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Administration of Tamoxifen but Not Flutamide to Hormonally Intact, Adult Male Rats Mimics the Effects of Short-Term Gonadectomy on the Catecholamine Innervation of the Cerebral Cortex

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ABSTRACT

Gonadectomy in adult male rats induces a series of changes in cortical catecholamine innervation that begins with a large, but transient decrease in the density of tyrosine hydroxylase- but not dopamine-β-hydroxylase-immunoreactive axons in sensory, motor, and association cortices. More recent studies have shown that estradiol maintains these presumed dopamine afferents but that supplementing acutely gonadectomized rats with dihydrotestosterone provides no protective effects for innervation. These findings suggest that the depression of mesocortical dopamine axons that follows gonadectomy is stimulated by changes in estrogen signaling. The studies presented here examined tyrosine hydroxylase and dopamine-β-hydroxylase innervation in hormonally intact adult male rats treated for 4 days with the nonsteroidal antiestrogen tamoxifen or with the nonsteroidal antiandrogen flutamide to probe for additional evidence for this selective hormone sensitivity and for insights into the intracellular mechanisms that may govern it. Qualitative and quantitative comparisons of innervation with corresponding data from control and acutely gonadectomized rats revealed that administration of the antiestrogen tamoxifen in hormonally intact rats produced deficits in catecholamine innervation that mirrored those induced by short-term gonadectomy. The antiandrogen flutamide, however, had no discernible impact on cortical afferents. When considered within the context of the known pharmacology and sites of action of tamoxifen, these findings not only provide additional support for an initial phase of selective estrogen sensitivity among the cortical catecholamines but also suggest that it is stimulation of intracellular estrogen receptors that confers this sensitivity in the adult rat cerebrum. J. Comp. Neurol. 431:444–459, 2001.

Indexing terms: dopamine; noradrenalin; antiestrogens; antiandrogens; prefrontal cortex

Gonadectomy in adult male rats produces a series of stereotyped changes in cortical catecholamine innervation that begins with a profound, albeit transient decrease in the density of tyrosine hydroxylase- (TH) but not dopamine-β-hydroxylase- (DBH) immunoreactive axons in sensory, motor, and association areas of the cerebrum (Kritzer et al., 1999; Kritzer, 2000). Although these presumed dopamine (DA) axons eventually and independently return to normal or even supranormal densities within a few weeks, hormone replacement is needed to prevent their initial gonadectomy-induced plummet (Kritzer et al., 1999). More recently, it has been shown that estradiol treatment in particular during this initial period is needed to maintain DA afferents effectively and

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that supplementing acutely gonadectomized rats with the nonaromatizable androgen dihydrotestosterone provides no protective effects for cortical innervation (Kritzer, 2000). These findings suggest that the initial depression of mesocortical DA afferents that follows gonadectomy is related principally to changes in estrogen signaling.

The studies presented here sought to extend these observations by examining the cortical catecholamine innervation in hormonally intact adult male rats treated with the nonsteroidal antiestrogen tamoxifen or with the nonsteroidal antiandrogen flutamide. Qualitative and quantitative analyses of axons immunoreactive for TH were carried out in representative sensory, motor, and association areas of the cerebral cortex and were compared with corresponding profiles in control and acutely gonadectomized rats to determine 1) whether deficits in innervation similar to those induced by acute (4-day) gonadectomy can be produced by short-term intracellular estrogen receptor blockade with tamoxifen; and 2) whether blocking intracellular androgen receptors for 4 days with flutamide has any discernible impact on cortical immunoreactivity. In both cases, DBH immunoreactivity was also analyzed to probe for hormone-independent effects of the antiestrogen or antiandrogen treatments on noradrenergic (NA) cortical afferents and to associate more definitively observed effects on TH immunoreactivity with changes in the DA and/or NA innervation of the adult rat cerebrum. In addition to providing insights into the intracellular mechanisms involved in gonadal hormone regulation of cortical catecholamines, these findings may also indicate end points in the central nervous system (CNS) that may need to be considered in relation to the therapeutic use of antiestrogens and antiandrogens in treating certain types of cancers.

MATERIALS AND METHODS

Animal subjects

Fifty adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used; ten of these animals (five controls; five gonadectomized rats) served as subjects in a previous study of cortical TH and DBH innervation (Kritzer, 2000). All procedures involving animals were approved by the Institutional Animal Care and Use Committee, SUNY at Stony Brook, and minimized both the use of animals and their discomfort.

All rats were housed with food and water freely available under a 12-hour light/12-hour dark cycle. Twenty-five animals were sham-operated; five of these were implanted with pellets that released biodegradable matrix, i.e., placebo, and served as controls (CTRL); ten were implanted with pellets that released tamoxifen (TAM); and five were implanted with pellets that released flutamide (FLU). These 20 animals were sacrificed 4 days after surgery. The remaining five sham-operated animals were implanted with pellets releasing tamoxifen and were sacrificed 7 days after surgery. Twenty-five additional rats were gonadectomized and implanted with pellets containing either biodegradable matrix, i.e., placebo (GDX) or tamoxifen (GDX+TAM). Ten rats from the GDX group and five rats from the GDX+TAM group were sacrificed 4 days after surgery; an additional five GDX and five GDX+TAM animals were also sacrificed 7 days after surgery.

TABLE 1. Mean Weight in Grams (± Standard Error of the Mean) of the Whole Body and Dissected Bulbocavernosus (BCN) Muscles, and the Percent of Whole Body Weight Represented by the Dissected Muscle Mass (BCN/Body)[1]

Animals (n = 5)	Body weight	BCN weight	BCN/body
Control[2]	258.4 ± 6.21	0.882 ± 0.052	.0034
4-day			
GDX[2]	258.1 ± 4.24	0.680 ± 0.020*	.0026*
TAM	250.3 ± 3.873	0.750 ± 0.018	.0030
FLU	262.0 ± 11.13	0.612 ± 0.018*	.0023*
GDX	236.3 ± 4.58*	0.496 ± 0.014*	.0021
TAM	250.5 ± 5.71	0.858 ± 0.057*	.0034
GDX + TAM	227.5 ± 5.18*	0.498 ± 0.38*	.0022*
7-day			
GDX	230.0 ± 3.16*	0.484 ± 0.027*	.0021*
TAM	244.1 ± 1.00	0.894 ± 0.058	.0037
GDX + TAM	228.3 ± 3.932*	0.472 ± 0.046*	.0020*

[1]The androgen-sensitive bulbocavernosus muscles showed expected decreases in acutely gonadectomized (4-day GDX, 7-day GDX) rats that are matched by decreases in muscle mass in animals treated for 4 days with flutamide (4-day FLU), or that were gonadectomized and tamoxifen-treated for 4 (4-day GDX + TAM) or 7 (7-day GDX + TAM) days. Corresponding measures obtained in intact animals that were treated with tamoxifen for 4 (4-day TAM) or 7 days (7-day TAM) lay within the normal weight range seen in the controls.
[2]Animal groups used as subjects in Kritzer (2000).
*Significantly different from control, ANOVA, followed by Student-Newman-Keuls, $P < 0.05$.

Surgical procedures

Surgical procedures were performed under aseptic conditions and a mixture of ketamine (0.09 ml/100 g) and xylazine (0.05 ml/100 g) to achieve appropriate levels of anesthesia. For both gonadectomies and sham operations, the sac of the scrotum and underlying tunica were incised. For the gonadectomies, the vas deferens was also bilaterally ligated and the testes were removed. Following both the gonadectomy and sham procedures, slow-release pellets were implanted within the scrotal sac (see below), and the surgical wounds were closed by using 6-0 silk sutures.

Hormone treatments

All rats were implanted with slow release pellets that contained either flutamide or tamoxifen in biodegradable matrix, or a placebo containing only the biodegradable matrix (cholesterol, microcrystalline cellulose, α-lactose, di- and tri-calcium phosphate, calcium and magnesium stearate, and stearic acid; Innovative Research of America, Toledo, OH). The tamoxifen-containing pellets released 0.2 mg of TAM per day, and the flutamide-containing pellets released 4.0 mg FLU per day.

Euthanasia

Four or 7 days after surgery, rats were weighed (Table 1) and then deeply anesthetized via intramuscular injection of ketamine (0.09 ml/100 g) and xylazine (0.05 ml/100 g). After corneal reflexes had disappeared, the chest cavity was opened, and the heart was injected with heparin (0.2 ml of a 1000-U/ml solution). The rats were then transcardially perfused with 50–100 ml of a 0.1 M phosphate-buffered saline (PBS, pH 7.4) flush followed by 4% paraformaldehyde in 0.1 M PB, pH 6.5 (flow rate 30 ml/min, duration 5 minutes), and then 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5 (flow rate 35 ml/min, duration 20 minutes). These fixation parameters were adhered to closely across animals to maximize consistent antigen preservation. Immediately after perfusion, the brains were removed, blocked, and cryoprotected in 0.1 M PB containing 30% sucrose. The bulbocavernosus muscles

were also dissected out and weighed at this time (Table 1). After the brain blocks had sunk in the sucrose solution, they were rapidly frozen in powdered dry ice and stored at −80°C prior to sectioning and immunocytochemical processing.

