Perinatal Gonadectomy Exerts Regionally Selective, Lateralized Effects on the Density of Axons Immunoreactive for Tyrosine Hydroxylase in the Cerebral Cortex of Adult Male Rats

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The catecholamine innervation of the cerebral cortex is essential for its normal operations and is implicated in cortical dysfunction in mental illness. Previous studies in rats have shown that the maturational tempo of these afferents is highly responsive to changes in gonadal hormones. The present findings show that perinatal hormone manipulation also has striking, region- and hemisphere-specific consequences for cortical catecholamines in adulthood.

The effect of perinatal gonadectomy on catecholamines was examined in representative sensory, motor, and association cortices of adult male rats by combining hormone manipulation with immunocytochemistry for tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis. Qualitative and quantitative comparison of immunoreactivity in rats perinatally gonadectomized or sham-operated revealed complex changes in gonadectomized subjects; in cingulate cortex, TH immunoreactivity was strongly and bilaterally diminished, in sensory

and motor cortices, axon density was decreased in left hemispheres, but was minimally affected on the right, and in a premotor cortex, gonadectomy was without significant effect in either hemisphere. Corresponding analyses in gonadectomized rats supplemented with testosterone revealed a protective influence, albeit one in which TH immunoreactivity so showed regional and hemispheric variability in responsiveness to hormone replacement. These complex patterns of TH sensitivity suggest highly asymmetric hormone stimulation of cortical catecholamines. Such discriminative action may contribute to sex differences in the functional maturation and lateralization of the cortex and may also have bearing on disorders such as dyslexia, which show sexual dimorphisms, and in which functional laterality of the cortex may be particularly at issue.

Key words: dopamine; neocortex; estrogen; androgen; tyrosine hydroxylase; cerebral hemisphere

Gonadal hormones exert important influence on developing cortical functions (Beatty, 1979; Bachevalier and Hager, 1991). Interestingly, however, hormones show a remarkable degree of regional and hemispheric selectivity in their actions. In infant monkeys (Clark and Goldman-Rakic, 1989; Bachevalier et al., 1990) for example, testicular hormones speed maturation of working memory skills computed within orbital prefrontal cortex, but retard acquisition of habit formation: a functional province of inferotemporal cortex. Sexual dimorphisms in developmental disorders such as dyslexia and schizophrenia also suggest roles for hormones in the lateralization of cortical functions (Gur, 1979; Geschwind and Behan, 1982; Tallal, 1991), a possibility consistent with hormone effects on tail posture in rats, a function bearing homology to handedness in man (Rosen et al., 1983), and in the sex-specific patterns of functional lateralization that normally distinguish the cortices of men and women (McGlone, 1980).

Regional stimulation of cortical structures are likely substrates for such patterned hormone influence over cortical functioning. Hormone stimulation of cortical thickness, for example, differentially affects the left and right hemispheres and further discrim-

inates among functionally specialized cytoarchitectonic subdivisions (for review, see Diamond, 1991). Other examples of regional and hemispheric anisotropies in structural endpoints include hormone stimulation of cortical neuron dendrite structure (Kolb and Stewart, 1991), an important component of cortical circuit structure. However, catecholamine afferents are also essential for cortical operations (Brozoski et al., 1979; Stam et al., 1989; Wilcott and Xuemei, 1990) and are repeatedly implicated in the cortical dysfunction of disorders that also display sexual dimorphisms (Davis et al., 1991). The present study explored whether regionally selective stimulation of these afferent systems may be an additional means by which gonadal steroids differentially influence cortical function and/or functional laterality.

Previous studies in laboratory animals have shown that gonadal steroids influence catecholamine innervation in juveniles (Stewart et al., 1991; Stewart and Rajabi, 1994) and in adults (Battaner et al., 1987; Adler et al., 1998). It is uncertain, however, whether changes in gonadal hormones early in life have long-term consequences for cortical neurochemistry. Furthermore, regional specificity in hormone stimulation of cortical catecholamines has never been rigorously examined. Resolving these issues has relevance both for the development of normal cortical functions, some of which mature only after puberty (Goldman-Rakic, 1987), and for sexually dimorphic cortical deficits in developmental disorders such as schizophrenia and Tourette's syndrome, which can dramatically change, e.g., stabilize or worsen, at similar life stages (DeLisi et al., 1989; Kurlan, 1992). Accordingly, catecholamine innervation, identified immunohistochemically with antibodies

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Table 1. Mean weight (grams $\pm$ SD) of whole body, bulbocavernosus muscles, and percent body weight represented by bulbocavernosus muscle mass

	Body weight	Bulbocavernosus	% Body weight
CTRL	336 $\pm$ 16.2	1.11 $\pm$ 0.18	0.0033
GDX-SO	277 $\pm$ 8.29	0.19 $\pm$ 0.09*	0.0007*
GDX-TP	330 $\pm$ 14.75	1.18 $\pm$ 0.12	0.0035

The androgen-sensitive bulbocavernosus muscles in adult male rats shrink by >80% after perinatal gonadectomy (GDX-SO). Muscle mass is not significantly different from normal in gonadectomized animals supplemented with testosterone propionate (GDX-TP). The wasting and sparing of bulbocavernosus muscle mass is a proven measure of circulating testicular hormones, and the changes seen in this study across animal groups indicate the efficacy of gonadectomy in reducing and testosterone propionate treatment in maintaining circulating hormone levels.

*Significantly different from control, ANOVA followed by Dunnett two-tailed *post hoc* comparison, $p < 0.05$.

recognizing the synthetic enzyme tyrosine hydroxylase, was qualitatively and quantitatively compared in representative sensory, motor, and association cortices of adult male rats that had been gonadectomized, gonadectomized and supplemented with testosterone, or sham-operated at birth. The region-specific consequences of gonadectomy that were revealed suggests that highly asymmetric hormone influences normally stimulate cortical catecholamine innervation. Such differential influence over these functionally critical afferents could be relevant for the sex differences that characterize the maturation, functional lateralization, and disease-related vulnerability of specific subsets of cortical functions.

MATERIALS AND METHODS

Animals

Eighteen adult male Sprague Dawley rats were used. All procedures involving animals have approval from the Institutional Animal Care and Use Committee of State University of New York at Stony Brook and have been refined to minimize the use of animals and their discomfort. All rats used were bred from dams that had resided in the Stony Brook colony for at least 3 weeks before mating.

