Gonadal hormones are known to broadly influence cortical information processing. Findings from this study in rats suggest that for androgens, this influence may include stimulation of underlying corticocortical connections. First, immunoreactivity for intracellular androgen receptors, while present in all regions and layers examined, was found to be particularly abundant in sensory and motor regions, and within these, within their major pyramidal cell layers, i.e., layers II/III and V/VI. Double labeling immunocytochemical studies for androgen receptors and for neuron-specific markers then confirmed that the majority of receptor-bearing cortical cells were pyramidal neurons. Finally, combined analyses of cortical receptor immunoreactivity and retrograde labeling produced by tracer injections made in specific subcortical (caudate, nucleus accumbens, superior colliculus, thalamus) areas yielded only isolated examples of receptor/tracer overlap. However, injections made within the cortex itself (sensory, motor, associational areas) retrogradely labeled cortical cells some 50% or more — especially within injected hemispheres, were receptor-immunoreactive. Thus, the regional, laminar, and cellular distributions of immunoreactivity in the rat cerebral cortex largely identify pyramidal neurons with connectional signatures aligning intracellular androgen receptors with the local, associational, and to a lesser degree, callosal circuits that interlink territories of the cortical mantle and play key roles in cortical information processing.

**Keywords:** estrogen, gonadal hormones, intracellular receptors, neocortex, pyramidal neurons, testosterone

**Introduction**

The cerebral cortices in humans, non-human primates, and rodents are sensitive to gonadal hormones; the presence of intracellular androgen and estrogen receptor proteins (e.g., Clancy *et al.*, 1992; Puy *et al.*, 1995; Finley and Kritzer, 1999; Burton-Jones and Tuszyński, 2002; Kritzer, 2002), mRNAs (e.g. Simerly *et al.*, 1990; Shughrue *et al.*, 1997), and hormone binding sites (Pfäff and Keiner, 1973; Sar and Stumpf, 1985; Sholl and Pomerantz, 1986; Sholl and Kim, 1990) within the cortical mantle are consistent with findings that both testicular and ovarian hormone-signaling pathways are active in influencing specific endpoints of cortical structure and/or function. However, in many instances these cortically directed influences are either non-overlapping or diometric. For example, in rats, estrogen but not androgen stimulation promotes neurite outgrowth in organotypic cortical cultures (see Toran-Allerand, 1991) and modulates the development of cortical catecholamine activity (Stewart and Rajabi, 1994), while androgens but not estrogens chronically stimulate cortical catecholamine innervation (Kritzer, 2000). The survival and differentiation of cultured embryonic rat cortical neurons (Zhang *et al.*, 2000), and stress-induced rates of dopamine and noradrenaline turnover in the prefrontal cortices of adult male rats (Handa *et al.*, 1997) on the other hand, are endpoints that are stimulated by estrogens but inhibited by androgens.

Although mechanisms are uncertain, one factor that could help define the spheres of influence and/or divisions of labor seen in hormone stimulation of the cerebral cortex could be the specific cortical distributions that intracellular, i.e., nuclear, estrogen (ER) and androgen receptors (AR) maintain. While clearly more abundant, however, less is known about the cortical distribution of intracellular AR as compared with ER proteins. Available evidence suggests, though, that at least in rats these two hormone pathways occupy distinct niches among cerebral cortical circuit structures. Specifically, while recent light microacopic studies have shown that ER proteins are present mainly within subsets of the nuclei of non-pyramidal interneurons (Blurton-Jones and Tuszyński, 2002; Kritzer, 2002), there is some evidence to suggest that androgen receptors may be preferentially aligned with the pyramidal neurons of the cortex. *In situ* hybridization studies, for example, have shown that cells containing mRNAs for androgen receptor proteins are particularly concentrated in cortical layers where pyramidal cells predominate (Simerly *et al.*, 1990), and immunocytochemical mapping of AR proteins, although limited to a single representative cortical level, described the morphology of at least some receptor bearing cells as pyramidal (Clancy *et al.*, 1992). If indeed intracellular androgen receptors are predominantly located within the projection neurons of the cortex then in order to fully define the anatomical and potential functional contexts of these target cells, precise information is needed not only about the regional, laminar, and cellular localization of these receptors but also about the connections that receptor-bearing neurons make. Accordingly, the studies presented here used single and double label immunocytochemistry, respectively, to map and phenotype intracellular AR-bearing cells in all major cortical fields of hormonally intact adult male and female rats. A second series of studies followed that combined receptor immunocytochemistry and retrograde tract tracing to explore the afferent connections of these receptor-bearing neurons. Some of this work has been presented in abstract form (Kritzer, 2005).