Immunocytochemistry

Tissue blocks that included tissue from the rostral caudate to the caudal septal nucleus were marked with subcortically placed artifacts in the left hemisphere and coronally sectioned on a freezing microtome (40 μm). Separate rostrocaudal series of sections from each animal were then immunoreacted by using antibodies recognizing either TH; Chemicon, Temecula, CA) or DBH; Protos Biotech, New York, NY). For these procedures, sections were first rinsed in 0.1 M PB, incubated in 1% H_2O_{22} (45 minutes) and then treated with 1% sodium borohydride in PB (45 minutes). After rinses in 50 mM Tri- buffered saline (TBS), pH 7.4, the sections were placed in blocking solution [50 mM TBS containing 10% normal swine serum (NSS)] for 2 hours, prior to incubation in anti-TH antibodies (2–3 days, diluted 1:1000 in TBS containing 1% NSS, 4°C) or anti-DBH antibodies (2–3 days, diluted 1:100 in TBS containing 1% NSS and 0.3% Triton X-100, 4°C). The tissue sections were then rinsed in TBS, incubated in biotinylated secondary antibodies (Vector, Burlingame, CA; 2 hours, room temperature, working dilution 1:50), rinsed again in TBS, and then placed in an avidin-biotin-complexed horseradish peroxidase solution (ABC, Vector; 2 hours, room temperature). After this step, sections were rinsed in Tris buffer, pH 7.6, and reacted by using 0.07% 3,3′-diaminobenzidine (DAB) as chromagen.

For control studies, immunocytochemical labeling was performed as above on representative sections with the omission of primary antisera or secondary antibodies. The specificity of immunolabeling for TH and for DBH was also supported by comparing patterns of cortical labeling in hormonally intact animals with descriptions documented in previous studies of cortical catecholamine innervation in rats (e.g., Morrison et al., 1978; Levitt and Moore, 1978; Lewis et al., 1979; Berger et al., 1985; Van Eden et al., 1987; see Results).

Silver/gold intensification

The DAB immunoreaction product was intensified by using the methods of Kitt et al. (1988). For this procedure, DAB-reacted, slide-mounted sections were first incubated in 1% silver nitrate (pH 7.0, 50 minutes, 55°C, in the dark), rinsed in running distilled H_2O, and then incubated in 0.2% gold chloride (15 minutes, room temperature, in the dark). Sections were then rinsed again in running distilled H_20, and fixed in a 5% sodium thiosulfate solution (10 minutes, room temperature). The silver/gold-intensified sections were then counterstained with 0.1% cresyl violet and placed under coverslips. Control sections (above) were intensified side by side with normally immunoreacted slides.

Qualitative evaluation

The laminar distribution, orientation, approximate density, and morphology of TH- and DBH-immunoreactive axons were assessed in animals of each experimental group from representative sections throughout the rostrocaudal extent of the left and right hemifields of the supragenual anterior dorsal cingulate cortex (area Cg1), the premotor cortex (area AGm), the primary motor cortex (area AGl), and the primary somatosensory cortex (area Par 1; Zilles, 1990; Donoghue and Wise, 1982). At least two series of sections (immunoreacted on different days) from each animal for each antigen were used for these evaluations.

Quantitative evaluation

Innervation in two layers (II/III and V) and a single anteroposterior cortical level, roughly transecting the midseptal nucleus, in areas Cg1 and AGl was examined for quantitative analyses. All the tissue sections used for these studies were prepared from all animals on the same day and were immunoreacted (by antigen) as single groups. Slides were coded prior to analysis, and a single observer performed all quantitative analyses for a given antigen and a given region (TH, DBH of area Cg1 for control, GDX, TAM, and FLU groups by M.F.K.; TH of areas Cg1 and AGl for 4-and 7-day GDX, TAM, and GDX+TAM cases by M.F.K.; TH, DBH for area AGl of control, GDX, TAM, and FLU groups by I.P.).

Quantitative estimates of fiber density were obtained from camera lucida drawings of immunoreactive fibers visualized under brightfield illumination by using a 63× oil immersion objective; section thickness was measured in the regions evaluated by roll-focusing from surface t -surface by using the calibrated fine-focus of the microscope (Zeiss Axioskop) to confirm uniformity in section breadth. In total, individual drawings subtended regions of approximately 300−600 μm across (measured parallel to the pial surface), heights that were dictated by layer thickness, and depths that assumed the thickness of the section. For every animal, layer, and hemisphere evaluated (for each antibody), two separate drawings were evaluated. Although areas that had been frankly damaged during processing were avoided, no other attempts were made to preselect the locations for these drawings within cytoarchitectonically identified cortical regions and layers. These drawing were then scanned (DeskScan 2.0) as digitized, binary images of black and white pixels (black and white drawings). Measures of mean pixel density were derived from these images following "skeletinization" (NIH Image 1.58) to yield fiber density estimates that are directly proportional to measures of two-dimensional line length (Kritzer and Kohama, 1998).

Statistical analysis

The sample sizes of all data sets analyzed in this study were equal; there were equal numbers of animal subjects for analyses of body and bulbocavernosus weights, and an equal number of drawings were obtained per layer, per hemisphere, per animal, and per antibody for mean pixel density measures. Descriptive statistical analyses were performed on each data set (Stat View 4.5) to evaluate sample distribution, mean, and variance. Measures of body and bulbocavernosus muscle weights were compared across groups by using analyses of variance (ANOVA), followed by Student-Newman-Keuls post hoc comparisons where indicated (Super ANOVA 1.11). Measures of mean axon density (mean pixel density) were compared across animal groups on a per-region basis by using ANOVAs with repeated measures designs (Super ANOVA 1.11). In both cases, hormone treatment was treated as an independent, between-group variable; weight and TH axon densities were entered as dependent or repeated within-group

variables, respectively. In addition, the individual factors included in these analyses were animal subjects for tests of both weights and TH innervation, and cortical hemisphere, cortical layer, and the two data points (drawings) obtained per layer for analyses of TH axon density. All ANOVAs were followed by Student-Newman-Keuls post-hoc comparisons where indicated.

RESULTS

Identification of control and gonadectomized animal subjects

Previous studies have identified very different effects of acute versus chronic gonadectomy on TH but not DBH innervation in association and sensorimotor cortices (Adler et al., 1999; Kritzer, 2000). This study delves further into the former, acute effects by describing data collected in hormonally intact adult male rats implanted for 4 days with pellets releasing either the antiestrogen tamoxifen (TAM) or the antiandrogen, flutamide (FLU). Tissue from five animals implanted with FLU and five rats implanted with TAM were prepared in parallel, from surgery to immunolabeling, with that from acutely operated control and GDX animals that served as subjects in an earlier investigation (Kritzer, 2000). The TH and DBH axon density values from these control and GDX groups are presented here in parallel with data from FLU and TAM rats for purposes of comparison, but they have been published elsewhere (Kritzer, 2000). The data from additional cohorts of acutely GDX rats (4-day and 7-day operated animals) that were prepared in parallel with separate sets of hormonally intact rats treated with TAM (4-day and 7-day treatments) and with rats that were both gonadectomized and treated with TAM (4-day and 7-day groups) are presented here for the first time.

Treatment efficacy

The doses of TAM and FLU that were released per day were similar to those that have been successfully used in previous studies to block estrogen- or androgen-sensitive end points in the CNS, respectively (e.g., Dohler et al., 1986; Fitch et al., 1991). Additional measures of their effectiveness and hormone selectivity also come from analyses of the weights of the androgen-sensitive bulbocavernosus muscles in our animals. After only 4 days of hormone deprivation (gonadectomy), these muscles shrink considerably in size and become disproportionately small relative to total body mass (Collins et al., 1992; Adler et al., 1999; Kritzer et al., 1999). In the FLU rats, average bulbocavernosus muscle mass, in grams ± standard error (0.61 ± 0.018), almost exactly matched that of the gonadectomized cohorts (0.65 ± 0.018), and in both FLU and GDX groups, these muscles represented an average of about 0.2% of total body mass (Table 1). In contrast, the average weights of these muscles in the TAM group (0.75 ± 0.018) overlapped with controls (0.882 ± 0.052) and represented approximately 0.3% of total body weight (Table 1). These data thus indicate that flutamide was as effective as gonadectomy in blocking intracellular androgen receptor stimulation and that tamoxifen did not appreciably impinge on this signaling pathway.

Effects of acute treatment with tamoxifen or flutamide on cortical DBH innervation

The distribution of DBH-immunoreactive afferents has been previously described for sensory, motor, and association areas of the adult rat cerebrum (e.g., Morrison et al., 1978; Levitt and Moore, 1978; Lewis et al., 1979). Similar features marked the cortical DBH immunostaining obtained in both the TAM and FLU animals of this study. In both groups of treated animals, for example, immunoreactive axons were modestly dense in sensorimotor fields, where thin but heavily beaded fibers ran mainly parallel to the pial surface in layer I, perpendicular to cortical lamination in layers II/III, and coursed both radially and tangentially in layers V/VI (Fig. 1). Thus, neither short-term antiestrogen and antiandrogen administration produced any obvious, qualitative effects on DBH innervation. Quantitative analyses of axon density in layers II/III and V of the left and right cingulate and primary motor hemifields (Fig. 2) provided additional evidence for similarities in DBH innervation across animal groups. Specifically, average axon density measures in the FLU and TAM cohorts all lay within 20% of corresponding measures of controls, with half of all measures (8 of 16) lying within 5% of controls, and another quarter (4 of 16) falling between 5% and 10% of normal. Separate ANOVAs (see Materials and Methods) performed on data collected from cingulate and motor cortical areas that were inclusive of density measures from all four animal groups (CTRL, GDX, TAM, FLU) also failed to identify significant main effects of hormone treatment ($P > 1.0$ for both regions) on DBH innervation for either region and ruled out individual animals as significant sources of variance in the data ($P > 1.0$ for both regions).