Each of the males used in this study was either gonadectomized or sham-operated (see below) within 8 hr of birth. Gonadectomized animals were divided into two groups: those injected daily with vehicle (sesame oil, GDX-SO), and those injected daily with 2 μ g/kg testosterone propionate (Sigma, St Louis, MO; adjusted weekly for body weight) suspended in sesame oil (GDX-TP). Sham-operated control animals (CTRL) were injected with sesame oil vehicle only. All animals were maintained in litters that included one or two female siblings until weaning at 21 d of age. Thereafter, animals were housed in cages containing two or three males, with food and water available *ad libitum* under a constant 12 hr light/dark cycle.

Surgeries

Gonadectomies were performed on rat pups within 8 hr of birth. Neonates were anesthetized using a combination of intraperitoneal injection of buprenorphin (0.0045 mg in sterile H₂O) and ether inhalation. Lidocaine solution was then applied to the abdomen, and a midline incision was made. The testes were removed bilaterally, and the incision was sutured. Sham operations consisted of administration of anesthesia, a midline incision, and suturing of the skin.

Euthanasia

After an 8 week survival, rats were deeply anesthetized with a mixture of ketamine (0.09 ml/100 gm) and xylazine (0.05 ml/100 gm) injected intramuscularly. After deep reflexes had disappeared, animals were transcardially perfused first with 50–100 ml of 0.1 M phosphate buffer (PB) and then with a series of two fixative solutions: 4% paraformaldehyde in 0.1 M PB, pH 6.0 (flow rate 30 ml/min, duration 5 min), followed by 4% paraformaldehyde, in 0.1 M borate buffer, pH 9.5 (flow rate 35 ml/min,

Figure 1. Schematic diagram showing a lateral view of the rat cerebral cortex (*top*) and a representative cross section (*bottom*) taken at the level of approximately the anteroposterior midpoint of the septal nucleus. In the coronal section, the locations of area Cg1 and Par 1 of Zilles (1990) and of areas AgM and AgL (Donoghue and Wise, 1982) are shown. Qualitative analyses were performed at representative rostrocaudal levels within each of these four areas. Quantitative measures were derived from sections matching the cross section depicted. In each section used for quantitative study, camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers were obtained from layer II/III and from layer V of all four areas. In total, these drawings subtended virtually the entire span of areas Cg1, AgM, and AgL present within the section; the *stippled area* offset with *dashed lines* within area Par1 illustrates the approximate location of the subportion of this region from which quantitative measures were obtained. *olf*, Olfactory bulb; *cc*, corpus callosum; *cd*, caudate nucleus.

duration 20 min). Brains were then removed, blocked, and cryoprotected in 0.1 M PB containing 30% sucrose before rapid freezing in powdered dry ice and storage at -80°C .

The androgen-sensitive medial, ventral, and lateral bulbocavernosus muscles were dissected out immediately after euthanasia and weighed. Mean muscle weights, whole body weights, and percent of whole body weight represented in the dissected muscle mass are presented in Table 1 for the three animal groups.

Immunocytochemistry

Blocked brains were secured to the stage of a freezing microtome and serially sectioned at a thickness of 40 μ m in the coronal plane. Left hemispheres were marked with subcortically placed sectioning artifacts made with a 16 gauge hypodermic needle. Sections were immunoreacted according to standard procedures. Initially, sections were rinsed in 0.1 M PB and then incubated in 1% H₂O₂ for 30 min. After further rinses in PB, sections were placed in 1% sodium borohydride in PB for an additional 30 min, and then rinsed in 50 mM Tris-buffered saline (TBS). Sections were next placed in a blocking solution [50 mM TBS containing 10% normal swine serum (NSS)] for 2 hr, and then incubated in one of two commercially available primary antibodies (2–3 d, diluted in TBS containing 1% NSS, 4°C). Anti-tyrosine hydroxylase antibodies (Chemicon, Temecula, CA; Eugene Tech International, Ridgefield Park, NJ) were used at working dilutions of 1:2000 and 1:500, respectively. After exposure to primary antibodies, the tissue sections were rinsed in TBS, incubated in biotinylated secondary antibodies (Vector Laboratories,

Figure 2. Representative camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers within the dorsal anterior cingulate cortex (area Cg1). The left (L) and right (R) hemispheres of a control (CTRL) animal, an animal gonadectomized on the day of birth (GDX-SO), and an animal gonadectomized at birth and supplemented with testosterone propionate (GDX-TP) are shown. The approximate borders of cortical layers are marked by the roman numerals appearing on the left. The immunostaining represented was obtained using a commercially available antibody purchased from Chemicon. Comparison across the animal groups represented illustrates the stark loss of axon density in both hemifields of the cingulate cortex of gonadectomized animals and the appearance of more normal levels of innervation in gonadectomized animals that received injections of testosterone propionate. In all animal groups, seemingly normal, layer-specific patterns of tyrosine hydroxylase-immunoreactive fiber orientation are preserved. *w.m.*, White matter.

Burlingame, CA; 2 hr, room temperature, working dilution 1:100), rinsed again in TBS, and then incubated in avidin–biotin–complexed horseradish peroxidase (ABC, Vector; 2 hr, room temperature). Sections were then rinsed thoroughly in Tris buffer, pH 7.6, and reacted using 0.07% 3,3'-diaminobenzidine (DAB) as chromagen.

Silver–gold intensification

The DAB reaction product was intensified according to methods of Kitt et al. (1988). Briefly, DAB-reacted, slide-mounted sections were incubated in 1% silver nitrate (pH 7.0, 45 min, 55°C), briefly rinsed in running distilled H₂O, and then incubated in 0.1% gold chloride (10 min, room temperature). Sections were then rinsed again in distilled H₂O, fixed in 5% sodium thiosulfate (10 min, room temperature), counterstained with 2% cresyl violet, and placed under coverslips.

Control experiments

The immunocytochemical labeling procedures above were performed on representative sections from hormonally intact and manipulated animals with omission of primary antiserum or secondary antibodies. Treated control sections were silver–gold-intensified side-by-side with normally immunoreacted slides.