**Materials and Methods**

**Tissue**

Single and double label immunocytochemistry was carried out in a total of 10 hormonally intact, adult (200–400 g) Sprague–Dawley rats (four males, six females); all were subjects in a previous study of cortical estrogen receptors (Kritzer, 2002). For the females, vaginal cytology smears were used to verify that animals were either in pro-
estrus (n = 3) or diestrus (n = 3) at the time of euthanasia. An additional 21 animals (17 males, four females) were used in experiments that combined retrograde tracing and receptor immunocytochemistry; the males were subjects in an unrelated tract-tracing study.

**Surgical Procedures: Craniotomy and Intracerebral Injection**

Rats were anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively, injected i.m.) and placed in a stereotaxic apparatus. Next, a midline incision was made over the skull, and burr holes were drilled over stereotaxically determined loci (Paxinos and Watson, 1986). Pulled glass micropipettes filled with the retrograde tracer, cholera toxin (inactive B-subunit conjugated to colloidal gold, List Biologicals, Campbell, CA), diluted to 0.025% in sterile water were then lowered to specific depths, and tracer was ejected into either the cingulate (θ = 3), primary motor (θ = 5) or primary somatosensory cortex (n = 3), the medial or lateral caudate nucleus (n = 4), the nucleus accumbens (core and shell, n = 2), the superior colliculus (n = 2) or the ventrolateral/ventral anterior nuclei of the thalamus (n = 4). At each site, tracer was pressure-injected in roughly 10 nl increments over a 15–20 min period using visual monitoring of the meniscus within the calibrated micropipettes to measure the volume of tracer ejected; total volumes ranged from 50 to 300 nl. After injecting, pipettes were left in place for 15 min and were then slowly withdrawn to minimize tracer efflux up the pipette track. A 7–10 day survival period followed the surgery, after which animals were transcardially perfused.

**Perfusion**

Perfusions were deeply anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively, injected i.m.). After deep reflexes could no longer be elicited, animals were transcardially perfused with ~100 ml of phosphate-buffered saline (PBS), followed by 500–1000 ml of 2% paraformaldehyde containing 15% saturated picric acid in 0.1 M PBS, pH 7.4. Following perfusion, brains were removed and placed in 0.1 M PBS containing 10, 20 and then 30% sucrose for cryoprotection. After the brains had sunk in the 30% sucrose solution, they were frozen in powdered dry ice, and serially sectioned on a freezing microtome (40 μm).

**Histology**

**Single Label Androgen Receptor (AR) Immunohistochemistry**

To visualize AR-immunoreactivity, sections were rinsed in 0.1 M phosphate buffer (PBS), pH 7.4, incubated in 1% H2O2 in PBS for 45 min, rinsed again in 0.1% PBS, and then placed in 1% sodium borohydride in PBS (45 min). Sections were then rinsed thoroughly in 0.1 M PBS, fixed in 2.5% sodium thiosulfate (10 min), washed extensively again in 0.1 M PBS, and were finally slide mounted, dehydrated and placed under coverslips. Some sections were counterstained with 1% cresyl violet.

