36 ± 0.35	6.91 ± 0.85	6.45 ± 0.22	4.21 ± 0.62
12[2]	6.35 ± 0.80	5.32 ± 0.24	6.42 ± 0.61	5.97 ± 0.32	3.88 ± 0.84
9[2]	6.87 ± 0.26	5.70 ± 0.53	7.36 ± 0.67	5.80 ± 0.59	4.58 ± 0.78

[1]Means ± standard errors from all normal (nml), ovariectomized (ovx), ovariectomized and estrogen-treated, (ovxE), and ovariectomized and estrogen plus progesterone-treated (ovxE + P) animals are shown. Animal age is on the left.
[2]Indicates animals euthanized in 1993; all others were euthanized in 1996.

innervated with CHAT-immunopositive axons that displayed seemingly normal morphology, orientation, and density (Fig. 3K, L). Quantitative analyses confirmed that in ovxE animals, axon density was between 91% (measured in layer III) and 107% of normal (measured in layer II), and that in ovxE+P cases, axon density fell between 94% of normal (in layer IV) and 109% of normal (in layer II); these axon density measures were not statistically different from controls in any layer (Fig. 8).

DISCUSSION

Growing evidence for functional interactions between neurotransmitters, e.g., serotonin stimulation of dopamine release (e.g., Rollema et al., 1997), and anatomical findings such as the localization of dopamine receptors on cholinergic axon terminals (e.g., Maura et al., 1992), indicate that the complex computations of the frontal lobe rely, at least in part, on the interplay between multiple neurotransmitter systems. The present findings of qualitatively different effects of ovariectomy on the density of axons immunoreactive for CHAT, DBH, and 5-HT in the prefrontal cortices of adult rhesus monkeys suggests that ovarian hormones may play important roles in maintaining the neurochemical balance necessary for these functional interactions.

Technical considerations

Several arguments can be made that support the conclusion that observed changes in the density of immunoreactive axons reflect sensitivity of frontal lobe afferents to circulating ovarian steroids. First, methodological approaches were taken to maximize interanimal consistency in antigen preservation, immunolabeling, and regional sampling (see Materials and Methods). In addition, the changes in axon density that were revealed occurred in the absence of obvious treatment-induced shifts in fiber orientation. This is an important point, because the axon measures used for comparisons were obtained from two-dimensional camera lucida representations of three-dimensional cortical space and are sensitive to fiber orientation. Axons oriented steeply with respect to the cut