Effects of acute treatment with tamoxifen or flutamide on cortical TH innervation

In hormonally intact rats, immunolabeling for TH corresponds to region-specific patterns of axon labeling that distinguish the sensory, motor and association cortices (e.g., Berger et al., 1985; Van Eden et al., 1987). Axons showing morphologies, orientations, and densities that corresponded closely to these normal patterns of innervation were also seen in the cerebral cortices of the hormonally intact rats treated with FLU (Figs. 3A,C, 4A,C, 5A,C). However, in the TAM rats even cursory inspection revealed conspicuous decreases in TH immunoreactivity in the cingulate, primary motor, and primary sensory areas. In the supragranular layers of all three areas, for example, sparse populations of relatively isolated immunoreactive fibers took the place of what would normally be enriched axonal plexuses (Figs. 3A,B,D, 5A,B,D). There was a notable exception, however, in area AGm, where TH immunoreactivity in both the FLU and TAM cohorts appeared to be intact (Fig. 4A,B,D).

Assessments of axon density were made in representative cortical regions in TAM and FLU animals. Comparison of these measures with densities previously obtained in GDX and control animals provided further evidence for similarities between control and FLU rats and striking parallels in the innervation of GDX and TAM rats (Fig. 6). In the FLU-treated animals, for example, average axon densities measured in area AGl ranged between roughly 84% (layer V, left hemisphere) and 110% (layer II/III, left hemisphere) of controls (Fig 6, top panel) and between

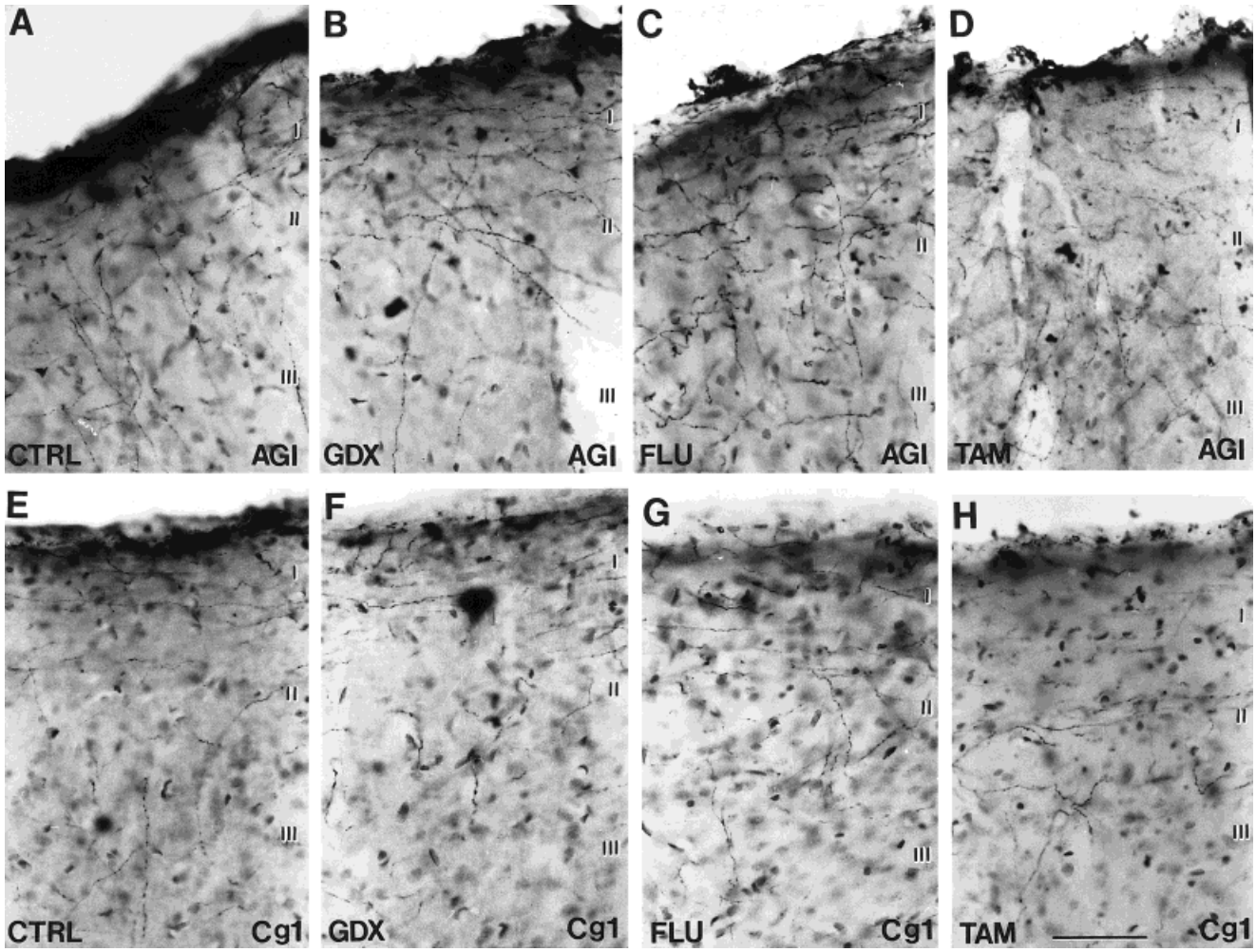

Fig. 1. Representative brightfield photomicrographs showing the morphology, orientation, and density of axons immunoreactive for dopamine-β-hydroxylase in layers I–III of the primary motor cortex (AGl, **A–D**) and the dorsal anterior cingulate cortex (Cg1, **E–H**) in sham-operated control rats (CTRL; A,E), in acutely (4-day) gonadectomized rats (GDX; B,F) and in hormonally intact rats given flutamide (FLU; C,G) or tamoxifen (TAM; D,H) for 4 days. All the sections shown are counterstained with 1% cresyl violet to reveal cellular architecture; none of the cells visible in any of the panels are immunoreactive for dopamine-β-hydroxylase. This figure illustrates that patterns of immunoreactivity typical of hormonally intact (CTRL) animals are also present in both area AGl (A–D) and area Cg1 (E–H) in intact animals treated with FLU (C,G) or with TAM (D,H). The illustration of the preservation of dopamine-β-hydroxylase immunoreactivity in acutely gonadectomized rats (B,F) is also included for comparison. Scale bar = 100 μm in H (applies to A–H).

approximately 89% (layer II/III, right hemisphere) and 114% (layer V, right hemisphere) of corresponding measures of the sham operated rats in area Cg1 (Fig. 6, bottom panel). Quantitative analyses in the TAM rats, on the other hand, revealed levels of innervation that more closely matched axon densities measured in GDX animals. In area AGl, for example, innervation in the supragranular layers of GDX animals was approximately 20% of normal in the left hemisphere, and 16% of control on the right, whereas corresponding values in TAM rats were 28% and 17% of normal, respectively (Fig. 6, top panel).

Interestingly, in comparison with the supragranular layers, TAM administration was less effective than GDX in depleting TH innervation in infragranular laminae. For example, in area AGl of GDX rats, average TH innervation density in layer V was reduced to about 17% and 19% of normal on the left and right, respectively, whereas corresponding measures in TAM rats were 37% and 32% of controls (Fig. 6, top panel). In area Cg1, average infragranular innervation dropped to 33% and 40% of control on the left and right in GDX rats, and to 46% and 60% of controls on the left and right, respectively, in the TAM animals (Fig. 6, bottom panel). Nonetheless, statistical analyses indicated that none of the axon density values in GDX and TAM rats were significantly different from one another in either cortical region, hemisphere, or layer evaluated. Thus, ANOVAs (see Materials and Methods) that separately compared results from cingulate and motor cortices in control, GDX, TAM, and FLU subjects both identified significant main effects of treatment (motor, $F_{3,16} = 19.88$, $P < 0.0001$: cingulate $F_{3,16} = 26.85$, $P < 0.0001$). Analyses in cingulate cortices additionally identified a significant main effect of cortical layer ($F_{1,3} = 148.30$, $P < 0.0001$) and a significant layer-by-treatment