**Double Label Immunohistochemistry**

For double labeling experiments, antigens were immunolabeled sequentially beginning with the anti-AR polyclonal antibody. After the DAB/nickel reaction, sections were rinsed in TBS and replaced in blocking solution (TBS with 1% NSS, 2 h) prior to being incubated in one of five primary monoclonal antibodies (1–2 days, 4°C). The monoclonal antibodies used were recognized either neuron specific enolase (NSE; Chemicon International Inc., Temecula, CA, working dilution 1:100), glial fibrillary acidic protein (GFAP; Chemicon International, working dilution 1:500) an oligodendrocyte-specific moiety [anti-RP developed by Dr Susan Hockfield, Yale University, New Haven, CT, obtained from the Developmental Studies Hybridoma Bank, a facility maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NO1-HD-7-3263 from the National Institute of Child Health and Development (NICHD), working dilution of 1:5], parvalbumin (PV, Sigma Chemical Corp., St Louis, MO, working dilution 1:1000) or calbindin (CB, Sigma, working dilution 1:1000). After incubations in biotinylated secondary antibodies and in ABC solution, sections were rinsed in TBS, then rinsed in TB, pH 7.6, and finally reacted a second time using DAB as chromagen.

**Tracer Visualization**

Sections were reacted to visualize the gold-conjugated cholera toxin using a silver enhancement technique. For this procedure, sections were first rinsed in 0.1 M PBS, and were then silver enhanced for 90–120 min in a solution composed of equal volumes of the Intense Initiator and Enhancer solutions (Amersham, Arlington Heights, IL). Sections were then rinsed in 0.1 M PBS, fixed in 2.5% sodium thiosulfate (10 min), washed extensively again in 0.1 M PBS, and were finally slide mounted, dehydrated and placed under coverslips. Some sections were counterstained with 1% cresyl violet.

**Combined AR-immunocytochemistry and Tracer Visualization**

To combine AR-immunocytochemistry and retrograde tracing, prior to the silver enhancement procedure above, sections were rinsed in 1% H2O2 for 30 min. After the enhancement procedure, sections were rinsed in TBS, pH 7.4, blocked for 2 h in TBS containing 10% NSS and then immunoreacted as described for the single procedure (above).

**Control Experiments**

All immunohistochemical labeling protocols (single, double and in combination with retrograde tracing) were carried out on representative sections from male and female animals with the omission of primary or secondary antibodies, or using antibodies against AR that had been preabsorbed for 5 h with a 10 M excess of the immunizing AR peptide (AR-21, provided by Dr G. Greene, Ben May Institute; sc-816 P, Santa Cruz Biotechnology). In the injected animals, receptor immunoreactivity was also carried out in representative sections that were not silver enhanced to determine whether this procedure affected detectability of AR-immunoreactivity.

**Data Analysis**

**Cellular Phenotyping**

The sections used to assess the cellular identity of AR-immunoreactive (AR-IR) cells in the rat cerebrum (double label immunocytochemistry) were taken at roughly 400 μm intervals from the rostral to caudal poles of the brains of uninjected animals. Within these sections, sensory, motor, association, and allocortical regions were examined. Colocalization between AR-immunoreactivity and immunoreactivity for NSE, GFAP, RIP, PV or CB was defined as a cell that had a blue/black DAB/nickel labeled nucleus that lay within the same focal plane as a surrounding brown, DAB labeled cytoplasm (see Kritzer, 1997; Creutz and Kritzer, 2002).

**Receptor Mapping**

For mapping of AR-immunoreactivity, cytoarchitectonic and laminar boundaries were visualized using nissl counterstaining (1% cresyl violet) in an adjacent series of the tissue sections, and in some representative immunoreacted sections. Cortical regions and layers were identified according to Zilles and Wree (1985). Detailed assessments of labeling distribution were made that included evaluation of the
Connections of AR-IR cells: Qualitative and Quantitative Analysis

Cases of injected animals were selected for analysis based on the positioning of injection sites determined in a 1-in-6 series of silver enhanced and cresyl violet counterstained sections. Using a Zeiss Axioskop and a 5× objective, darkfield illumination revealed injection sites as dense accumulations of silver-enhanced gold particles within which cellular labeling was completely obscured (see Venkatesan and Kritzer, 1999); brightfield optics were used to precisely place sites with respect to cytoarchitecture. The target regions injected were the caudate nucleus (n = 4), nucleus accumbens (n = 2), thalamus (n = 4), superior colliculus (n = 2) and the sensory (n = 5), motor (n = 3) and cingulate (n = 3) cortex.