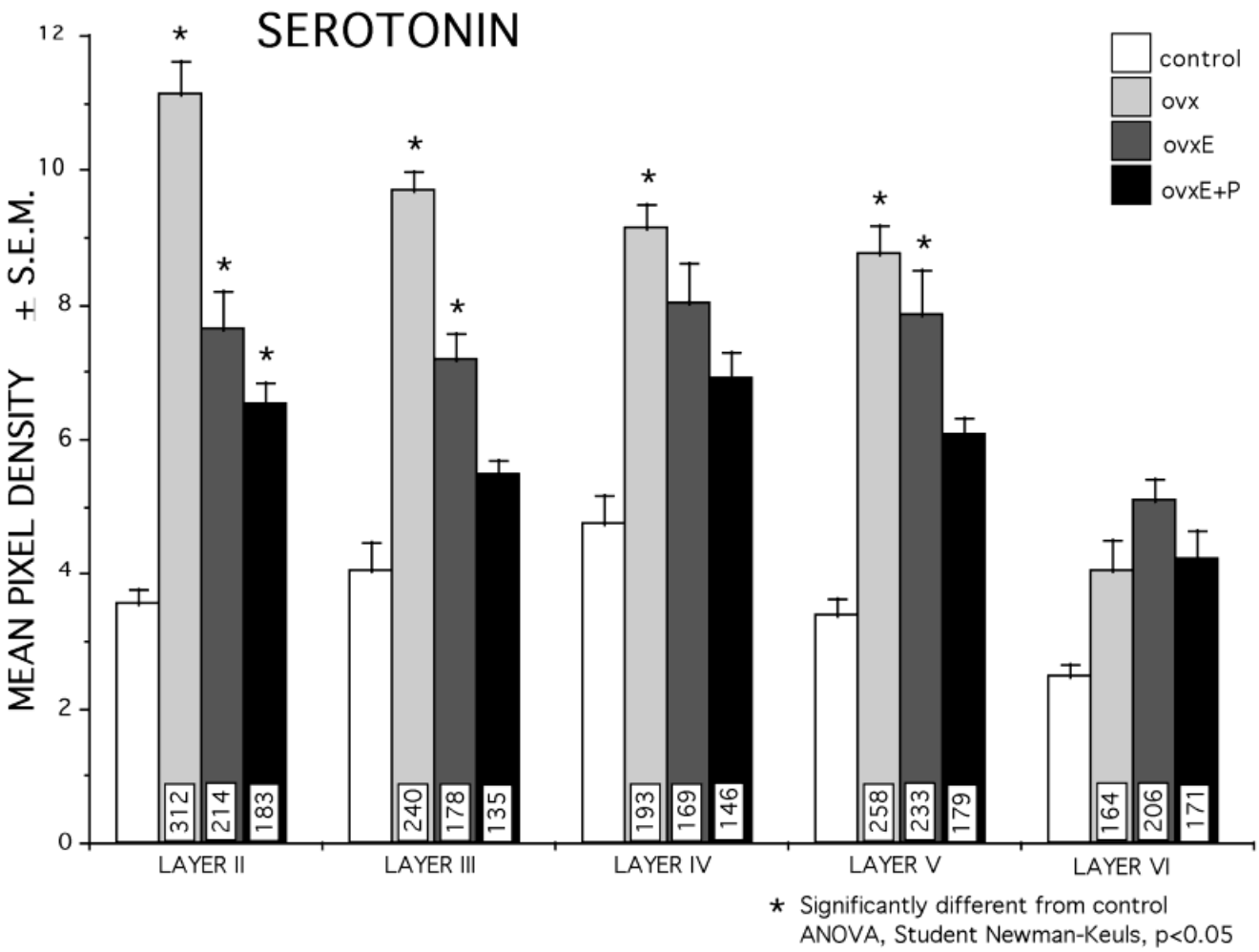

Fig. 6. Bar graphs of group mean pixel densities measured from digitized camera lucida drawings of serotonin (5-HT)-immunoreactive axons in identified cortical layers of intact controls (nml), ovariectomized animals (ovx), ovariectomized animals treated with estrogen (ovxE), and ovariectomized animals treated with estrogen plus progesterone (ovxE+P). Mean density values expressed as percent of normal appear in the white boxes at the base of graph bars. In each of the major cellular layers, fiber density in ovariectomized animals (ovx) was considerably higher than normal, with intermediate fiber density values in ovx E and ovxE+P cases. These graphs illustrate the layer-specific increases in 5-HT innervation that follow ovariectomy, a limited sensitivity of cortical 5-HT innervation to estrogen replacement, and a greater responsiveness of these axons to estrogen plus progesterone replacement.

surfaces of coronal sections, for example, are significantly foreshortened in these two-dimensional representations. Because there was no qualitative and quantitative evidence for obvious treatment-induced shifts in axon orientation, however, this parameter is an unlikely source of group-specific bias in axon density measures (also see Kritzer and Kohama, 1998). Statistical analyses (ANOVA) also failed to identify main effects of animal age or tissue storage time on axon density measures, effectively ruling out these factors as potential confounds. Finally, for all three systems examined, the density of innervation more closely approximated control values in ovariectomized animals that received hormone replacement compared to animals that were only ovariectomized. In total, these factors sum to conclude that the quantitative differences in innervation that distinguished the ovariectomized animals from hormonally intact groups are consequences of experimentally induced changes in circulating ovarian steroids.

For the control animals, it was only known with certainty that one of the three animals was in the follicular phase of the estrous cycle, and that neither of the other two were in estrous at the time of perfusion. Thus, at the time of euthanasia, none of the animals were in the portion of the menstrual cycle when hormone levels are at their lowest (estrous). However, the nonestrous phases of the 28-day primate menstrual cycle are differentiated by relatively modest and more gradually occurring fluctuations in circulating ovarian steroids. That there was basic uniformity in axon measures obtained in the three control animals in spite of probable differences in menstrual status within this range suggests that these fluxes are not sufficient to induce large changes in prefrontal monoaminergic or cholinergic innervation. This conclusion is similar to that suspected for the dopamine innervation of the primate prefrontal cortex, and shown more directly in a study of dopamine levels in the frontal cortices in female

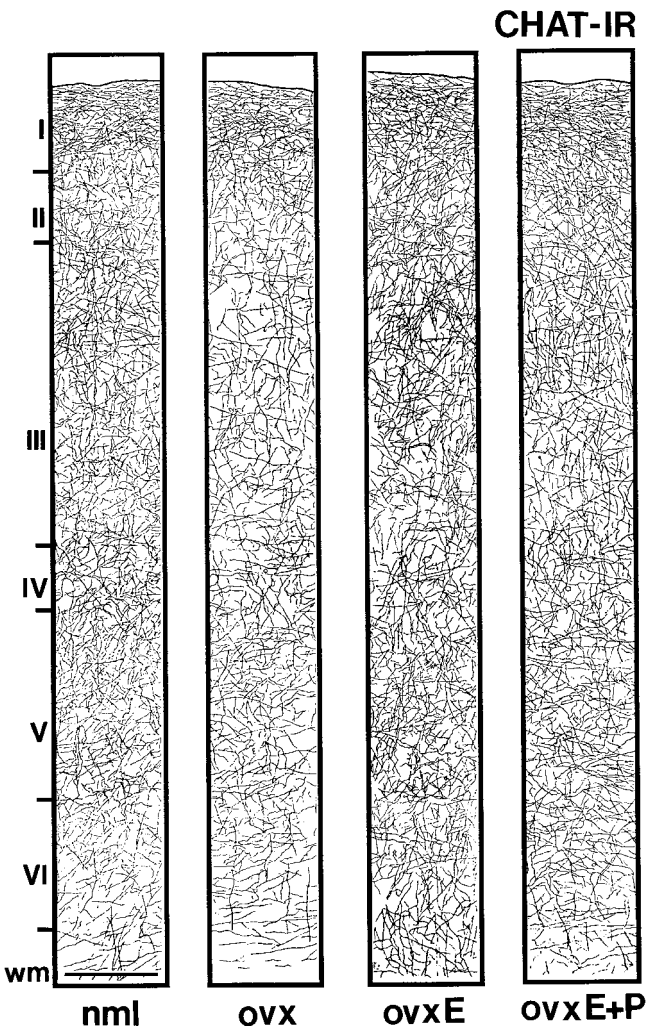

Fig. 7. Representative camera lucida drawings of choline acetyltransferase (CHAT)-immunoreactive (IR) fibers from the dorsolateral prefrontal cortex of a control subject (nml), an ovariectomized (ovx) animal, an ovariectomized animal treated with estrogen (ovxE), and an ovariectomized monkey treated with estrogen followed by progesterone (ovxE+P). Cortical layers are identified by roman numerals on the left. A modest decrease in CHAT axon density following ovariectomy is discernible. In both ovxE and ovxE+P cases, however, there are no obvious differences in immunolabeling compared to controls. wm, white matter. Scale bar = 250 μm.

rats across the four- to five-day estrous cycle (Glaser et al., 1990).