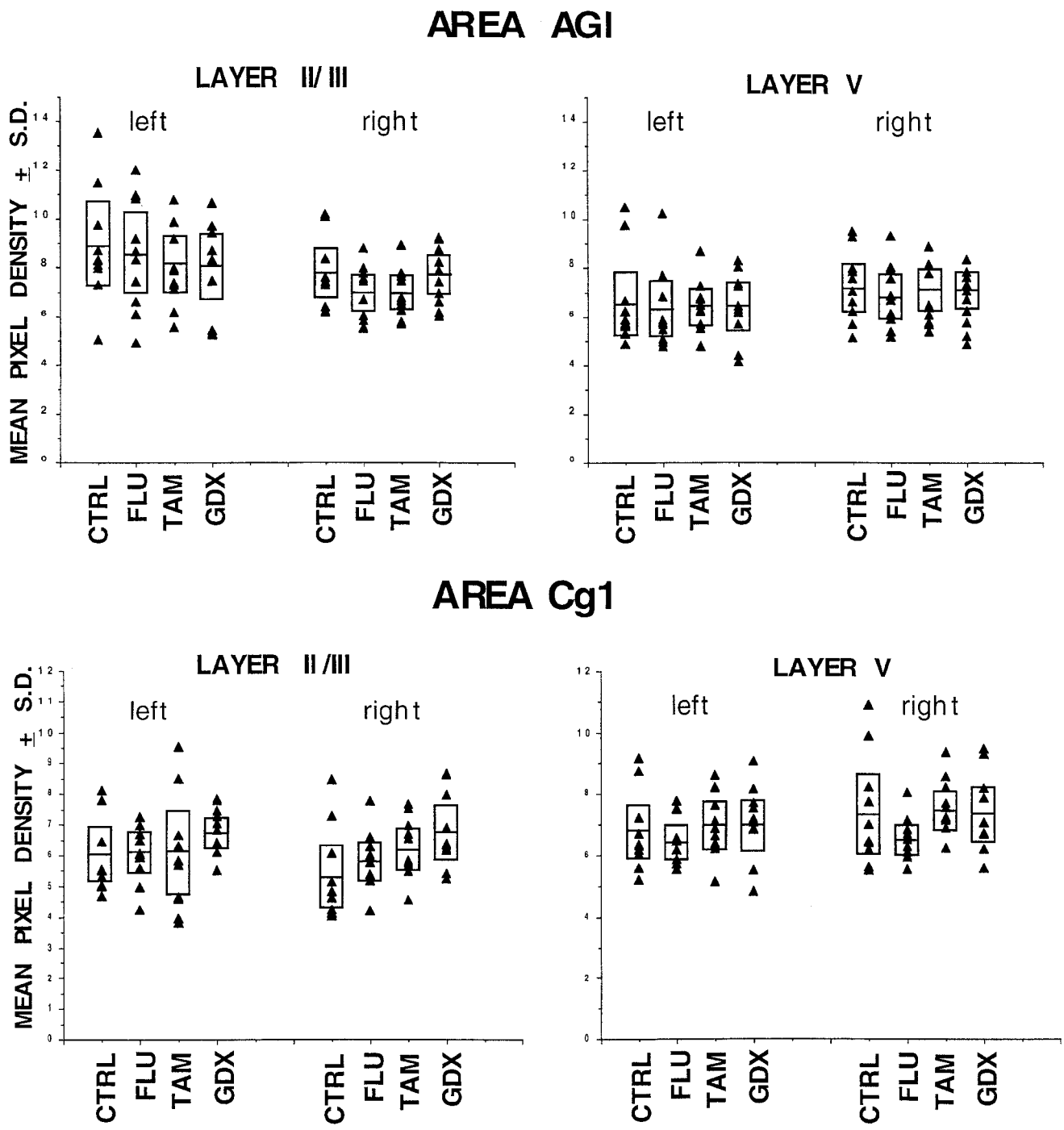

Fig. 2. Scatter plots of mean pixel density measures obtained from digitized camera lucida drawings of dopamine-β-hydroxylase-immunoreactive axons in layers II/III and V from the left and right hemifields of primary motor cortex (AGl, top graphs) and of dorsal anterior cingulate cortex (Cg1, bottom graphs) in sham-operated controls (CTRL), in hormonally intact rats given flutamide (FLU) or given tamoxifen (TAM), and in acutely (4-day) gonadectomized rats; all animals had postoperative survivals of 4 days. Each plot is inclusive of all measures obtained in all individual animals in each group, with each point represented as a triangle. The rectangles that surround these points mark off the standard deviation of the data and are bisected by a horizontal line that marks the group mean. Values for CTRL and GDX groups shown here have been presented elsewhere (Kritzer, 2000) and are included for comparison only. These graphs show that density measures from both regions, layers, and hemispheres evaluated were overlapping for all the animal groups and were not significantly different from one another.

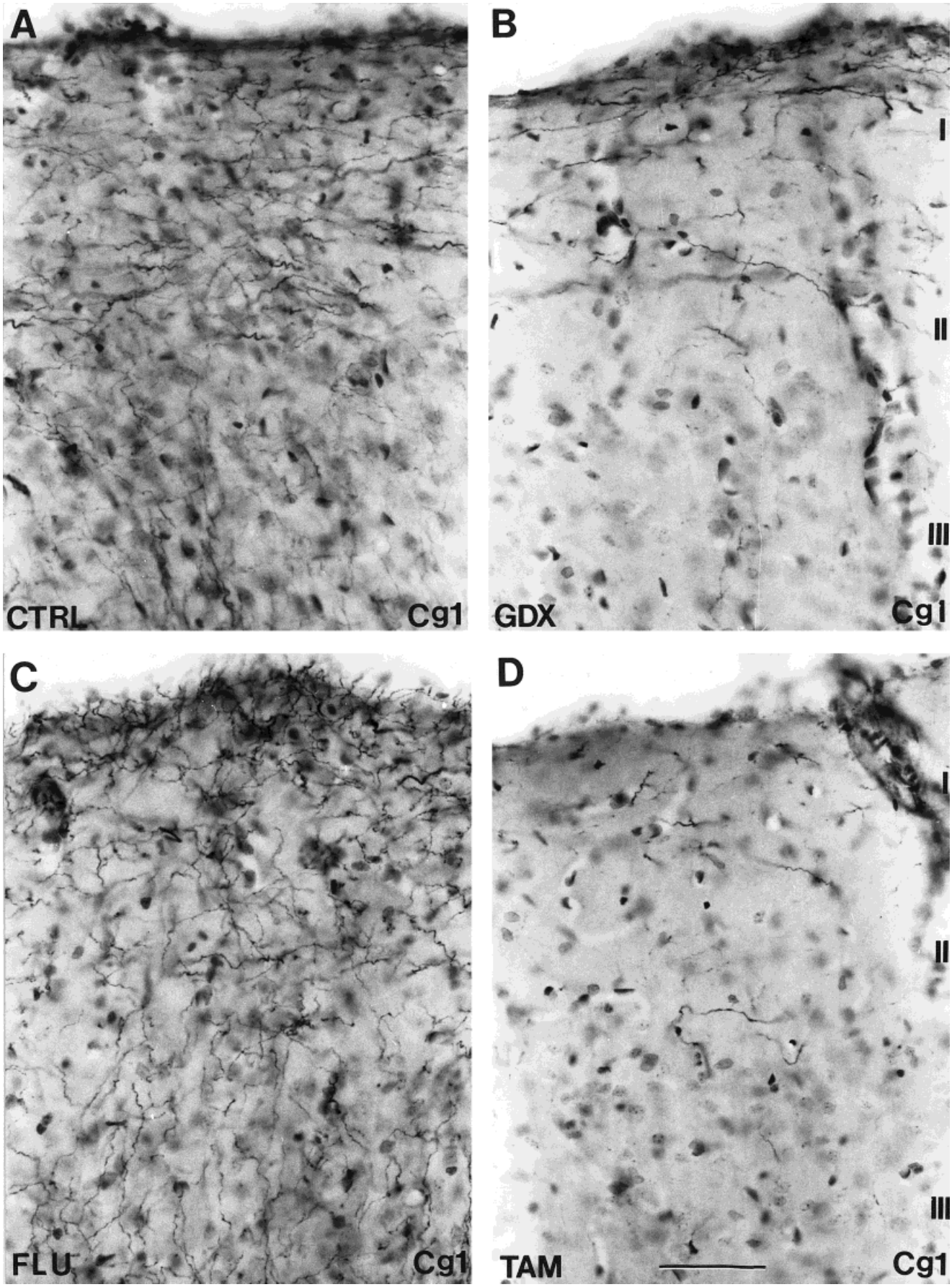

Fig. 3. Representative brightfield photomicrographs showing the morphology, orientation, and density of axons immunoreactive for tyrosine hydroxylase in layers I–III of the dorsal anterior cingulate cortex (Cg1) in sham-operated control rats (CTRL; **A**), in acutely (4-day) gonadectomized rats (GDX; **B**), and in hormonally intact rats given flutamide (FLU; **C**) or tamoxifen (TAM; **D**) for 4 days. All sections are counterstained with 1% cresyl violet to reveal cellular architecture; none of the cells visible are immunoreactive for tyrosine hydroxylase. This figure illustrates that patterns of immunoreactivity typical of hormonally intact (CTRL) animals, e.g., dense plexuses of thick and thin, randomly oriented axons in the supragranular layers, are preserved in intact animals treated with FLU (C), but that severe axonal deficits that are similar to those produced by acute gonadectomy (B) are present in rats given TAM (D). Scale bar = 50 μm in D (applies to A–D).