Qualitative evaluation

Detailed examination of the laminar distribution, the course, approximate density, and the morphology of TH-immunoreactive axons was performed in representative sections throughout the rostrocaudal extent of dorsal anterior cingulate cortex (area Cg1), primary motor cortex (area AgL), primary somatosensory cortex (area Par1), and premotor cortex (area AgM) in each of the three groups of animals (Donoghue and

Wise, 1982; Zilles, 1990; Fig. 1). Two antibodies directed against TH were used in initial studies. At least two series of sections, immunoreacted on different days, was obtained from each animal used in this study.

Quantitative evaluation

A superior signal and signal-to-noise ratio was consistently obtained using the antibody from Chemicon, in the paraformaldehyde-fixed tissue. This prompted the decision to use this antibody exclusively for quantitative studies. Because subtle gradients in the density of catecholamine innervation in the rat cerebral cortex have been identified along major axes (anteroposterior, mediolateral; Van Eden et al., 1987), the decision was made to focus on a single anteroposterior level for quantitative evaluation. The level selected, lying at roughly the midseptal nucleus (Fig. 1), was chosen for its optimal representation of the four regions examined. For these studies, two to three tissue sections from this level were cut for all animals on the same day and immunoreacted as a group. Slides were coded before quantitative analyses, and all analyses were performed by a single observer (M.F.K.).

Quantitative analyses of fiber density and orientation were performed on camera lucida drawings made of immunoreactive fibers visualized under bright-field illumination using a 63× oil immersion objective. In each of the four areas examined, layers II/III and V were analyzed in left and right hemifields. Cresyl violet counterstaining was used to identify cortical regions and layers. Section thickness within the region to be evaluated was measured beforehand by roll-focusing from section surface-to-surface using the calibrated fine-focus of the microscope (Zeiss Axioskop).

Innervation density. Individual camera lucida drawings subtended widths of ~100–300 μm measured parallel to the cortical surface, a height dictated by the thickness of the layer, and a depth spanning the

AREA Cg1

Figure 3. Scatterplots of mean pixel density measures derived from camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers (Chemicon) in layers II/III and V of the left and right anterior dorsal cingulate hemifields of control (*CTRL*), gonadectomized (*GDX-SO*), and gonadectomized, testosterone propionate-supplemented (*GDX-TP*) animals. The points plotted (triangles) correspond to raw data points that are inclusive of all measures obtained from each of the six individual animals comprising each of the three experimental groups. Horizontal bars mark the group means of the pixel density measures. The decreases in axon density in *GDX-SO* animals are proportionately similar across layers and hemispheres, and values in both are significantly less than controls. Treatment of gonadectomized animals with testosterone propionate yields statistically normal innervation density in layer II, but produces axon density levels that fall between and are significantly different from *GDX-SO* and control values in layer V.

thickness of the tissue section. Three nonoverlapping drawings were obtained from each cortical layer, from each area of interest, from each animal. In total, the drawings from areas Cg1, AgM, and AgL subtended nearly the entire cytoarchitectonic field present in tissue sections at the level selected for analysis (Fig. 1). In area Par1, a specific subregion was defined for analysis by (1) an approximate 3 o'clock position within the hemisphere and (2) its radial alignment with a local maxima in the width and cell-packing density of layer IV. Other than these constraints, no attempts were made to preselect the location of drawings.

Camera lucida drawings were digitized as black and white photograph quality images (Hewlett Packard 4C Scanner, Desksan 2.0 software; Palo Alto, CA), and imported into NIH Image 5.8 software. Fiber density estimates were obtained from these imported images by first skeletonizing images to replace lines of varying width with ones of uniform thickness and then measuring mean pixel density (NIH Image 5.8). This automated measure is directly proportional to two-dimensional fiber length (Kritzer and Kohama, 1998).

Fiber orientation/trajectory. Fiber orientation relative to the cut surfaces of the tissue sections was quantitatively evaluated in the same sections used to analyze fiber density. For these analyses, separate sets of camera lucida drawings were made tracing portions of fiber segments that were in focus within single focal planes. At least 100 fiber segments were traced from each area, from each hemisphere, from each of two layers, in each individual animal. The two dimensional lengths of these segments were then measured using calibrated stereological software (MacStereology, Runturly Microsystems). Populations of length measurements were statistically compared among animal groups (Kolmogorov–Smirnov, nonparametric analysis). Mean lengths and the percentage of fibers <15 μ m in length in individual animals were also compared.

RESULTS

Efficacy of hormone treatments

The surgical procedures and injection schedules used in this study are identical to methods previously used to deplete and maintain physiological levels of gonadal hormones in rats (Sodersten, 1984). Their effectiveness was confirmed in this study by weighing the androgen-sensitive bulbocavernosus muscles, which are sensitive index to circulating testicular hormones (Wainman and Shipounoff, 1941; Collins et al., 1992). As anticipated (Collins et al., 1992), bulbocavernosus muscle mass in *GDX-SO* animals dropped to ~17% of controls (Table 1). Although total body weights were also lower than normal (~82% of normal), when expressed as a percent of total body mass, an 80% was still evident in bulbocavernosus mass in the gonadectomized group (Table 1). In contrast, total body weight, bulbocavernosus weight, and percent body weight represented in bulbocavernosus muscle was 98, 105, and 106% of normal, respectively, in gonadectomized animals supplemented with testosterone propionate (*GDX-TP*, Table 1). Statistical evaluation (ANOVA followed by Dunnett two-tailed *post hoc* comparisons; $p < 0.05$) revealed that all of the weights in *GDX-SO* animals and none of the weights in *GDX-TP* animals were significantly different from controls.

Figure 4. Representative bright-field photomicrographs illustrating the morphology and orientation of axons immunoreactive for tyrosine hydroxylase in layer II of the anterior dorsal cingulate cortex (area *Cg1*, *A–C*) and layer III of the primary motor cortex (area *AgL*, *D–F*) in controls (*CTRL*), gonadectomized rats (*GDX-SO*), and rats gonadectomized and supplemented with testosterone propionate (*GDX-TP*). All sections were immunoreacted for tyrosine hydroxylase (antibody from Chemicon) and are counterstained for Nissl substance with 1% cresyl violet. None of the cellular staining depicted in any of the panels corresponds to tyrosine-immunopositive somata. In both regions represented, the same features of axon morphology and orientation that characterize control animals are also found in the immunoreactivity of gonadectomized and gonadectomized, testosterone-supplemented animals; distinctive features such as the short, tortuous axons segments in layer II of the cingulate cortex (*A–C*), and the long radial fibers that are prominent in layer III of the primary motor cortex (*D–F*) are preserved across experimental groups. Clear decreases in the number of immunoreactive axon segments, however, readily distinguish the *GDX-SO* animal from control and *GDX-TP* cases. Scale bars: *A–C*, 100 μm ; *E, F*, 500 μm .