For each of the 21 selected cases, a separate 1-in-6 series of sections were silver enhanced and immunoreacted for AR; from these qualitative analyses of the two labels were made, and five sections located at and anteriorly and posteriorly adjacent to the apparent central foci of the cortical field or fields of labeling were quantitatively analyzed. Because the seemingly most densely labeled sections were purposefully selected for assessment, these analyses did not utilize a systematic random sampling approach. However, in including a series of sections located anterior and posterior to these central starting points, in all cases the labeling evaluated included central to peripheral portions of the labeled fields. Neurons within these sections were identified as retrogradely labeled if the number of gold particles in their somata exceeded four-times background levels. All such cells located within the cerebral cortex were then recorded in camera lucida drawings using darkfield optics and a 20× or 63× objective, and were assigned to cortical regions and layers identified in nissl counterstaining in the same or adjacent sections. Separate drawings were made of labeling in injected and where applicable, uninjected hemispheres, and superimposed on these were the locations of AR-IR nuclei visualized using brightfield illumination. All cells with potential receptor/tracer overlap were further examined using brightfield optics and a 40× or 63× objective to verify cellular colocalization, i.e. that immunoreactive nuclei were situated at appropriate planes with respect to silver grain-containing cytoplasmics. For each injection site evaluated the drawings produced included totals of between 200 and 600 retrogradely labeled cells per animal subject, per hemisphere, per injection site. From each of these drawings the proportions (percentages) of the total numbers of retrogradely labeled cells identified that were also AR-immunoreactive were calculated. Because for each injection site evaluated the proportions from the five sections evaluated all lay within a 5% of one another, cell counts were summed across sections and the overall proportion of doubly labeled cells was calculated on per hemisphere bases; from these figures, mean proportions and standard deviations were then computed for labeling produced by comparably placed injection sites across animal subjects. For subcortical injection sites, labeling in supragranular and infragranular layers was considered together; for cortical injections, labeling in upper versus lower cortical layers was evaluated separately.

Photomicroscopy

All of the images presented were captured using a Zeiss Axioscam digital camera and a Zeiss Axiplan II light microscope interfaced with Axiosvision Software (Carl Zeiss, Inc., Thornwood, New York). Some adjustments were made to the brightness and contrast of captured images. All images of androgen receptor immunoreactivity were generated using the PG-21 antibody, and all examples shown are from male animal subjects.

Results

Appearance, Specificity and Potency of Immunoreactivity

With the exception of the PG-21 antibody, all of the antibodies used in this study are commercially available, and all gave rise to expected patterns of labeling. Thus, the AR antibodies both produced labeling that was localized mainly over cell nuclei in the cerebral cortex and adjacent allocortical and subcortical structures in patterns matching previous descriptions of receptor distribution (e.g. Simerly et al., 1990; Xiao and Jordan, 2002). Immunoreactivity for oligodendrocytes (anti-RIP, Fig. 2B), astrocytes (anti-glial fibrillary acidic protein) and neurons (anti-neuron-specific enolase, anti-parvalbumin, anti-calbindin) also labeled subsets of cells that displayed morphological features and distributions consistent with previous descriptions of identified cortical glial cells and neurons (e.g. see Debus et al., 1983; Friedman et al., 1989; Marone et al., 1995; Hof et al., 1999). In addition, no patterned labeling was evident in control experiments in which the primary or secondary antibodies were omitted from immunolabeling protocols, or in control studies in which anti-AR antibodies were preabsorbed with immunizing peptides (see Materials and Methods). Taken together, these findings support the selectivity of the antibodies used to label intended antigens.

It has been previously noted that the double labeling immunocytochemical method used to coincidently visualize intracellular gonadal hormone receptors and cytoplasmic markers does not appreciably diminish the immunostaining of either antigen (Kritzer, 1997; Creutz and Kritzer, 2002). Because the combination of hormone receptor immunocytochemistry and silver enhancement of the gold-conjugated retrograde tracer was used here for the first time, initial studies were conducted on tissue sections from injected rats to compare patterns of retrogradely and immunoreacted cells visualized separately on adjacent sections or co-labeled sequentially in a single section. These assessments revealed some attenuation of the intensity of AR-immunoreactivity in the combined procedure. However, there were no appreciable effects on the density of either AR-IR nuclei or of retrogradally labeled cells.