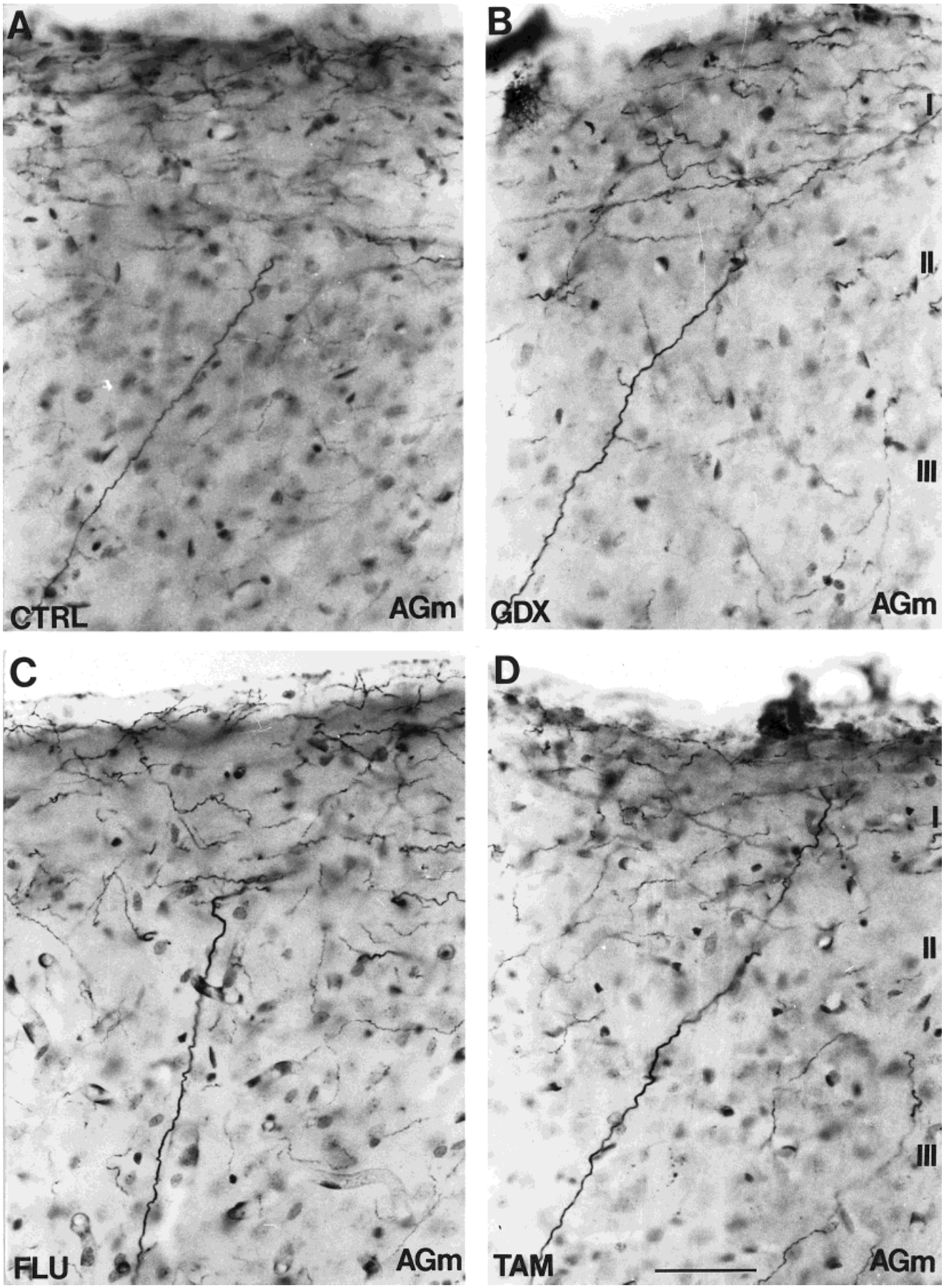

Fig. 4. Representative brightfield photomicrographs showing the morphology, orientation, and density of axons immunoreactive for tyrosine hydroxylase in layers I–III of the premotor cortex (AGm) in sham operated control rats (CTRL; **A**), in acutely (4-day) gonadectomized rats (GDX; **B**), and in hormonally intact rats given flutamide (FLU; **C**) or tamoxifen (TAM; **D**) for 4 days. The sections have been counterstained with 1% cresyl violet to reveal cellular architecture; none of the cells visible in any of the panels are immunoreactive for tyrosine hydroxylase. This figure illustrates that patterns of immunoreactivity typical of hormonally intact (CTRL) animals, e.g., innervation in the supragranular layers that is dominated by thick, smooth, and predominantly radially oriented axons, are preserved in intact animals treated with FLU (C) or with TAM (D). An illustration of the preservation of tyrosine hydroxylase immunoreactivity in acutely gonadectomized rats (B) is also included for comparison. Scale bar = 50 μm in D (applies to A–D).

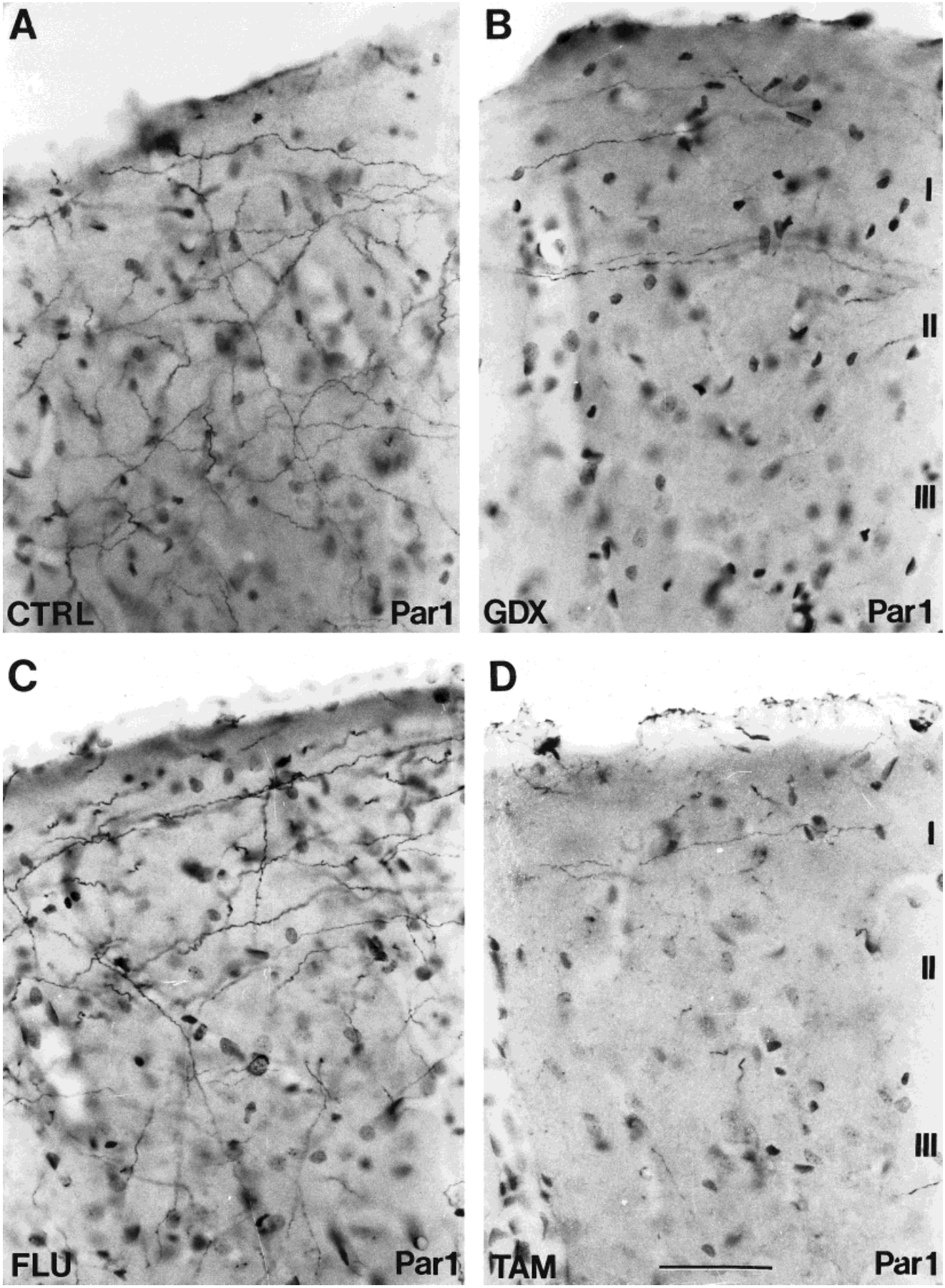

Fig. 5. Representative brightfield photomicrographs showing the morphology, orientation, and density of axons immunoreactive for tyrosine hydroxylase in layers I–III of the primary somatosensory cortex (Par1) in sham-operated control rats (CTRL; **A**), in acutely (4-day) gonadectomized rats (GDX; **B**), and in hormonally intact rats given flutamide (FLU; **C**) or tamoxifen (TAM; **D**) for 4 days. The sections shown have been counterstained with 1% cresyl violet to reveal cellular architecture; none of the cells visible are immunoreactive for tyrosine hydroxylase. This figure illustrates that patterns of immunoreactivity typical of hormonally intact (CTRL) animals are preserved in intact animals treated with FLU (C) but that decreases in immunoreactivity similar to that produced by acute gonadectomy (B) characterize the immunolabeling in rats given TAM (D). Scale bar = 50 μm in D (applies to A–D).