Hormone stimulation of cortical neurotransmitters: Comparison to previous studies

Each of the neurotransmitter system markers examined showed unique responses to ovariectomy and/or to ovariectomy followed by hormone replacement. Among the monoamine markers examined, the density of DBH- and 5-HT-immunoreactive axons were both significantly increased in ovariectomized monkeys. These stimulatory effects of hormone deprivation have parallels in the small number of existing studies in adult rats examining gonadal hormone influence over cortical monoamines. In adult male rats, for example, gonadectomy yields a small, albeit nonsignificant, mean increase in the levels of norepinephrine (NE) and 5-HT in parietal cortex (Battaner et al., 1987). The observed differential effectiveness of estrogen verses estrogen plus progesterone replacement in attenuating the effects of ovariectomy on monoamines in monkeys also has some precedent in the rodent literature. In pregnant female rats, for example, cortical levels of the serotonin metabolite 5-hydroxyindoleacetic acid are negatively correlated with circulating progesterone, but not estrogen levels (Glaser et al., 1990). This finding may be consistent with the particular effectiveness of progesterone (following estrogen priming) to suppress 5-HT overinnervation in the frontal lobes of ovariectomized macaques. Finally, the present study also revealed that ovariectomy affected CHAT-immunoreactive axons, albeit in a qualitatively and quantitatively different manner than the monoamines. Thus, in contrast to ovariectomy-induced up-regulation of 5-HT and DBH, CHAT-immunoreactive axons were modestly decreased in ovariectomized monkeys but were not obviously different from controls in both ovxE and ovxE+P cases. These findings may offer a parallel to results obtained in adult rats in which ovariectomy in females decreased CHAT activity and high affinity choline uptake in an estrogen-reversible manner in frontal cortex (e.g., Luine, 1985; O'Malley et al., 1987; Singh et al., 1994). However, although these depressed biochemical measures seem at least intuitively consistent with the decreased CHAT immunoreactivity in the primate frontal lobe, it must also be borne in mind that, unlike in primates, cholinergic axons in rat cortex are derived from intrinsic and well as extrinsic sources (see Emson and Lindvall, 1986), and it is uncertain whether biochemical findings in rodents reflect changes in one or both of these systems.

In sum, numerous studies document gonadal hormone effects on the levels, turnover, release, and/or receptor binding of cholinergic and monoaminergic neurotransmitter systems in whole brain or subcortical structures (see Vaccari, 1980; e.g., Miller, 1983); although information is more fragmentary, the limited data available largely suggest that gonadal steroids are also potent regulators of

TABLE 3. Mean Pixel Densities From Digitized Camera Lucida Drawings of Choline Acetyltransferase-Immunoreactive Axons in Identified Layers (LYR) of Brodmann's Area 46[1]

	LYR				
	II	III	IV	V	VI
nml (age in yrs)					
9	14.49 ± 0.82	13.37 ± 1.06	13.40 ± 0.87	13.24 ± 0.92	11.38 ± 0.68
8	17.63 ± 1.25	17.36 ± 1.9	13.71 ± 0.64	12.81 ± 1.01	11.02 ± 0.73
17	19.67 ± 2.34	13.08 ± 0.52	14.42 ± 1.41	13.75 ± 0.53	11.30 ± 0.69
ovx (age in yrs)					
11[2]	16.07 ± 0.88	10.83 ± 0.92	12.47 ± 0.65	10.12 ± 0.75	9.35 ± 1.07
15	15.93 ± 0.87	10.52 ± 0.19	12.18 ± 0.50	11.48 ± 1.02	9.68 ± 1.56
7	13.94 ± 0.90	10.32 ± 0.36	10.91 ± 0.85	9.69 ± 1.31	8.69 ± 0.81
ovxE (age in yrs)					
12[2]	18.14 ± 0.47	12.36 ± 0.55	13.64 ± 1.19	11.66 ± 0.87	10.67 ± 0.21
13	19.19 ± 1.18	12.29 ± 0.79	13.30 ± 0.48	11.58 ± 0.69	11.03 ± 0.84
6[2]	17.99 ± 0.46	11.54 ± 0.84	13.47 ± 1.63	12.52 ± 0.93	12.34 ± 1.03
ovxE + P (age in yrs)					
7	18.79 ± 1.91	12.37 ± 0.72	13.03 ± 0.69	14.14 ± 0.36	10.69 ± 0.61
12[2]	19.99 ± 2.19	12.98 ± 0.72	13.24 ± 0.38	13.91 ± 0.59	11.64 ± 0.80
9[2]	17.53 ± 2.25	12.68 ± 0.24	13.14 ± 0.46	11.82 ± 0.43	12.56 ± 0.74

[1]Means ± standard errors from all normal (nml), ovariectomized (ovx), ovariectomized and estrogen-treated, (ovxE), and ovariectomized and estrogen plus progesterone-treated (ovxE + P) animals are shown. Animal age is on the left.
[2]Indicates animals euthanized in 1993; all others were euthanized in 1996.