Cortical AR-IR Cells

The subjects used in these studies included hormonally intact adult male and female rats. Visual comparisons, however, revealed no obvious qualitative or quantitative differences in either single or double labeling immunocytochemistry between these groups. Thus, the descriptions below apply to males, diestrus females and proestrus females alike. Separate assessments were also made of labeling in the left and right hemispheres; as these revealed no obvious hemispheric asymmetries in receptor distribution or density, the data below also reflect receptor distributions in the left and right cortical hemispheres.

Cellular Phenotype

Representative sections of various levels of the cortex that were immunoreacted for AR proteins revealed labeled profiles that corresponded mainly to cell nuclei, with staining in the surrounding cytoplasm usually remaining near background levels (Fig. 1A–C). Most of these immunoreactive nuclei were round, although occasionally more oblong shaped profiles were observed in layer I (Fig. 1A). The nuclei in layer V also stood out as noticeably larger (10–15 µm in diameter, Fig. 1C) than the nuclei present in other layers that were usually between 2–5 µm in diameter (Fig. 1B). Even these smaller dimensions, however, fell within a range more typical of the nuclei of cortical neurons rather than glial cells. Thus, it seemed likely that AR-IR nuclei belonged to cortical neurons. This was verified in experiments that combined immunoreac-
tivity for AR with that for the neuronal marker neuron-specific enolase (NSE) that showed that virtually all of the cortical receptor-bearing nuclei were surrounded by cytoplasm that was immunoreactive for NSE (Fig. 2A, B). As further verification, double labeling for immunoreactivity for AR and for astrocytes (anti-glial fibrillary acidic protein, GFAP, Fig. 2F) or for oligodendrocytes (anti-RIP) was carried out; these analyses failed to reveal any examples of cortical AR-IR cells that were also immunoreactive for these glial markers.

The somatic and dendritic morphology revealed in the NSE-immunoreactivity identified many AR/NSE-IR cells as pyramidal neurons (Fig. 2A, B). The predominantly pyramidal character of AR-IR cortical cells was also supported in double labeling experiments combining AR-immunoreactivity and immunoreactivity for the calcium binding protein calbindin (CB), which showed that as many as half of the smaller receptor-bearing neurons in the supragranular layers were small, CB-IR pyramids (Fig. 2C). Further, in most areas of the cortex virtually none of the non-pyramidal cells labeled by immunoreactivity for CB (Fig. 2D) or for the calcium binding protein parvalbumin (PV, Fig. 2E) were found to be immunoreactive for the intracellular hormone receptor. There were, however, two exceptions to this rule. Specifically, in the proisocortical areas of the piriform cortex and entorhinal cortices, up to three quarters of the AR-IR cells present were either CB- or PV-IR, non-pyramidal neurons. In the remainder of the cortical mantle, it was only the small numbers of AR-IR neurons present in layer I — where all neurons are presumed GABAergic (Jones and Powell, 1970), that were also likely to be non-pyramidal cells.

**Regional and Laminar Distribution**

The distribution of AR-immunoreactivity was next mapped in relation to cytoarchitectonic divisions of the rat cerebrum (Zilles and Wree, 1985). Virtually all of the cortical areas and layers examined contained at least some AR-IR neurons. However, across the cortex differences in the laminar distributions and/or apparent densities of AR-IR cells coincided with certain recognized anatomical subdivisions of the cortical mantle. The most obvious was the broad division of the cortex into densely versus much more diffusely immunoreactive zones — a separation that followed the division of the rat cerebral cortex into its isocortical versus periallocortical/proisocortical domains. As described below, within each of these two cortical categories there were also instances of more subtle regional
differences in receptor distribution that distinguished some of the individual component areas.