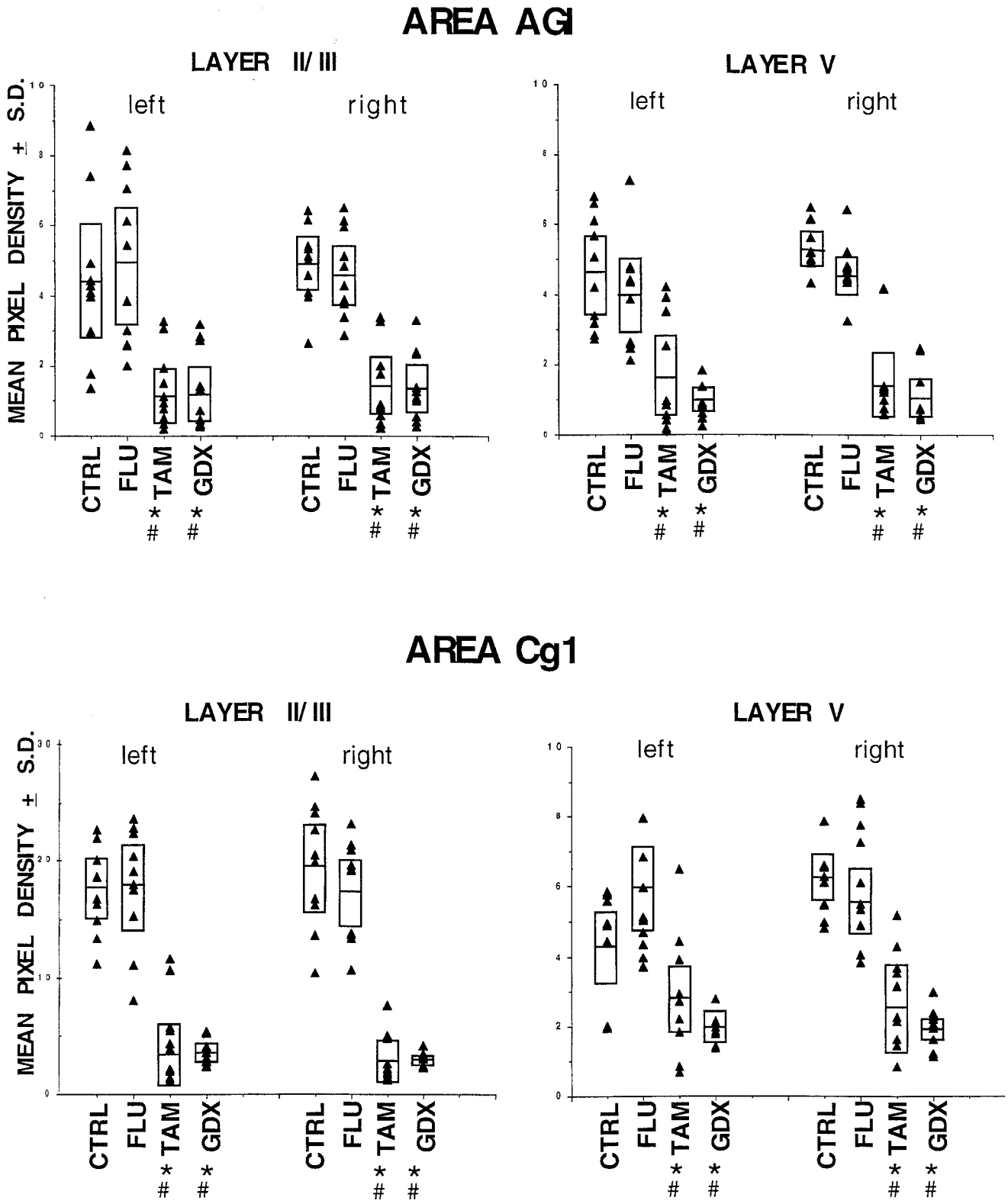

Fig. 6. Scatter plots of mean pixel density measures obtained from digitized camera lucida drawings of tyrosine hydroxylase-immunoreactive axons in layers II/III and V from the left and right hemifields of primary motor cortex (AGl, top graphs) and of dorsal anterior cingulate cortex (Cg1, bottom graphs) in sham-operated controls (CTRL), in hormonally intact rats given flutamide (FLU) or given tamoxifen (TAM), and in acutely (4-day) gonadectomized rats; all animals had postoperative survivals of 4 days. Each plot is inclusive of all measures obtained in all individual animals in each group, with each point represented as a triangle. The rectangles that surround these points mark off the standard deviation of the data and are bisected by a horizontal line that marks the group mean. Values for CTRL and GDX groups shown here have been presented elsewhere (Kritzer, 2000) and are included for comparison only. Density measures from both regions, layers, and hemispheres evaluated were overlapping for the control and flutamide-treated animal groups and for the gonadectomized and tamoxifen-treated cohorts. However, for every cortical compartment evaluated, measures in the gonadectomized and tamoxifen-treated animals were significantly different from those in controls (marked by *) as well as in flutamide-treated rats (marked by #).

interaction ($F_{3,16} = 32.03$, $P < 0.0001$). The allowed post hoc comparisons (Student-Newman-Keuls, $P < 0.05$ accepted as significant) revealed that in every region, layer, and hemisphere evaluated, TH axon density was not significantly different between control and FLU animals, or between GDX and TAM animals, but that the innervation values of these two groupings (control and FLU rats versus GDX and TAM rats) were all significantly different from each other (Fig. 6).

Effects of combined tamoxifen treatment and acute gonadectomy on cortical TH innervation

The axon densities of TH immunoreactive afferents were also assessed in additional groups of rats in which gonadectomy and tamoxifen treatment were combined. Comparisons were first made between TH axon density in layers II/III and V of cingulate and primary motor cortices in rats that were hormonally intact and TAM treated for 4 days, gonadectomized and placebo-treated for 4 days, or gonadectomized and TAM-treated over the same 4-day period (Figs. 7, 8, left side). Qualitatively, there were no obvious differences in innervation among these three groups, and statistical analyses (ANOVA; see Materials and Methods) carried out on the density data obtained from these cohorts failed to identify any main effects of hormone treatment for either cortical region ($P > 1.0$ for both cortical areas). This same approach was also applied to animals that were TAM-treated, gonadectomized, or gonadectomized and TAM-treated for 7 days prior to sacrifice. Previous studies have shown that by 1 week after gonadectomy, TH innervation begins to climb from the very low levels of innervation seen at the 4-day mark toward higher, nearer normal levels of innervation in cingulate and motor areas (Adler et al., 1999), and in this study, as expected, higher mean densities were obtained in the 7- compared with the 4-day GDX groups (compare left and right sides, Figs. 7, 8). Comparisons of these relatively elevated GDX axon density baselines with values obtained in the 7-day TAM-treated and 7-day GDX+TAM groups (ANOVA, repeated measures design), however, revealed no significant differences in axon density measures among the three (Figs. 7, 8, right sides).

DISCUSSION

Gonadectomy in adult male rats induces a striking, albeit transient decrease in the density of TH- but not DBH-immunoreactive afferents in primary sensory, primary motor, and association areas of the cerebral cortex within days of hormone deprivation. Previous studies have suggested that this acute drop in presumed DA innervation is a consequence of experimentally reducing estrogen stimulation, as axonal deficits are rescued in acutely gonadectomized rats supplemented with estradiol, but not in rats given the nonaromatizable androgen DHT (Kritzer, 2000). The present study showed that administration of the antiestrogen tamoxifen to hormonally intact rats also produced catecholamine axon deficits that mirrored those induced by short-term gonadectomy. Administration of the antiandrogen flutamide, on the other hand, had no discernible impact on either TH- or DBH-immunoreactive afferents. As discussed below, when these findings are considered within the context of the known pharmacology and sites of action of these nonsteroidal ligands, they not only support an initial phase of selective estrogen sensitivity among cortical DA afferents but provide insights into the intracellular mechanisms that may confer this sensitivity in the adult rat cerebrum.

Androgen receptor stimulation plays no apparent role in the acute regulation of cortical catecholamines

Qualitative and quantitative assessments of TH and DBH immunoreactivity in the cerebral cortices of hormonally intact rats treated for 4 days with the antiandrogen flutamide revealed patterns of innervation that were indistinguishable from controls. The amount of flutamide released from the pellets implanted in these animals was approximately 4 mg/day, which is comparable to the dose used in previous studies to demasculinize successfully, for example, the size of the corpus callosum in rats (Fitch et al., 1991). The effective antagonism of intracellular androgen receptors was also indicated in our studies in the diminished weights of the androgen-sensitive bulbocavernosus muscles in the FLU rats. Thus, in the FLU cohorts, the bulbocavernosus muscles were proportionately smaller than controls and were similar in weight to musculature in acutely gonadectomized, placebo-treated rats (Table 1). Because flutamide crosses the blood-brain barrier (Neri et al., 1972), and because there is only a single, flutamide-sensitive androgen receptor subtype known in the CNS (Wilson et al., 1991), it seems reasonable to assume that androgen receptors were also effectively blocked in the brains of the FLU animals. Thus, the negative findings reported here are unlikely to be attributable to an inadequate dose of flutamide, nor to a failure to block androgen actions at flutamide-insensitive CNS sites, rather, the intact TH and DBH innervation that we show here remaining in the FLU animals dovetails with previous evidence of an inability to replace DHT in acutely gonadectomized rats to protect cortical innervation (Kritzer, 2000). In sum, it appears that neither pharmacological blockade of intracellular androgen receptors nor their decreased stimulation by deprivation of endogenous ligand(s) contributes to the striking changes in cortical catecholamine innervation that are induced by acute changes in the hormonal milieu in adult male rats.