Specificity of immunostaining

The morphology, distribution, and the density of TH-immunoreactive axons was examined at representative rostrocaudal levels in the dorsal anterior cingulate cortex (area *Cg1*): an association area, the primary motor cortex (area *AgL*), a premotor cortical region (area *AgM*), and the primary somatosensory cortex (area *Par1*; Donoghue and Wise, 1982; Zilles, 1990; Fig. 1). Two commercially available anti-TH antisera were used, and both produced patterns of immunostaining in control animals (Fig. 2;

see Figs. 6, 8, 10) that were similar to previous immunocytochemical (Berger et al., 1985a; Van Eden et al., 1987; Papadopoulos et al., 1989) and radioautographic (Descarries et al., 1987) studies of cortical TH- and dopamine-containing axons in rats. Immunoreactive axons also displayed morphologies and orientations anticipated in previous studies (Berger et al., 1985a; Febvret et al., 1991; see Fig. 4*A, D*). These qualitative parallels support the specificity of immunostaining in this study for TH-containing fibers. This specificity was further supported by weak, nonpat-

Table 2. Fiber orientation was quantitatively evaluated by measuring two-dimensional fiber lengths within a single focal plane

	Left	Right
Cg1 layer II		
CTRL	1.03 ± 0.58	1.11 ± 0.62
GDX-SO	1.08 ± 0.68	1.00 ± 0.61
GDX-TP	1.20 ± 0.35	0.98 ± 0.49
Cg1 layer V		
CTRL	1.74 ± 0.99	1.66 ± 0.99
GDX-SO	1.35 ± 0.97	1.42 ± 0.92
GDX-TP	1.36 ± 0.70	1.72 ± 1.26
AgM layer III		
CTRL	2.23 ± 1.22	2.43 ± 1.88
GDX-SO	1.66 ± 1.22	2.02 ± 1.57
GDX-TP	1.99 ± 1.50*	1.99 ± 1.26**
AgM layer V		
CTRL	1.59 ± 0.87	2.02 ± 6.24
GDX-SO	1.52 ± 0.87**	1.67 ± 1.14
GDX-TP	1.48 ± 0.85	1.52 ± 0.87
AgL layer III		
CTRL	2.42 ± 1.77	2.38 ± 1.89
GDX-SO	1.96 ± 1.51**	2.06 ± 1.44**
GDX-TP	1.92 ± 1.46**	2.03 ± 1.47*
AgL layer V		
CTRL	1.73 ± 0.97	1.72 ± 0.98
GDX-SO	1.46 ± 0.88	1.66 ± 1.08
GDX-TP	1.52 ± 0.96	1.59 ± 0.92
Par1 layer II		
CTRL	2.15 ± 1.67	2.42 ± 2.00
GDX-SO	2.06 ± 1.57*	1.97 ± 1.37*
GDX-TP	1.84 ± 1.19	2.34 ± 6.32**
Par1 layer V		
CTRL	1.68 ± 0.93	1.74 ± 0.99
GDX-SO	1.54 ± 1.02	1.56 ± 1.19**
GDX-TP	1.51 ± 0.89	1.57 ± 0.82

Mean length ± SD from the left and right hemispheres is given for each layer for each of the three animal groups. Those axon segment populations that were statistically invariant (Kolmogorov-Smirnov nonparametric comparison, * $p < 0.05$; ** $p < 0.01$) are marked.

terned staining in control experiments in which primary or secondary antibodies were omitted. Although it has been argued that TH immunoreactivity has some selectivity for dopamine axons in rodent cortex (Berger et al., 1985b), this question of selectivity was not addressed in this study. Thus, although dopaminergic axons were undoubtedly in the majority in cingulate cortex (Lewis et al., 1979), the relative contributions of dopaminergic and noradrenergic axons to TH immunoreactivity was uncertain, particularly in lateral cortices where these inputs are more similarly dense (Levitt and Moore, 1978; Morrison et al., 1978).

TH immunocytochemistry in gonadectomized animals

Areas Cg1, AgM, AgL, and Par 1 each displayed expected patterns of dense to moderate TH immunoreactivity in control animals. In age-matched rats gonadectomized as neonates (GDX-SO), however, visual inspection alone revealed markedly abnormal patterns of catecholamine innervation. It was also evident that there were regional differences in the effects of gonadectomy on cortical catecholamine innervation among sensory, motor, and association areas. Gonadectomy either profoundly and symmetrically diminished TH innervation in the left and right hemi-

spheres, markedly decreased immunoreactivity in one cerebral hemisphere but not the other, or was without obvious effect in either hemisphere. These three outcomes, described separately below, occurred side-by-side in adjacent cytoarchitectonic cortical fields and were discernible in sections immunoreacted with each of the two anti-TH antisera used; supporting quantitative data were obtained using the antibody from Chemicon exclusively. A final subsection describes TH immunoreactivity in corresponding regions of neonatally gonadectomized animals that were supplemented with testosterone propionate (GDX-TP).

Hemispherically symmetric depletion of TH immunoreactivity: dorsal anterior cingulate cortex

Gonadectomy-induced decreases in catecholamine innervation were most striking in the dorsal anterior cingulate cortex (area Cg1; Fig. 2). In control animals, this region was densely labeled, with prominent bands of immunoreactivity standing out over layers Ia and II (Berger et al., 1985a, 1988; Febvret et al., 1991). In GDX-SO animals, however, TH immunoreactivity was clearly diminished in the left and right hemifields (Fig. 2). The dense tangle of immunoreactive fibers that normally mark the supragranular layers (see Fig. 4A), for example, was replaced in gonadectomized animals by a loose mesh of TH-immunoreactive processes (see Fig. 4B). Innervation was also diminished in the infragranular layers, where immunoreactivity dropped from the moderate levels in intact animals to sparse innervation in GDX-SO rats (Fig. 2). Quantitative evaluation of TH-immunoreactive axon density in representative supragranular (layer II) and infragranular (layer V) layers more precisely defined these effects (Fig. 3). First, in control animals baseline immunoreactivity was approximately twofold greater in supragranular compared with infragranular layers. Corresponding measures in GDX-SO rats revealed that effects of hormone manipulation were proportionate in these two strata. In the left hemisphere, mean TH axon density was 28.8 and 27.7% of normal in the supragranular and infragranular layers, respectively, and on the right, mean TH axon density dropped to 32.8% of normal in layer II and 38.2% of normal in layer V (Fig. 3).