**Periallocortex/Proisocortex**

The periallocortical/proisocortical fields in rats include the cingulate and retrosplenial areas of the medial cortical wall, and the ventrolaterally located insular and perirhinal cortices. Compared with the rest of the cortex, AR-immunoreactivity in these regions was noticeably sparse, and was weakest overall in the insular (Fig. 1E) and perirhinal cortices. In each of these three areas, the very few AR-IR cells that were present were scattered across all of the principal cellular layers of these areas (Fig. 1F). This contrasted the pattern observed in remaining allocortices, where receptors overall appeared to be somewhat more prevalent, and more clearly concentrated in the supragranular layers; in cingulate cortex (Cg1, 2, 3) AR-IR nuclei were most plentiful in layers II and upper III (Fig. 1F), and in retrosplenial areas (RSG, RSA), immunoreactive nuclei were concentrated mainly in component layer II (Fig. 1G).

The entorhinal and piriform cortices of the medial temporal lobe were also evaluated in this study. Like the prefrontal and retrosplenial areas (above), AR-immunoreactivity in these regions was sparse. In both piriform and entorhinal areas (Fig. 1D), immunoreactive nuclei were scattered among all major cellular layers and did not show any obvious preference or concentration in any particular layer or layers.

**Isocortex**

In rats, the isocortices occupy the lateral cortical surface and include all major primary and secondary sensory and motor areas. In each of these regions, AR-IR cells were much more numerous than in the allocortical fields, and were prominent in both supra- and infragranular cortical layers. These marked differences in the density of immunoreactivity highlighted the dorsomedial (Fig. 1F) and ventrolateral (Fig. 1E) boundary regions where areas of allo- and isocortices abut. The border separating area Cg1 from the premotor cortex (area Fr2), for example, coincided with a clear rise in the number and density of AR-IR neurons in layers II/IIIa, and the emergence of a second band of strong receptor-immunoreactivity in layers V and VI (Fig. 1F). This broad bilaminar pattern of labeling continued from premotor areas without obvious interruption into the laterally adjacent primary motor fields (areas Fr1, FL, HL; Fig. 5A). At the borders separating motor and sensory cortices, however, labeling took on a more conspicuously stratified pattern (Fig. 5C). Thus, in the primary and secondary somatosensory (Par1, Par2), auditory (Te1, Te2) and visual cortices (Oc1, Oc2) alike, AR-immunoreactivity conformed to a dense stratum of cells in layers II/IIIa, and a second, deeper and more discrete band of labeling that was dominated by nuclear immunoreactivity within the large pyramidal neurons of the lower half of layer V (Fig. 3B). Often seen especially within the sensory cortices as well was a periodic waxing and waning in the apparent density of supragranular labeling. Although varying in crispness from region to region, from animal to animal, and in some cases, from section to section, in virtually all subjects it was possible to discern clusters of denser labeling, usually 500–750 µm across, that alternated with narrower zones (∼250 µm wide) of reduced AR-immunoreactivity (Fig. 3D, E). There was no obvious tangential organization to the labeling in infragranular layers.

**Connections of Cortical AR-IR Neurons**

The abundance of AR-IR pyramids in layers II/III, V and to a lesser extent layer VI suggested that the afferent targets of these cells could include the neostriatum, thalamus, superior colliculus and/or cerebral cortex, as each of these regions receives a major cortical input from cells located in one or more of these layers (e.g. Donoghue and Parham, 1983; Olavarria and Van Sluyters, 1983; Isseroff et al., 1984; Miller and Vogt, 1984; Killackey et al., 1989; Hayama and Ogawa, 1997; Desbois and Villanueva, 2001; Ding et al., 2001). These predictions were tested by co-mapping and quantifying the retrograde labeling produced by injections made in either the thalamus (ventrolateral and ventral anterior nuclei; Fig. 4A), the superior colliculus (Fig. 4D), the caudate/putamen (all quadrants; Fig. 4B), the nucleus accumbens (core and shell; Fig. 4C), or in the somatosensory, primary motor, or cingulate (Fig. 4E) divisions of the cerebral cortex (see Fig 4), with AR-immunoreactivity (e.g. Fig. 5).