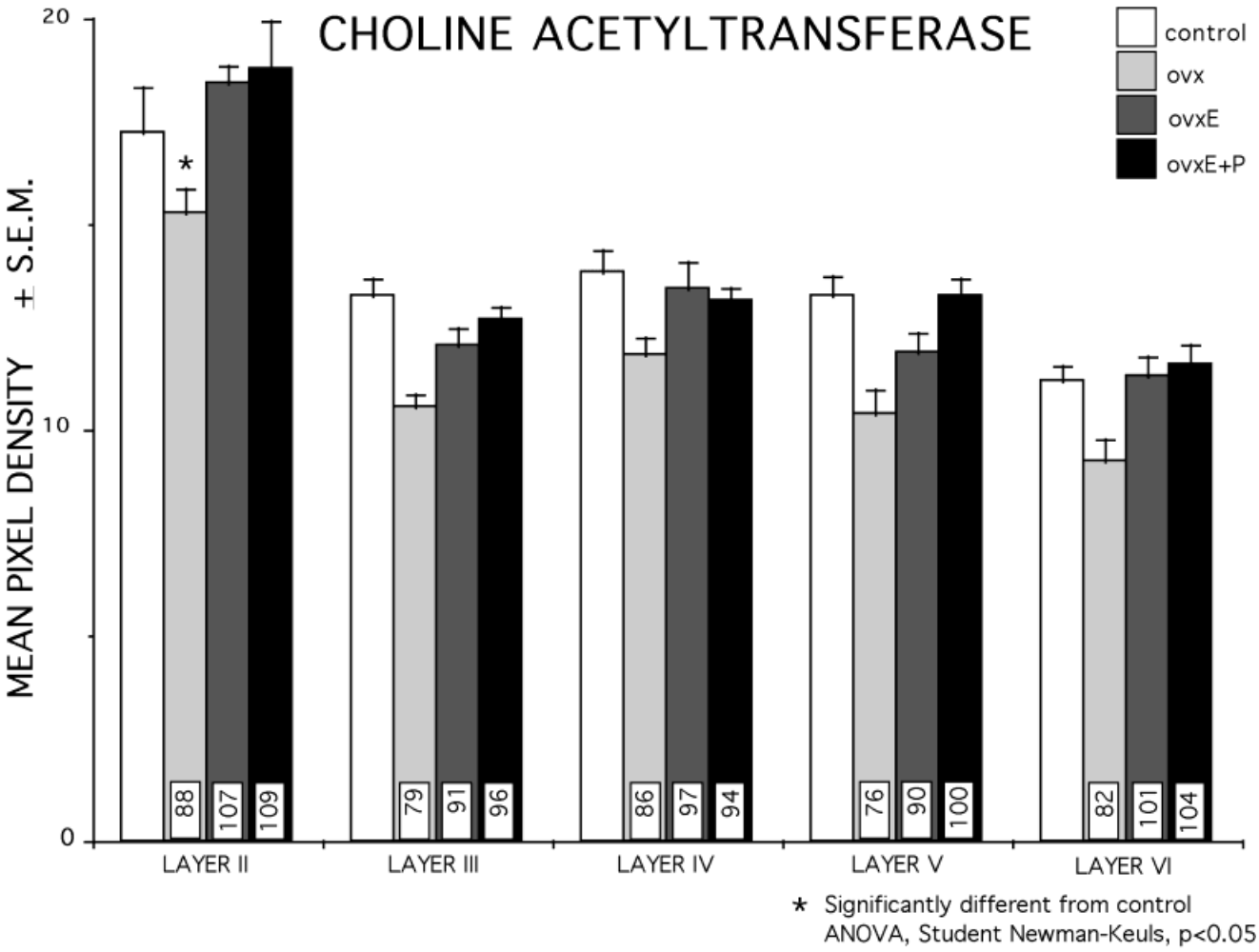

Fig. 8. Bar graphs of group mean pixel densities measured from digitized camera lucida drawings of choline acetyltransferase (CHAT)-immunoreactive axons in identified cortical layers of intact controls (nml), ovariectomized animals (ovx), ovariectomized animals treated with estrogen (ovxE), and ovariectomized animals treated with estrogen plus progesterone (ovxE+P). Mean density values expressed as percent of normal appear in white boxes at the base of graph bars. In all layers, fiber density in ovariectomized animals (ovx) was, on average, lower than normal, but was only significantly so in layer II. There were no apparent quantitative differences in any of the layers of ovxE and ovxE+P cases compared to the hormonally intact control group. These graphs illustrate the small albeit consistent decrements in CHAT fiber density in ovx animals, and the capacity of both estrogen and estrogen plus progesterone replacement to prevent this modest axon density decline.

neurotransmitters at the level of the cerebral cortex. In view of the evidence presented in this study for possible transmitter-specific effects of ovariectomy, it may be particularly important to identify the mechanisms governing these cortical endpoints of ovarian hormone stimulation in the primate frontal lobe.