The reliability of density estimates was verified in an ANOVA with a repeated measures design. This analysis (which included GDX-TP animals), identified significant main effects of treatment ($F_{(2,15)} = 31.36$; $p < 0.0001$) and layer ($F_{(1,15)} = 110.47$; $p < 0.0001$), the latter consistent with near the twofold differences in axon density in the supragranular and infragranular layers. However, individual animals were excluded ($p > 1.0$) as significant sources of variance in the data. Effects of hemisphere ($F_{(1,15)} = 0.24$; $p < 0.88$) and hemisphere-by-treatment interactions ($F_{(2,15)} = 2.29$; $p < 0.13$) were also nonsignificant, reinforcing the symmetry of the response of axons to hormone manipulation. Subsequent *post hoc* comparisons (Dunnett two-tailed; $p < 0.05$), confirmed that differences in axon density between GDX-SO and control animals were significant in both layers and hemispheres evaluated (Fig. 3).

The quantitative effects of neonatal gonadectomy took place in the absence of obvious morphological disturbance. Although clearly reduced in number, the TH-immunopositive axons of GDX-SO animals displayed to expected morphologies (Fig. 4B). Furthermore, TH-positive axons displayed normal patterns of layer-specific orientations, e.g., a predominance of horizontally running fibers in layer I and more randomly oriented, short axon segments in layers II/VI (Figs. 2, 4A,B). This second point of similarity was relevant for quantitative changes in axon density

Figure 5. Scatterplots of the percentages of axon arbor measured at a single focal plane that are $<15 \mu\text{m}$ in two-dimensional length in the supragranular and infragranular layers of cingulate (*CgI*), premotor (*AgM*), primary motor (*AgL*), and primary somatosensory (*ParI*) cortex. For each plot, points represent raw data inclusive of all measures obtained from each of the six control animals (large black squares), the six gonadectomized animals (small open squares), and the six gonadectomized animals supplemented with testosterone propionate (small open circles) analyzed in this study. The relative high percentage of short fiber segments in layer II of the cingulate cortex is consistent with its signature pattern of innervation by short, highly branched, randomly oriented axon segments. In all other regions and layers, shortest axon segments, which could include segments oriented steeply with respect to the plane of the tissue section and, thus, most significantly foreshortened in two-dimensional drawings, comprised $\sim 10\%$ or less of the total axon arbor sampled. Data collected for all three animal groups is largely overlapping. Area *AgM* represents an exception in which a disproportionate number of GDX-SO animals had more short axon segments than GDX-TP or control animals.

because the units for comparison, two-dimensional fiber length, are sensitive to fiber orientation. For example, an increase in the percentage of fibers coursing at steep angles with respect to the tissue section surface, which would be most attenuated in two-dimensional drawings, could lead to underestimation of fiber content. Although the qualitative similarities noted in fiber orientation among GDX-SO and control rats argue against such a confound (Figs. 2, 3A–C) for further confirmation, fiber orientation was quantitatively evaluated (see Materials and Methods). Although these analyses indicated that axon arbors sampled were not statistically invariant (Kolmogorov–Smirnov nonparametric comparison; $p < 0.01$), mean fiber lengths were similar among animal groups (Table 2). Equally important, the percentages of arbor sampled that corresponded to shortest axon segments ($<15 \mu\text{m}$) were overlapping for control and GDX-SO animals (Fig. 5). These findings argue against method-introduced foreshortening of axon measures in GDX-SO rats.

Unilateral depletion of TH immunoreactivity: primary motor and somatosensory cortices

The distribution and density of TH-immunoreactive axons in the primary motor (*AgL*) and somatosensory (*ParI*) cortices of control rats were similar to previous descriptions of cortical catecholamine innervation (Berger et al., 1985a; Febvre et al., 1991; Fig. 6; see Fig. 8). Both regions showed a moderate innervation, characterized by horizontally running processes in layer I, long, radially oriented fibers in the supragranular layers, and shorter, more randomly oriented axons in infragranular layers. In GDX-SO rats, this innervation was markedly reduced, but in a hemisphere-specific manner. Whereas axon density in the left hemispheres of GDX-SO animals was well below normal in the right motor and sensory hemifields, gross inspection suggested no

obvious decrement in immunoreactivity (Fig. 6; see Fig. 8). However, quantitative evaluation revealed small but consistent decreases in the density of TH immunoreactivity; in the right area *AgL*, axon density measures were consistently $\sim 80\%$ of normal (Fig. 7), and in area *ParI*, axon density in layers II/III and V represented 63 and 67% of normal, respectively (Figs. 8, 9). These declines were small compared with effects in the left hemisphere, where in the motor cortex, for example, TH innervation dropped on average to 38.8 and 30.9% of normal in the supragranular and infragranular layers, respectively (Fig. 7). Similarly, in the left somatosensory area, mean axon density in GDX-SO rats was 22.6 and 30.5% of normal in corresponding strata (Fig. 9). Thus, in sensory and motor areas, the effects of gonadectomy within a given hemisphere were proportionate for supragranular and infragranular layers but were remarkably different across hemispheres.