Injections made in each of the subcortical target areas evaluated produced fields of retrogradely labeled pyramidal cells that conformed to expected patterns and that at least partially overlapped with fields of AR-immunoreactivity (Figs 6–8). Thus, large injections in the medial and lateral caudate back-
labeled cells in layer V and to a lesser extent, layers III and VI of ipsilateral cingulate and sensorimotor cortices, respectively, as well as in corresponding layers of the contralateral hemispheres (Fig. 6); injections in the nucleus accumbens most strongly labeled the infragranular layers of ipsilateral cingulate and insular cortex (Fig. 7A,B) and yielded much sparser labeling in these areas contralaterally; injections in the superior colliculus produced labeling mainly in layers V and VI of the ipsilateral retrosplenial cortex, and layer V of ipsilateral visual and adjacent auditory cortices (Fig. 7C,D); and the thalamic injections retrogradely labeled dense populations of cells in layers V and VI of ipsilateral and to a minimal extent, contralateral somatosensory and pre- and primary motor regions (Fig. 8). For each of the subcortical sites evaluated, however, cell-by-cell examinations carried out across a series of five sections revealed on average <5% coincidence between retrogradely labeled cortical cells and those bearing AR-immunoreactivity (Fig. 9A). Although such doubly labeled cells were readily identified by their blackened nuclei and surrounding grain-filled cytoplasm (Fig. 5A,B), the thalamic injections tended to produce the greatest coincidence among labeling which rarely exceeded more than to two or three double labeled cells within a given section (Figures 8 and 9A). More typical for the subcortical sites evaluated were sections from for example, animals in which the nucleus accumbens or caudate nucleus had been injected in which the two labels marked non-overlapping subsets of pyramidal neurons (Figs 5C–E, 6, 7 and 9A).

In other sets of animals, injections were made in cingulate (deep layers only), primary motor, and primary somatosensory cortex (supragranular and infragranular injections). As expected, each site produced cortical labeling that involved specific cytoarchitectonic fields in both cerebral hemispheres, and specific sets of cortical layers (e.g. Donoghue and Parham, 1983; Olavarria and Van Sluijters, 1983; Isseroff et al., 1984;
Androgen Receptors in Rat Cerebral Cortex • Kritzer

Miller and Vogt, 1984). When examined for registry with AR-immunoreactivity, considerably higher proportions of these retrogradely labeled cells were found to be immunoreactive for AR compared with those labeled by injections made any of the subcortical target areas investigated (Figs 9–11). Some apparent differences in the prevalence of AR-immunoreactive retrogradely labeled cells were also noted, depending on the cortical region injected and on the cortical region, layer, and/or hemisphere evaluated (Fig. 9B). Injections made in infragranular cingulate cortex for example, produced retrograde labeling in the medial prefrontal cortex that was most dense in layers III and V, and more scattered in layer II (Figs 9B and 11A, B). In the supragranular layers where receptor immunoreactivity was also abundant between 45 and 54% of the retrogradely labeled cells sampled within the injected hemisphere were also AR-immunoreactive (Fig. 9B). More modest degrees of coincidence were seen, however, in the deeper layers where retrogradely labeled cells were plentiful but AR-IR cells were scarce (Figs 9B and 11A, B). The callosal labeling produced by cingulate injections also occupied layers II, III and V, but overall was lighter than the labeling present in the injected hemisphere, and in both supragranular and infragranular strata showed slightly lower degrees of overlap with AR-immunoreactivity as well, with roughly 35% of the callosally labeled cells in the supragranular layers and between 6 and 8% of the cells sampled in the infragranular layers being AR-immunopositive (Figs 9B and 11A, B).