Hormone stimulation of cortical neurotransmitters: Possible mechanisms

Previous studies in monkeys revealed large decreases in tyrosine-hydroxylase immunoreactivity in the prefrontal cortex following ovariectomy, a partial restoration of dopamine innervation by estrogen replacement, and a return to control levels of axon density in ovariectomized animals treated with estrogen followed by progesterone (Kritzer and Kohama, 1998). These studies, carried out on tissue sections from the same animals examined here, round out a list of transmitter-specific outcomes of ovarian hormone manipulation for each of the four major extrathalamic sources of innervation to the frontal lobe. The primary division among these four afferent systems uncovered, i.e., that some are increased and others decreased by ovariectomy, argues against observed changes in immunoreactivity as secondary to global, perhaps nonspecific effects of hormone manipulation on brain metabolism. Similarly, changes in cortical volume, thickness, or cell density that may be anticipated from studies in rat cortex to be on the order of around 10% (see Diamond, 1991) would also be unlikely to account for qualitatively different and proportionately larger effects on axon density observed. The further division of neurotransmitter systems by the relative effectiveness of estrogen replacement verses a combined treatment of estrogen plus progesterone in attenuating the effects of ovariectomy suggests that divergent

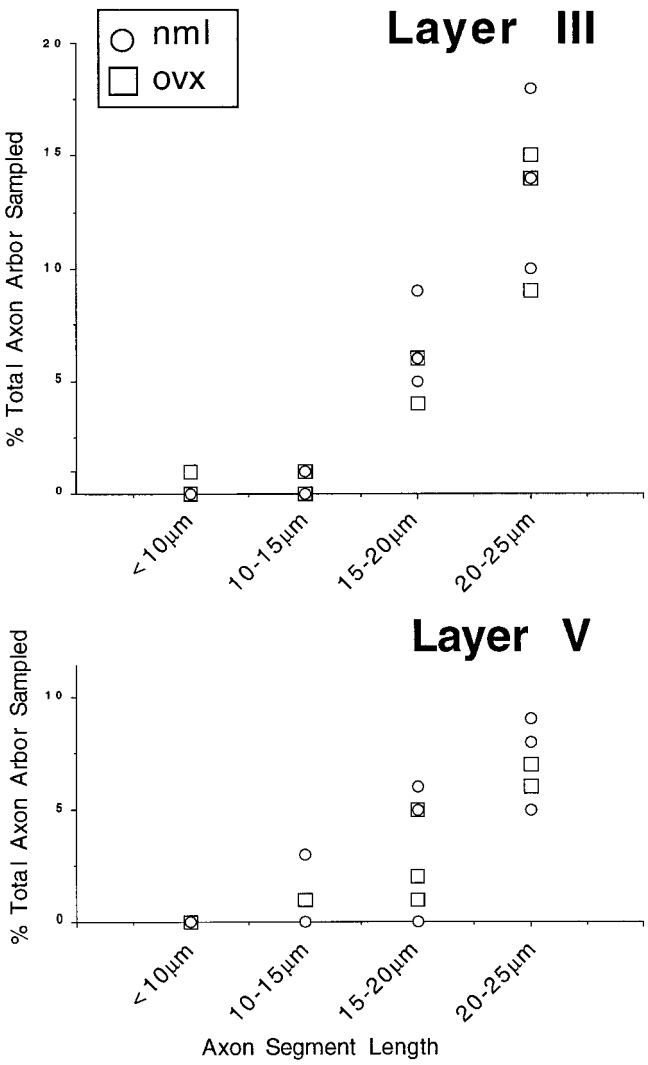

Fig. 9. Scatter plots of the percentage of choline-acetyltransferase (CHAT)- immunopositive axon arbor measured at a single focal plane that were less than 25 µm in two-dimensional length from layer III and layer V of the dorsolateral prefrontal cortex of ovariectomized (ovx) and control (nml) animals. These shortest axon segments include those oriented most steeply with respect to the plane of the section and which would be most foreshortened in two-dimensional drawings. For each plot, the points plotted are inclusive of all measures obtained from each of the three control animals (open circles), and the three ovariectomized animals(open squares) analyzed in this study. In both layers, the percentage of arbor corresponding to shortest axons segments was either similar for control and ovx animals, or was slightly higher in individual control cases. These data argue against a contribution of method-induced underestimation of fiber content in the lower mean densities of CHAT axons in ovx compared to control cases.

endocrine signaling pathways may underlie the complex effects on cortical immunoreactivity.

It has been previously suggested that the requirement for progesterone in stimulating prefrontal TH immunoreactivity may indicate that the changes in axon density observed are secondary to changes in circulating levels of substances such as prolactin or β-endorphin, which are also stimulated by estrogen plus progesterone but not by estrogen alone (e.g., Wardlow et al., 1982; Kohama et al., 1992; Kohama and Bethea, 1995; see Kritzer and Kohama, 1998). The present findings indicate that a similar argument may be applicable to ovarian hormone regulation of prefrontal 5-HT, but not to immunoreactivity for CHAT or DBH which are both as responsive to estrogen as they are to estrogen plus progesterone replacement. Recent findings concerning estrogen receptors in the cerebral cortex, and concerning ovarian hormone regulation of neurotrophic factors, however, may also be relevant to discussion of candidate mechanisms for ovarian hormone stimulation of cortical neurotransmitter systems. For example, until recently, genomic regulation mediated by intracellular ovarian hormone receptors at the level of the cerebral cortex could have been considered unlikely because immunocytochemical, mRNA, and/or receptor binding evidence all indicate that cortical estrogen and progestin receptors are nearly undetectable in the mature cerebrum (e.g., Warembourg et al., 1986; Blaustein et al., 1988; Miranda and Toran-Allerand, 1992). However, recent studies in adult rats have identified an abundance of cortical mRNA encoding for beta estrogen receptors (ER-β; Shughrue et al., 1997), suggesting in turn that estrogen receptor-mediated signaling pathways may persist in the adult cerebral cortex.