Axon density measures were statistically analyzed in ANOVAs; each identified main factors of treatment (motor: $F_{(2,15)} = 26.62$, $p < 0.0001$; sensory: $F_{(2,15)} = 46.04$, $p < 0.0001$), and hemisphere (motor: $F_{(1,15)} = 98.44$, $p < 0.0001$; sensory: $F_{(1,15)} = 16.89$, $p < 0.0001$) as significant contributors to variance in the data. Significant treatment-by-hemisphere interactions (motor: $F_{(2,15)} = 96.16$, $p < 0.0001$; sensory: $F_{(2,15)} = 16.07$, $p < 0.0001$) were also identified, consonant with the difference in the degree to which immunoreactivity was affected in left and right hemifields. Variation among individual animals, however, was not a significant source ($p > 1.0$) of variance in density measures. Subsequent *post hoc* comparisons (Dunnett two-tailed; $p < 0.05$) confirmed that large gonadectomy-induced axon density reductions on the left, as well as the more modest decreases in the right sensory and motor hemifields were significant in upper and lower cortical layers (Figs. 7, 9).

Figure 6. Representative camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers within the primary motor cortex (area AgL). The left (L) and right (R) hemispheres of a control (CTRL) animal, an animal gonadectomized on the day of birth (GDX-SO), and an animal gonadectomized at birth and supplemented with testosterone propionate (GDX-TP) are shown. The approximate borders of cortical layers are marked by the *roman numerals* appearing on the left. The immunostaining represented was obtained using a commercially available antibody purchased from Chemicon. Comparison across the animal groups represented illustrates the pronounced reduction in axon density in the left primary motor hemifield in gonadectomized animals, and the appearance of more normal levels of innervation in the right hemifield of gonadectomized animals, and in both hemispheres of gonadectomized animals that received injections of testosterone propionate. In all animal groups, seemingly normal, layer-specific patterns of tyrosine hydroxylase-immunoreactive fiber orientation are preserved in both hemispheres. *w.m.*, White matter.

Despite quantitative differences, axon morphology and orientation were preserved in GDX-SO rats (Figs. 4*E*, 6, 8). For example, the supragranular layers of the left and right sensory and motor hemifields were innervated mainly by long, radially oriented, moderately varicose axons (Figs. 4*D,E*, 6, 8). The similarities in orientation in particular suggested that gonadectomy-induced shifts in fiber orientation were unlikely to account for group differences in axon density. Quantitative analyses of fiber orientation reinforced this conclusion; axon arbor was either statistically indistinguishable from controls (Table 2), or frequency analysis of axon segment length revealed extensive overlap in the percentage of fibers corresponding to shortest axon segments among gonadectomized and control animals (Fig. 5). Finally, although one individual GDX-SO animal had some of the highest percentages of fibers measuring 15 μm or less, in no cases did shortest segments account for more than $\sim 10\%$ of total axon arbor. Thus, bias introduced in measurements as a function of fiber orientation could make at most a fractional contribution to the group differences in TH innervation observed.

Minimal effects on TH innervation: premotor cortex

In contrast to the other areas examined, TH immunoreactivity in the premotor cortex (area AgM) appeared normal in both hemispheres of GDX-SO animals (Fig. 10). Quantitative analyses also revealed extensive overlap in measures of axon density obtained in GDX-SO rats and controls (Fig. 11). However, a small but consistent decrease in TH innervation in GDX-SO animals relative to controls was revealed in which axon density varied between 84 (layer V, right hemisphere) and 88% (layer II/III, right hemisphere) of normal (Fig. 11). An initial ANOVA revealed a weak main effect of treatment ($F_{(2,15)} = 3.937$; $p < 0.0422$) but unambiguously excluded individual animals ($p > 1.0$) and hemispheres ($F_{(2,15)} = 2.09$; $p < 0.1687$) as significant sources of variance in the data. Subsequent *post hoc* comparisons revealed that axon density differences between control and GDX-SO rats were not significant (Dunnett two-tailed, $p < 0.05$; Fig. 11).

Quantitative analysis of fiber orientation revealed layers in which fiber populations in GDX-SO rats were statistically indistinguishable from controls (Table 2). For the others, however,

AREA AgL

Figure 7. Scatterplots of mean pixel density measures derived from camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers (Chemicon) in layers II/III and V of the left and right primary motor hemifields of control (*CTRL*), gonadectomized (*GDX-SO*), and gonadectomized, testosterone propionate-supplemented (*GDX-TP*) animals. The points plotted (triangles) correspond to raw data points that are inclusive of all measures obtained from each of six individual animals comprising the three experimental groups. Horizontal bars mark numerical group means of the pixel density measures. The large decreases in axon density in left motor hemifields of *GDX-SO* animals are proportionately similar across layers and are significantly less than controls in both layers II/III and V. More modest, albeit statistically significant, decreases in density mark the supragranular and infragranular layers of the right motor hemifield of *GDX-SO* animals. Treatment of gonadectomized animals with testosterone propionate yields statistically normal innervation density in layers II/III and V of the left primary motor cortex but has seemingly no effect on TH axon density in these layers on the right.

frequency analysis indicated that individual *GDX-SO* animals had among the highest proportions of short axon segments (Fig. 5). Although tempered by fact that shortest axon segments accounted for a small percentage of axon arbor overall, even a potentially modest method-related foreshortening of axon segments in *GDX-SO* cases should be kept in mind when considering the small, nonsignificant differences in axon density measures in these animals.

TH immunocytochemistry in gonadectomized animals after hormone replacement

To determine whether gonadectomy-induced changes in cortical TH innervation were sensitive to hormone replacement, corresponding analyses of immunoreactivity were performed in gonadectomized animals that were supplemented with testosterone propionate. In most cases, hormone replacement provided obvious protective effects against gonadectomy-induced decreases in TH immunoreactivity (Figs. 2, 4C,F, 6, 8, 10). Quantitative analyses, however, suggested subtle regional differences in the effectiveness of hormone replacement. In area Cg1, for example, axon densities in *GDX-TP* animals closely approximated control levels in layer II (99 and 93% of normal in the left and right hemifields, respectively), but in layer V axon density only reached ~65% of normal (Fig. 3). These intermediate values were significantly

different (ANOVA, Dunnett two-tailed *post hoc* comparison; $p < 0.05$) from both control and *GDX-SO* densities (Fig. 3).