Fig. 7. Scatter plots of mean pixel density measures obtained from digitized camera lucida drawings of tyrosine hydroxylase-immunoreactive axons in layers II/III and V from the left and right hemifields of dorsal anterior cingulate cortex (Cg1). The animals represented in the left-hand graphs (top and bottom) are acutely (4 day) gonadectomized rats, hormonally intact rats given tamoxifen (TAM) for 4 days, and acutely (4-day) gonadectomized rats given tamoxifen (GDX+TAM); the animals represented in the right-hand graphs (top and bottom) are acutely (7-day) gonadectomized rats, hormonally intact rats given tamoxifen (TAM) for 7 days, and acutely (7-day) gonadectomized rats given tamoxifen (GDX+TAM). Each plot is inclusive of all measures obtained in all individual animals in each group, with each point represented as a triangle. The rectangles that surround these points mark off the standard deviation of the data and are bisected by a horizontal line that marks the group mean. Density measures from both layers and hemispheres evaluated were overlapping for all the 4-day and all the 7-day animal groups and were not significantly different from one another. As anticipated in a previous study (Adler et al., 1999), axon density values are elevated in all the 7-day groups relative to the 4-day treatment cohorts.

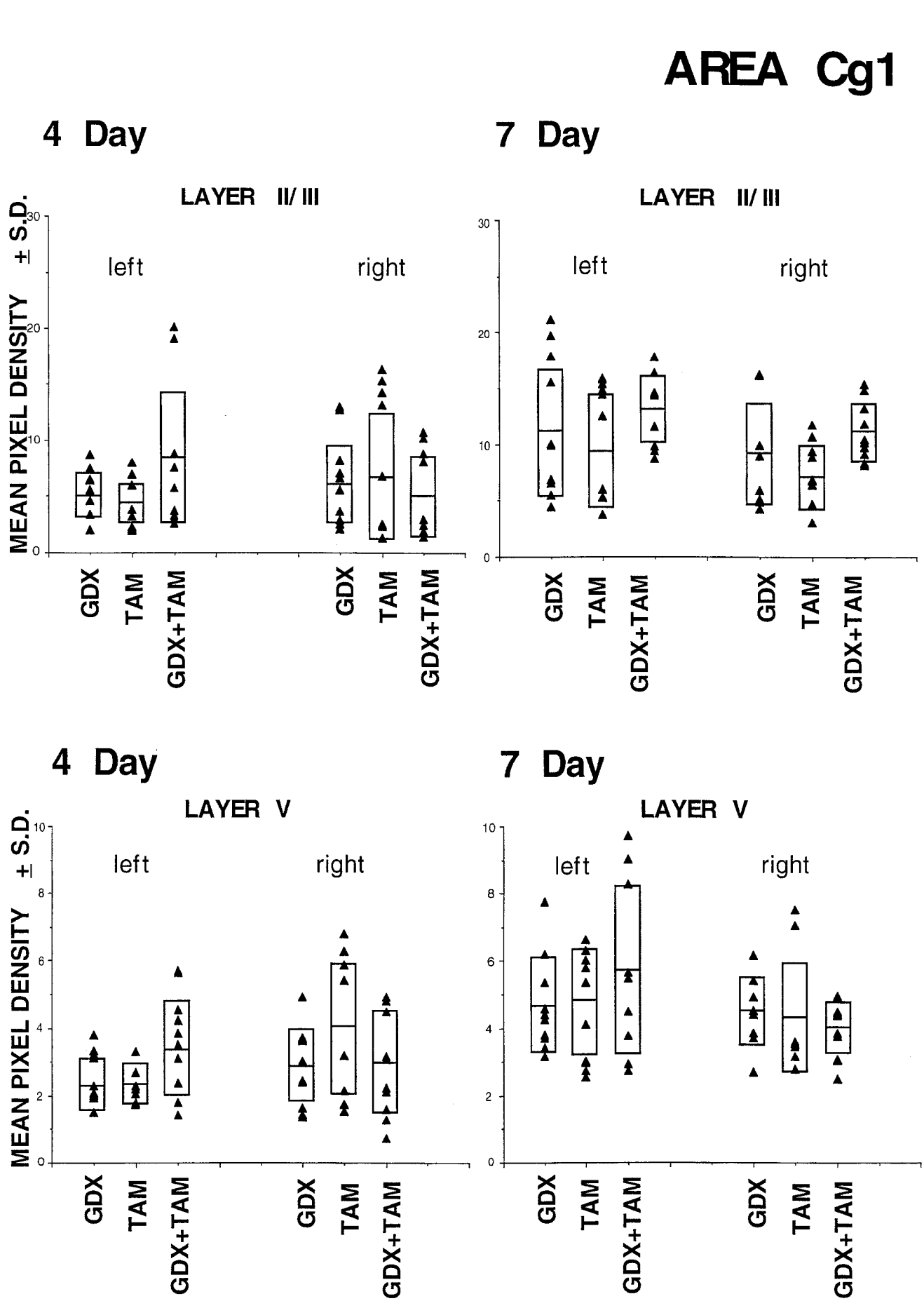
AREA Cg1
4 Day
7 Day
LAYER II/III
LAYER V
MEAN PIXEL DENSITY ± S.D.
left
right
GDX
TAM
GDX+TAM

Figure 7

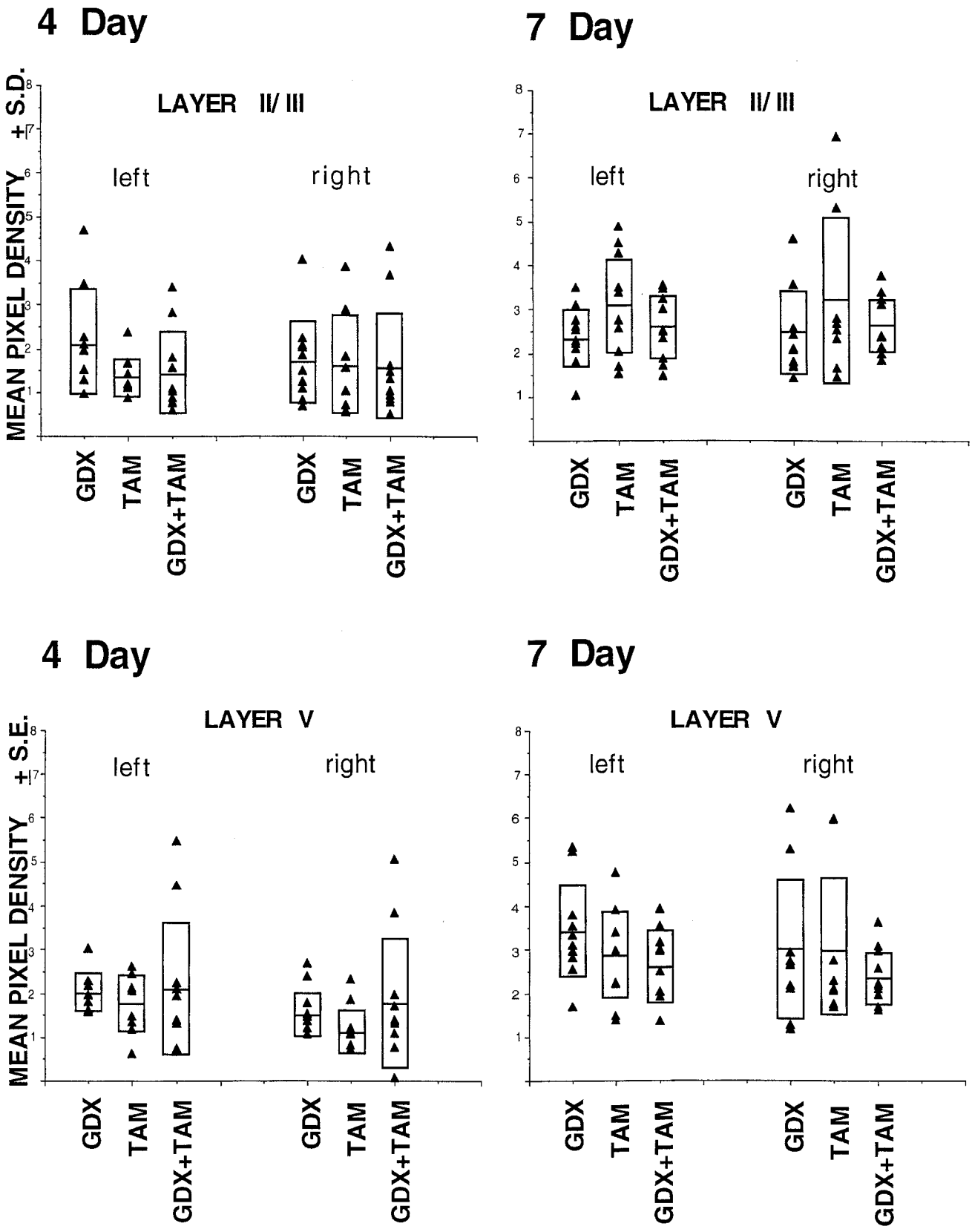
4 Day
7 Day
LAYER II/III
LAYER V
MEAN PIXEL DENSITY +S.D.
MEAN PIXEL DENSITY +S.E.
left
right
GDX
TAM
GDX+TAM

Figure 8

Cortical catecholamines are not regulated by estrogen-independent actions of tamoxifen

The studies presented here used the antiestrogen tamoxifen. Although other compounds with antiestrogen activity are known, tamoxifen is one of the few that is both commercially available and that crosses the blood-brain barrier. These features were important in our selection of this ligand, as it enabled us to use implanted slow-release pellets for compound delivery. We used slow-release pellets to administer hormone replacement in all our preceding studies mainly because they obviate the need for daily handling/ injecting of animals—procedures that could introduce confounds in our data, as cortical DA systems show striking regional selectivity in their sensitivity to even mild stressors (Deutch, 1993).