Other potential targets of genomic hormone action include the midbrain and brainstem cells of origin of the extrathalamic systems examined. In rats, for example, catecholamine neurons in the locus coeruleus (e.g., Heritage et al., 1977; Sar and Stumpf, 1981) and cholinergic cells in the basal forebrain concentrate ^{3}H-estradiol (e.g., Toran-Allerand et al., 1992), and in monkeys, immunocytochemistry for progestin receptors is colocalized with immunoreactivity for 5-HT in the raphae nuclei (Bethea, 1993). The mRNA encoding of ER-β has also been identified in the region of the locus coeruleus, raphae nuclei, and the nucleus Basalis of Meynert, although mRNA-containing cells were not chemically identified (Shughrue et al., 1997). This localization of intracellular hormone receptors within midbrain and brainstem cells of origin suggests that the changes in cortical immunoreactivity (that presumably reflect increases or decreases in intracellular antigen levels) could be an end result of hormone regulation of the transcription of transmitter synthesizing enzymes (e.g., Gibbs et al., 1994; Kohama and Bethea, 1995). However, the observed effects may also be consequences of hormone stimulation of neurotrophic factors. Ovariectomy in rats, for example, decreases cortical mRNA levels for both nerve growth factor (NGF, Gibbs et al., 1994) which promotes the survival of cholinergic afferents (e.g., Hefti, 1986), as well as mRNA for brain-derived neurotrophic factor (BDNF, Singh et al., 1995; Cavus and Duman, 1997), which stimulates subsets of cholinergic and midbrain dopaminergic neurons (e.g., Alderson et al., 1990; Hyman et al., 1991; Knusel et al., 1991), as well as cortical serotonin innervation (e.g., Suiciak et al., 1997). Interestingly, whereas estradiol treatment of ovariectomized animals stimulates NGF (Gibbs et al., 1994), as for the prefrontal cortical 5-HT axons examined in this study, this replacement regimen has limited restorative capacity for cortical BDNF mRNA levels (Singh et al., 1995).

The question remains as to whether and to what extent the observed changes in axon innervation reflect structural verses metabolic endpoints of hormone stimulation. Regardless of mechanism, however, ovarian hormone manipulation in adult rhesus monkeys seems to impose unique

sets of consequences for the density of cholinergic, noradrenergic, dopaminergic, and serotonergic innervation in the dorsolateral prefrontal cortex. Together, these transmitters provide an extrathalamic innervation for the frontal lobes that is essential for their healthy operation, and is repeatedly implicated in their dysfunction in disease. The complex patterns of the sensitivity of these four neurotransmitter systems to ovarian hormones observed in this study suggest that gonadal steroid stimulation may provide for differential and perhaps independent regulation of these important chemically identified prefrontal inputs. The positive effects of estrogen replacement therapies on cognitive performance in postmenopausal women (Kimura, 1995) and the reversible learning deficits demonstrated in ovariectomized rats concomitant with decreases in frontal cortical CHAT activity (Singh et al., 1994) indicate that there may indeed be functionally relevant consequences of this regulation. Accordingly, understanding how this is achieved could be important for the sex differences noted in normal prefrontal function and perhaps even the prefrontal dysfunction in disorders such as schizophrenia.

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LITERATURE CITED

Alderson RF, Alterman AL, Barde YA, Lindsey RM. 1990. Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron 5:297–306.

Araneda R, Andrade R. 1991. 5-Hydroxytryptamine 2 and 5-hydroxytryptamine 1A receptors mediate opposing responses on membrane excitability in rat association cortex. Neuroscience 40:399–412.

Arnsten AF. 1997. Catecholamine regulation of the prefrontal cortex. J Psychopharmacol 11:151–162.

Arnsten AFT, Goldman-Rakic PS. 1985. a2-Adrenergic mechanisms in prefrontal cortex associated with cognitive decline in aged nonhuman primates. Science 230:1273–1276.

Bachevalier J, Hagger C. 1991. Sex differences in the development of learning abilities in primates. Psychoneuroendocrinology 16:177–188.

Bartus RT, Dean RLI, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–417.

Battaner E, Del Casillo AR, Guerra M, Mas M. 1987. Gonadal influences on spinal cord and brain monoamines in male rats. Brain Res 425:391–394.

Berger B, Trottier S, Verney C, Gaspar P, Alvarez C. 1988. Regional and laminar distribution of the dopamine and serotonin innervation in the macaque cerebral cortex: a radioautographic study. J Comp Neurol 273:99–119.

Bernard BK, Paolino RM. 1974. Time-dependent changes in brain biogenic amine dynamics following castration in male rats. J Neurochem 22:951–956.

Bethea CL. 1993. Colocalization of progestin receptors with serotonin in raphe neurons of macaque. Neuroendocrinology 57:1–6.

Blaustein JD, King JC, Toft DO, Turcotte J. 1988. Immunocytochemical localization of estrogen-induced progestin receptors in guinea pig brain. Brain Res 474:1–15.

Brozoski TJ, Brown RM, Rosvold HE, Goldman PS. 1979. Cognitive deficit caused by regional depletion of dopamine in prefrontal cortex of rhesus monkey. Science 205:929–932.

Cavus I, Duman RS. 1997. Estradiol and a 5-HT2A agonist regulate the expression of BDNF mRNA in the hippocampus. Soc Neurosci Abstr 27: 661.