In areas Par1 and AgL, differences in the effects of TP were also observed. In left hemifields, TH innervation in *GDX-TP* rats was between 78% of normal (layer II/III of area Par 1) and 93% of normal (layer V of area AgL) and statistically indistinguishable from controls (ANOVA, Dunnett two-tailed *post hoc* comparison; $p < 0.05$; Figs. 7, 9). On the right, however, axon densities in both sensory and motor cortices were overlapping with and statistically indistinguishable from measures obtained in *GDX-SO* animals (Figs. 7, 9). Testosterone treatment also showed minimal effect on TH immunoreactivity in area AgM, where axon densities of control, *GDX-SO*, and *GDX-TP* animals were all overlapping and statistically indistinguishable (ANOVA, Dunnett two-tailed *post hoc* comparison; $p < 0.05$; Fig. 11).

DISCUSSION

Sex differences and/or hormone modifiability in the maturation and lateralization of cortical functions (McGlone, 1980; Rosen et al., 1983; Clark and Goldman-Rakic, 1989; Bachevalier et al., 1990) and in the incidence of specific constellations of cortical deficits in sexually dimorphic developmental disorders, e.g., schizophrenia and dyslexia (Geschwind and Behan, 1982; Seeman

Figure 8. Representative camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers within the primary somatosensory cortex (*Par1*). The left (*L*) and right (*R*) hemispheres of a control (*CTRL*) animal, an animal gonadectomized on the day of birth (*GDX-SO*), and an animal gonadectomized at birth and supplemented with testosterone propionate (*GDX-TP*) are shown. The approximate borders of cortical layers are marked by the *roman numerals* appearing on the *left*. The immunostaining represented was obtained using a commercially available antibody purchased from Chemicon. Comparison across the animal groups represented illustrates a striking decrease in tyrosine hydroxylase-immunoreactive axon density in the left somatosensory hemifield in gonadectomized animals and normal appearing innervation in the right hemifield of gonadectomized animals, and in both hemispheres of gonadectomized animals that received testosterone propionate treatment. In all animal groups, qualitatively normal, layer-specific patterns of tyrosine hydroxylase-immunoreactive fiber orientation are preserved. *w.m.*, White matter.

and Lang, 1991; Tallal, 1991) indicate functionally relevant, selective patterns of hormone influence within the maturing cerebrum. This study revealed highly differentiated effects of perinatal gonadectomy on cortical catecholamine innervation, an innervation important for both cortical function and dysfunction (Stam et al., 1989; Wilcott and Xuemei, 1990; Davis et al., 1991). That these effects were attenuated by treatment with testosterone propionate suggested that changes in hormone levels were primarily responsible for the complex series of changes observed. This causal implication was strengthened by the fact that technical factors that could yield variability in axon quantification were minimized. For example, consistent preservation of TH antigens was maximized by holding parameters of fixation constant, consistency in immunostaining was optimized by reacting tissue across subjects in parallel, and regional sampling strategies were strictly defined to ensure comparison of corresponding cortical regions, subregions, and layers. Steps were also taken to control for error introduced in the method of axon quantification. First, section thickness was measured to verify that sampled areas were of comparable breadth. Furthermore, quantitative analysis of fiber orientation was performed to determine whether compression of information in two-dimensional drawings along the *z*-axis biased measures across groups; these studies revealed little evidence for treatment-induced shifts in the percentage of fibers that

would be most foreshortened in two-dimensional representations (Fig. 5). These elements of experimental design and analysis thus support the reliability of quantitative evaluation of TH immunoreactivity and reinforce conclusions that catecholamine axons in the adult rat cerebrum are profoundly sensitive to changes induced in the perinatal hormone environment. Comparative analyses further revealed that across cortical regions, catecholamine axons are differentially sensitive to hormone stimulation.

Of the four regions examined, TH innervation was most affected in the cingulate cortex, where axon density was strikingly and bilaterally diminished. In primary somatosensory and motor cortices, TH immunoreactivity was significantly depleted in the left hemisphere but was only modestly reduced on the right, the latter effects only being brought to light in quantitative analyses of axon density. In the premotor area, however, catecholamine afferents appeared qualitatively and quantitatively normal in gonadectomized animals, thus providing these studies with a fortuitous internal control.

There was also regional variation in the effectiveness of hormone replacement. Overall, those regions that were most affected by gonadectomy tended to be those most responsive to hormone replacement. In primary somatosensory and motor cortices, for example, testosterone treatment yielded values of axon density that were indistinguishable from normal in left hemifields but that

AREA Par 1

Figure 9. Scatterplots of mean pixel density measures obtained from camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers (Chemicon) in layers II/III and V of the left and right primary somatosensory hemifields of control (*CTRL*), gonadectomized (*GDX-SO*), and gonadectomized, testosterone propionate-supplemented (*GDX-TP*) animals. The points plotted (triangles) correspond to raw data points that are inclusive of all measures obtained from each of six individual animals from each of the three experimental groups. Horizontal bars mark the numerical group means of the pixel density measures. The large decreases in axon density in left somatosensory cortex in *GDX-SO* animals are proportionately similar in supragranular and infragranular layers, with both values being significantly different from controls. More modest, yet statistically significant, decreases in density also mark the supragranular and infragranular layers of the right somatosensory cortex in *GDX-SO* animals. Treatment of gonadectomized animals with testosterone propionate yields statistically normal innervation density in layers II/III and V of the left primary somatosensory cortex but has minimal effect on TH axon density in corresponding layers of the right somatosensory field.

were largely overlapping with those of gonadectomized animals on the right. This variable effectiveness may be related to differences between experimentally maintained and native hormone environments; whereas testosterone replacement yielded physiological levels of gonadal steroids, the sustained pulse delivered differed markedly from the stereotyped peaks and valleys in circulating hormone levels that normally bathe the postnatal male brain (Resko et al., 1968; Weisz and Ward, 1980). Accordingly, although hormone exposure alone seemed a sufficient stimulus in many regions, in others, e.g., layer V of cingulate cortex, the right sensory and motor hemifields, precise temporal patterning of hormone exposure may also be part of an adequate stimulus for catecholamine innervation. It is also possible, however, that some of the decline in TH innervation observed in *GDX-SO* animals occurred secondarily to removal of the testes but independently of gonadal steroid influence.