For the sake of procedural continuity as well as to avoid unnecessary sources of stress to our animals, we opted here to take advantage of the availability of tamoxifen in slow-release pellet form. We are mindful, however, that the use of tamoxifen has potential drawbacks. Perhaps most significant is that tamoxifen not only antagonizes estrogen's activities at intracellular estrogen receptors in peripheral and CNS tissues (e.g., Chazal et al., 1975; see Roy, 1979) but it also has actions in the CNS that are independent of estrogen and estrogen receptor antagonism. For example, tamoxifen binds with high affinity to estrogen noncompetable, so-called antiestrogen binding sites that are distributed throughout the brain (Sudo et al., 1983; Gray and Ziemian, 1992), inhibits protein kinase C (O'Brian et al., 1985) and calmodulin (Lam, 1984) activity in brain homogenates, and competes with ligands such as ^{3}H-spiperone for presumed dopamine D2 binding sites in striatal membrane preparations (Hiemke and Ghraf, 1984).

There are, however, several reasons to believe that the relevance of these estrogen-independent actions to the effects on cortical DA innervation observed here are minimal. First, there are striking qualitative parallels in the consequences of surgical hormone deprivation (gonadectomy) and the pharmacological blockade of hormone receptors (tamoxifen treatment), i.e., gonadectomy yields signature patterns of cortical catecholamine axon depletions that are matched, essentially detail for detail, by tamoxifen administration in intact animals. For example, both gonadectomy and tamoxifen depress TH axon density in cingulate and primary motor cortices, while leaving innervation in the premotor area (AGm) in between them relatively undisturbed. This region-specific pattern of influence is difficult to reconcile with the distribution of antiestrogen or even DA D2 bindings sites, which are more ubiquitously distributed in the forebrain. Furthermore, the effects of both gonadectomy and tamoxifen treatment lessen over time; whereas both treatments generally depress innervation to between roughly 20% and 40% of controls after 4 days of treatment, by 7 days, axon density values in both GDX and TAM animals more typically range between approximately 50% and 70% of controls (see below). Finally, neither TAM treatment nor gonadectomy affected cortical DBH innervation, indicating parallels in the neurochemical selectivity of these two experimental treatments as well.

The effects of GDX and TAM on TH-immunoreactive axon density also appear to be nonadditive. Thus, in both cingulate and motor cortex, 4-day treatment with tamoxifen, 4-day gonadectomy, and 4-day gonadectomy plus tamoxifen treatment all lowered innervation in supragranular and infragranular layers to statistically invariant degrees. Quantitative analyses of axon density also indicated similarities among animals treated with tamoxifen and/or gonadectomized for 7 days. The negative findings obtained in these latter, 1-week treatment groups are especially relevant because TH innervation in both cingulate (Adler et al., 1999) and motor cortices (Fig. 7) of gonadectomized animals has begun to return (rise) to normal levels. Accordingly, baseline densities in gonadectomized rats are slightly higher relative to the 4-day groups, where extremely low levels of innervation in GDX and TAM cohorts could have attenuated the quantitative (statistical) identification of group density differences. Furthermore, if tamoxifen were to depress TH innervation in a hormone-independent manner, this depression should oppose the stimulatory influences at work on innervation in the 7-day gonadectomized rats—again favoring detection of group differences. However, among the 7-day cohorts, no group differences in axon density were detected in either cortical region, hemisphere, or layer evaluated.

Thus, acute gonadectomy and acute tamoxifen treatment both yield effects on cortical catecholamine innervation that are regionally, neurochemically, temporally, and quantitatively similar, as well as nonadditive. Together these findings suggest that tamoxifen's impact on cortical innervation is probably mediated via its antiestrogen activity or activities.

Cortical catecholamines and tamoxifen's actions at intracellular estrogen receptors

Estrogen receptors currently include two broad classes. Although there may be additional, novel estrogen binding sites in the brain (e.g., Asaithambi et al., 1997; Singh et al., 2000), established receptor subtypes in the CNS include intracellular nuclear estrogen receptors, which when bound to estradiol act as ligand-dependent transcription factors, and membrane-bound estrogen binding proteins, which mediate more rapid, nongenomic responses of estrogen (McEwen, 1991). Both genomic and nongenomic means of estrogen influence have been identified in relation to nigrostriatal and hypothalamic DA

Fig. 8. Scatter plots of mean pixel density measures obtained from digitized camera lucida drawings of tyrosine hydroxylase-immunoreactive axons in layers II/III and V from the left and right hemifields of primary motor cortex (AGl). The animals represented in the left-hand graphs (top and bottom) are acutely (4-day) gonadectomized rats, hormonally intact rats given tamoxifen (TAM) for 4 days, and acutely (4-day) gonadectomized rats given tamoxifen (GDX+TAM); the animals represented in the right-hand graphs (top and bottom) are acutely (7-day) gonadectomized rats, hormonally intact rats given tamoxifen (TAM) for seven days, and acutely (7-day) gonadectomized rats given tamoxifen (GDX+TAM). Each plot is inclusive of all measures obtained in all individual animals in each group, with each point represented as a triangle. The rectangles that surround these points mark off the standard deviation of the data and are bisected by a horizontal line that marks the group mean. Density measures from both layers and hemispheres evaluated were overlapping for all the 4- and and all the 7-day animal groups and were not significantly different from one another. The axon density values of the latter 7-day cohorts are also elevated relative to the 4-day treatment groups.

systems. In the hypothalamus, for example, genomic estrogen stimulation provides acute regulation of TH gene transcription in tuberoinfundibular neurons (Blum et al., 1987), whereas in nigrostriatal neurons, nongenomic estradiol action stimulated increased cell excitability (Chiodo and Caggiula, 1980), potentiated DA synthesis and release (Thompson and Moss, 1994; Paqualini et al., 1995) and increased intracellular calcium levels (Beyer and Raab, 1998). As has been demonstrated for nongenomic end points in other systems, (e.g., hippocampus; Gu and Moss, 1994), these rapid, membrane receptor-mediated effects are not typically blocked by tamoxifen or other tamoxifen-like antiestrogens (Mermelstein et al., 1996; Beyer and Karolczak, 2000)—a striking contrast to tamoxifen's potency in antagonizing estradiol-dependent transcription when bound to intracellular estrogen receptors (e.g., Webb et al., 1995; Paech et al., 1997). According to this dissociation, the tamoxifen sensitivity of mesocortical DA afferents shown in this study probably indicates that underlying mechanisms involve intracellular nuclear receptors rather than membrane-bound, estrogen-binding proteins.

Although tamoxifen sensitivity may help to narrow the field of mechanistic possibilities to intracellular receptors, it does little to distinguish relevant signaling pathways from among the multiplicity of intracellular estrogen receptor subtypes currently known in the CNS. In addition to what is now termed the classical or ERα intracellular site, many structures of the CNS also contain the more recently identified ERβ receptor subtype. Because tamoxifen antagonizes transcriptional activation mediated by both of these nuclear receptors, its effectiveness in our study falls short of delineating specific roles for one or the other of these sites in estrogen's stimulation of cortical DA. In perhaps the simplest scenario in which the midbrain DA neurons are themselves principal targets of hormone action, recent in situ hybridization (e.g., Simerly et al., 1990; Shughrue et al., 1997) studies indicating little to no mRNA for ERα but significant amounts of mRNA encoding the ERβ subtype (Shughrue et al., 1997) in the adult rat substantia nigra, pars compacta, and ventral tegmental area might predict a role for ERβ. However, additional experiments are needed to identify definitively which of the estrogen receptor subtypes are involved and which response elements these receptors use in stimulating transcription. Because gonadal hormone stimulation is ultimately able to affect DA innervation differentially in motor versus cognitive areas of the cerebral cortex (Kritzer et al., 1999), such clarification of underlying signaling pathways could ultimately prove useful in clinical settings, including the control of motor side effects associated with DA-inhibiting treatments in psychotic illness, or in ameliorating the psychotic symptoms that are sometimes produced by DA-potentiating therapies in movement disorders (Davis et al., 1991; Deutch, 1993). These findings may also be relevant in identifying and perhaps avoiding unwanted CNS side effects in the current use of antiestrogens including tamoxifen in the treatment of breast cancers.

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