Clark AS, Goldman-Rakic PS. 1989. Gonadal hormones influence the emergence of cortical function in nonhuman primates. Behav Neurosci 103:1287–1295.

Diamond MC. 1991. Hormonal effects on the development of cerebral lateralization. Psychoneuroendocrinology 16:121–129.

Emson PC, Lindvall O. 1986. Neuroanatomical aspects of neurotransmitters affected in Alzheimer's disease. Br Med Bull 42:57–62.

Felten DL, Sladek JR Jr. 1983. Monoamine distribution in primate brain V. Monoaminergic nuclei: anatomy, pathways and local organization. Brain Res Bull 10:171–285.

Flor-Henry P. 1990. Influence of gender in schizophrenia as related to other psychopathological syndromes. Schizophrenia Bull 16:211–228.

Fuster J. 1989. The pre-frontal cortex: anatomy, physiology and neuropsychology of the frontal lobe. Raven Press: New York.

Fuxe K, Cintra A, Agnati LF, Harfstrand A, Wikstrom A-C, Okret S, Zoli M, Miller LS, Greene JL, Gustafsson J-A. 1987. Studies on the cellular localization and distribution of glucocorticoid receptor and estrogen receptor immunoreactivity in the central nervous system of the rat and their relationship to the monoaminergic and peptidergic neurons of the brain. J Steroid Biochem 27:159–170.

Gibbs RB, Wu D, Hersh LB, Pfaff DW. 1994. Effects of estrogen replacement on the relative levels of choline acetyltransferase, trkA, and nerve growth factor messenger RNAs in the basal forebrain and hippocampal formation of adult rats. Exp Neurol 129:70–80.

Glaser J, Russell VA, deVilliers AS, Searson JA, Taljaard JJ. 1990. Rat brain monoamine and serotonin S2 receptor changes during pregnancy. Neurochemical Res 15:949–956.

Goldberg E, Podell K. 1993. Sex differences in the lateralization of frontal lobe functions. Soc Neurosci Abstr 19:560.

Goldman-Rakic PS. 1987. Circuit basis of cognitive function in non-human primates. In: Iversen S, Strauss J editors. Cognitive neurochemistry. New York: Oxford University Press. p 91–110.

Halbreich U, Lumley LA. 1993. The multiple interactional biological processes that might lead to depression and gender differences in its appearance. J Affective Disorders 29:159–173.

Hefti F. 1986. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 6:21565–2162.

Heritage AS, Grant LD, Stumpf WE. 1977. 3H-estradiol in catecholamine neurons of rat brain stem: Combined localization by autoradiography and formaldehyde-induced fluorescence J Comp Neurol 176:607–630.

Hyman C, Hofer M, Barde Y, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM. 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230–232.

Kapur S, Remington G. 1996. Serotonin-dopamine interaction and its relevance to schizophrenia. Am J Psychiatry 153:466–476.

Kimura D 1995. Estrogen replacement therapy may protect against intellectual decline in postmenopausal women. Hormone Behav 29:312–321.

Kitt CA, Mitchell SJ, DeLong MR, Wainer BH, Price DL. 1987. Fiber pathways of basal forebrain cholinergic neurons in monkeys. Brain Res 406:192–206.

Kitt CA, Levey AI, Friedmont DP, Walker LC, Koliatsos VE, Raskin LS, Price DL. 1988. Immunocytochemical visualization of cholinergic fibers in monkey neocortex: enhanced visualization using silver nitrate. Abstr Soc Neurosci 14:631.

Knusel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, Hefti F. 1991. Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci USA 88:961–965.

Kohama SG, Bethea CL. 1995. Steroid regulation of tyrosine hydroxylase messenger ribonucleic acid in dopaminergic subpopulations of monkey hypothalamus. Endocrinology 136:1790–1800.

Kohama SG, Freesh F, Bethea CL. 1992. Immunocytochemical colocalization of hypothalamic progestin receptors and tyrosine hydroxylase in steroid-treated monkeys. Endocrinology 131:509–517.

Kritzer MF, Kohama SG. 1998. Ovarian hormones influence the morphology, distribution and density of tyrosine hydroxylase immunoreactive axons in the dorsolateral prefrontal cortex of adult rhesus monkeys. J Comp Neurol 395:1–17.

Levitt P, Rakic P, Goldman-Rakic PS. 1984. Region specific distribution of catecholamine afferents in primate cerebral cortex: a fluorescence histochemical analysis. J Comp Neurol 227:23–36.

Lewis DA. 1991. Distribution of choline acetyltransferase-immunoreactive axons in monkey frontal cortex. Neuroscience 40:363–374.

Lewis DA, Morrison JH. 1989. Noradrenergic innervation of monkey prefrontal cortex: a dopamine β-hydroxylase immunohistochemical study. J Comp Neurol 282:317–330.

Liskowsky DR, Potter LT. 1985. Muscarine-binding sites localized to cortical dopamine terminals. Neurosci Lett 58:229–233.

Luine VN. 1985. Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp Neurol 89:484–490.

Lynch MR. 1997. Selective effects in prefrontal cortex serotonin by dopamine D3 receptor agonism: interaction with low-dose haloperidol. Prog in Neuro-Psychopharm Biol Psychiatry 21:1141–1153.

Marchi M, Raiteri M. 1985. On the presence in the cerebral cortex of muscarinic receptor subtypes which differ in neuronal localization, function, and pharmacological properties. J Pharm Exp Ther 235:230–233.