Possible mechanisms

Mechanisms governing the complex patterns of hormone stimulation observed in this study were not directly explored. However, some inferences can be drawn from the patterns of hormone sensitivity observed. For example, regional hormone sensitivity does not appear readily explained by factors such as the maturational state, functional specialization, or axon density of target cortices. Whereas maturational gradients for most features of cortical development occur along mediolateral or rostrocaudal

planes (Smart, 1984), there were no systematic changes in hormone sensitivity along either of these major axes. Functional specialization was also not consistently related to hormone sensitivity. The functionally disparate sensory and motor cortices, for example, showed similar responses to gonadectomy, whereas two more closely related regions, motor and premotor cortex, responded very differently to hormone manipulation. Finally, gonadectomy-induced changes in axon density were not simply more or less detectable in densely or lightly innervated regions, because the two most heavily innervated cortices examined, areas Cg1 and AgM, were most and least affected by gonadectomy, respectively.

The distribution of intracellular hormone receptors in the midbrain and brainstem, however, suggests links between genomic hormone action levied at the cells of origin of cortical catecholamine afferents and their regional patterns of hormone sensitivity. Not only do subsets of catecholamine neurons in the substantia nigra, ventral tegmentum, and locus coeruleus contain intracellular estrogen ($ER-\alpha$ and $ER-\beta$) and/or androgen receptors (AR) (Heritage et al., 1981; Kritzer, 1997a; Shughrue et al., 1997) but, among midbrain neurons, $ER-\alpha$, $ER-\beta$, and AR are most abundant in the ventral tegmental area (Kritzer, 1997a; Shughrue et al., 1997), the major source of catecholamine input to the highly hormone-sensitive cingulate cortex. The distribution of cortical $ER-\alpha$, on the other hand, may provide more intriguing

Figure 10. Representative camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers within the premotor cortex (area *AgM*). The left (*L*) and right (*R*) hemispheres of a control (*CTRL*) animal, an animal gonadectomized on the day of birth (*GDX-SO*), and an animal gonadectomized at birth and supplemented with testosterone propionate (*GDX-TP*) are shown. The approximate borders of cortical layers are marked by the *roman numerals* appearing on the *left*. The immunostaining represented was obtained using a commercially available antibody purchased from Chemicon. Comparison across the animal groups represented illustrates the insensitivity of tyrosine hydroxylase-immunoreactive axons to gonadectomy, with and without testosterone propionate supplementation, in both hemifields. In all animal groups normal, layer-specific patterns of tyrosine hydroxylase-immunoreactive fiber density, distribution, and orientation are preserved. *wm*, White matter.

parallels with the lateralized consequences of hormone manipulation. Thus, whereas levels of ER- α peak during the first week of postnatal life (Shughrue et al., 1990; Miranda and Toran-Allerand, 1992), in lateral cortex (including sensory and motor areas) this transient peak coincides with a near twofold right over left difference in the number of intracellular binding sites (Sandhu et al., 1986). Interestingly, a decline in receptor levels and concomitant disappearance of hemispheric differences in maximal binding during the first two weeks of life (Sandhu et al., 1986) subtends the timeline emerging in preliminary studies in rats for the effectiveness of perinatal gonadectomy to stimulate lateralized deficits in TH innervation in motor cortex (Kritzer, 1997b). However, in addition to the possibilities above, cortical androgen receptors, which are highly lateralized in the cortex of fetal monkeys (Scholl and Kim, 1990), cortical ER- β , which seems to remain elevated within the cerebrum throughout the lifespan (Shughrue et al., 1997), as well as nongenomic and even transneuronal routes of hormone stimulation must also be considered among candidate endocrine signaling pathways in the hormone sensitivity of cortical catecholamine innervation.

Comparison with previous studies

Few studies have examined hormone effects on cortical catecholamine systems. To date the only previous study pairing perinatal gonadectomy with neurotransmitter analysis in adulthood revealed no changes in dopamine, noradrenalin, or serotonin levels in HPLC analyses of cortical homogenates (Siddiqui and Shah, 1997). These findings seem incongruous with changes reported here in immunoreactivity for catecholamine-synthesizing enzymes. However, among methodological differences separating these studies, one that may be relevant to these seeming discrepancies is that cortical homogenates comprised virtually the entire cortical mantle (Siddiqui and Shah, 1997). In light of present findings suggesting regional hormone sensitivity, analyses conducted on pooled cortical regions may be an inappropriate comparison.

Previous studies have also examined hormone effects on the maturation of catecholamine innervation in juvenile animals (Stewart et al., 1991; Stewart and Rajabi, 1994) and on transmitter levels or enzyme immunoreactivity in adults (DuPont et al., 1981; Battaner et al., 1987; Adler et al., 1998; Kritzer and Kohama,

AREA AgM

Figure 11. Scatterplots of mean pixel density measures derived from camera lucida drawings of tyrosine hydroxylase-immunoreactive fibers (Chemicon) in layers II/III and V of the left and right premotor hemifields of control (*CTRL*), gonadectomized (*GDX-SO*), and gonadectomized, testosterone propionate-supplemented (*GDX-TP*) animals. The points plotted (triangles) correspond to raw data points that are inclusive of all measures obtained from each of six individual animals comprising the three experimental groups. Horizontal bars mark the numerical group means of the pixel density measures. Overall, measures of axon density in control, *GDX-SO*, and *GDX-TP* animals are overlapping, and none of the differences in group mean values among the groups are statistically significant.

1998). Interestingly, gonadectomy in adult males produces an increase either in transmitter levels (Battaner et al., 1987) or immunoreactivity for related synthetic enzymes (Adler et al., 1998), an effect opposite to the present findings. These findings, coupled with preliminary investigations in this laboratory of the early postnatal period (Kritzer, 1997b) suggest that the outcomes of gonadectomy on cortical catecholamines can be markedly different at different stages of the lifespan. This, in turn, suggests that features such as critical periods and differential activational and organizational effects that are tenets of hormone influence in reproductive and neuroendocrine brain centers (for review, see Breedlove and Arnold, 1983) may also apply to gonadal steroid stimulation of cortical catecholamine innervation. It is possible that the highly discriminating actions of gonadal steroids identified in this study on cortical catecholamine innervation hold relevance not only for normal cortical maturation and lateralization, but also for disorders such as schizophrenia and dyslexia that show sexual dimorphisms, and in which functional laterality of the cortex is particularly vulnerable. Accordingly, it may be especially pressing to establish details of the timing as well as the relative permanence or plasticity of the effects of gonadal hormone stimulation on cortical catecholamines in future studies.

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