Matsumoto M, Yoshioka M, Togashi H, Mori K, Ueno K, Saito H. 1998. Effects of idazoxan on dopamine release in the prefrontal cortex of freely moving rats. Eur J Pharm 343:165–170.

Maura G, Andriolo GC, Cavazzani P, Raiteri M. 1992. 5-Hydroxytryptamine 3 receptors sited on cholinergic axon terminals of human cerebral cortex mediate inhibition of acetylcholine release. J Neurochem 58:2334–2337.

Mesulam M-M, Van Hoesen GW. 1976. Acetylcholinesterase-rich projections from the basal forebrain of the rhesus monkey to neocortex. Brain Res 109:152–157.

Mesulam M-M, Mufson EJ, Levey AI, Wainer BH. 1983. Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata) and hypothalamus in the rhesus monkey. J Comp Neurol 214:170–197.

Miller JC. 1983. Sex differences in dopaminergic and cholinergic activity and function in the nigrostriatal system of the rat. Psychoneuroendocrinology 8:225–236.

Miranda RC, Toran-Allerand CD. 1992. Developmental expression of estrogen receptor mRNA in the rat cerebral cortex: a nonisotopic in situ hybridization histochemistry study. Cerebral Cortex 2:1–15.

Mrzljak L, Goldman-Rakic PS. 1993. Low-affinity nerve growth factor receptor ($p75^{ngfr}$) and choline acetyltransferase (ChAT)-immunoreactive axons in the cerebral cortex and hippocampus of adult macaque monkeys and humans. Cerebral Cortex 3:133–147.

O'Malley CA, Hautamaki RD, Kelley M, Meyer EM. 1987. Effects of ovariectomy and estradiol benzoate on high affinity choline uptake, Ach synthesis, and release from rat cerebral cortical synaptosomes. Brain Res 403:389–392.

Overman WH, Bachevalier J, Schumann E, Ryan P. 1996. Cognitive gender differences in very young children parallel biologically based cognitive gender differences in monkeys. Behav Neurosci 110:673–684.

Porrino LJ, Goldman-Rakic PS. 1982. Brainstem innervation of prefrontal and anterior cingulate cortex in the rhesus monkey revealed by retrograde transport of HRP. J Comp Neurol 205:63–76.

Richardson RT, DeLong MR. 1986. Nucleus basalis of Meynert neuronal activity during a delayed response task in monkey. Brain Res 399:364–368.

Rollema H, Lu Y, Schmidt AW, Zorn SH. 1997. Clozapine increases dopamine release in prefrontal cortex by 5-HT1A receptor activation. Eur J Pharm 338:R3–R5.

Sar M, Stumpf WE. 1981. Central noradrenergic neurons concentrate 3H-oestradiol. Nature 289:500–503.

Sawaguchi T, Goldman-Rakic PS. 1991. D1 dopamine receptors in prefrontal cortex: involvement in working memory. Science 251:947–950.

Sawaguchi T, Goldman-Rakic PS. 1994. The role of D1 dopamine in working memory: local injections of dopamine antagonists into the prefrontal cortex of rhesus monkeys performing and oculomotor delayed-response task. J Neurophysiol 71:515–528.

Seeman MV, Lang M. 1990. The role of estrogens in schizophrenia gender differences. Schizophrenia Bull 16:185–191.

Shughrue PJ, Lane MV, Mercanthaler I. 1997. Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 388:507–525.

Singh M, Meyer EM, Millard WJ, Simpkins JW. 1994. Ovarian steroid deprivation results in reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res 644:305–312.

Singh M, Meyer EM, Simpkins JW. 1995. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. Endocrinology 136:2320–2324.

Siuciak JA, Lewis D, Alexander CR, Bailey KM, Ryan TE, Lindsey RM, Wiegand SJ. 1997. Co-infusion with a TrkB-Fc fusion protein enhances BDNF-induced sprouting of serotonergic axons in rat brain. Soc Neurosci Abstr 27:60.

Smiley JF, Goldman-Rakic PS. 1996. Serotonergic axons in monkey prefrontal cerebral cortex synapse predominantly on interneurons as demonstrated by serial section electron microscopy. J Comp Neurol 367:431–443.

Sprangers SA, West NB, Brenner RM, Bethea CL. 1990. Regulation and localization of estrogen and progestin receptors in the pituitary of steroid-treated monkeys. Endocrinology 126:1113–1142.

Toran-Allerand CD, Miranda RC, Bentham WDL, Sohragji F, Brown TJ, Hochberg RB, MacLusky NJ. 1982. Estrogen receptors colocalize with low-affinity nerve growth factor receptors in cholinergic neurons of the basal forebrain. Proc Natl Acad Sci USA 89: 4668–4672.

Vaccari A. 1980. Sexual differentiation of monoamine neurotransmitters. In: Parvez H, Parvez S, editors. Biogenic amines in development. Amsterdam: Elsevier Press. p 327–352.

Wardlow SL, Wehrenberg WB, Ferin M, Antunes JL, Frantz AG. 1982. Effect of sex steroids on β-endorphin in hypophyseal portal blood. J Clin Endocrinol Metab 55:877–881.

Warembourg M, Logeat F, Milgrom E. 1986. Immunocytochemical localization of progesterone receptors in the guinea pig central nervous system. Brain Res 384:121–131.

Wilson MA, Molliver ME. 1991. The organization of serotonergic projections to cerebral cortex in primates: regional distribution of axon terminals. Neuroscience 44:537–553.