Injections in somatosensory and motor areas produced characteristic, laterally widespread labeling; for motor injections, labeling tended to spread laterally into adjacent sensory territories, and for sensory injections, label spread medially into motor areas. For both areas, superficially placed injections produced discrete columns of labeling that were most cell dense in layers II/III, and narrower and more cell sparse in layers V/VI. Evaluation of double labeling in representative sections revealed that in the injected hemispheres, 31–51% of the cells making up these columns in motor cortex, and 70–82% of these cells in somatosensory fields, whether located adjacent (Figs 9B and 11C) or several millimeters lateral to the parent injection site (Fig. 11E), were also AR-immunoreactive. Callosal labeling, on the other hand, showed lower degrees of overlap with AR-immunoreactivity in both motor (22–25% of
sampled cells) and somatosensory cortex (21–33% of sampled cells; see Figs 9B and 11D). The more superficial as well as more deeply placed injections sites in sensory and motor cortices also produced strong bands of laterally extensive labeling in layers V and VI (Fig. 11F,G). In injected hemispheres, about a third of these labeled cells in layers V/VI motor cortex, and 75–80% of the labeled cells in layer Vb of somatosensory fields were AR-immunoreactive (Fig. 9B). However, there was little if any overlap observed between retrograde labeling and the relatively few AR-IR pyramids present in layers Va and VI of the somatosensory cortex (Fig. 11F). As for all cortical injections, the patterns of retrograde labeling in these infragranular strata were largely repeated in the un.injected hemisphere, albeit with a reduced density of retrograde labeling overall, and with markedly reduced degrees of overlap between retrograde labeling and hormone receptor immunoreactivity in both motor cortex (7–13% of sampled cells) and in somatosensory fields where overlap was negligible (Figs 9B and 11G).

Discussion

Androgens impact an array of cortical structures and functions both during development and in adulthood (e.g. Leranth et al., 2003; Kritzer et al., 2001; Kritzer, 2000; Christiansen and Knussman, 1987; Stenn et al., 1972). Despite the prevalence of intracellular nuclear androgen receptors, at least in comparison with other hormone receptors, little is known about where in the cortex this receptive machinery lies. As these receptors are likely to contribute at least in part to observed androgen effects on the cerebrum, it is important to determine where within the cortical mantle these signaling pathways may be brought to bear. To address this issue, a comprehensive series of studies was undertaken to map the distributions of intracellular AR proteins in the cerebral cortices of hormonally intact adult male and female rats. Using combined immunocytochemical and tract tracing methods, the regional, laminar, cellular and connectional signatures of AR-immunoreactivity were identified in all major divisions of the cortical mantle. In all, three basic patterns of receptor distribution were found; light, diffuse and concentrated mainly in the supragranular layers in association areas of the cortex; densely distributed in layers II/III and V/VI in motor areas; and dense in layers II/III and Vb in primary and secondary sensory cortical regions. As discussed below, however, a common theme to each of these
patterns was the prominent alignment of AR-immunoreactivity with cells making corticocortical and to a lesser extent corticothalamic connections — both pivotal among circuit systems contributing to cortical information processing.

**AR in the Cerebrum: Comparisons to Previous Studies**

Androgen receptors have been mapped in forebrain areas of rats including the cerebral cortices using methods of ligand binding (Sar and Stumpf, 1985), in situ hybridization (Simerly et al., 1990), and immunocytochemistry (Clancy et al., 1992).

All of these studies share basic conclusions concerning the cortical distributions of the intracellular AR markers examined. First, all agree that there is an abundance of androgen receptors in the adult rat cerebral cortex. Further, in studies where receptors were compared across males and females, no obvious sex differences in receptor distribution or apparent density were found (Simerly et al., 1990; Clancy et al., 1992).

Finally, many of the anatomical details of cortical receptor distribution are consistent. For example, similar to the regional differences noted in this study, previous in situ hybridization studies revealed moderate to dense numbers of AR mRNA containing cells in isocortical areas, and sparser receptor signals in piriform and entorhinal areas (Simerly et al., 1990). These studies also reported a bilaminar distribution of receptor mRNA in layers II/III and V/VI that matches the supragranular and infragranular concentrations of AR-immunoreactivity identified in this study for most lateral cortical regions.

The only previous immunocytochemical investigation of AR in rat cerebral cortex was limited to a single cortical level and used a different antibody to label receptor proteins than either of the two used here; while this study described receptor immunoreactivity only in infragranular layers, the receptor-immunopositive cells within these layers were described as pyramidal or fusiform in morphology (Clancy et al., 1992), which is consistent with the appearance AR/NSE-IR neurons identified in deep cortical strata in this study.

In contrast to studies in rats, studies of AR-immunoreactivity in the cortex of human and non-human primates have produced results that are quite different from those obtained in rodents. For example, in rats there are fewer androgen receptors in prefrontal compared with sensorimotor cortices (Simerly et al., 1990, present study), whereas in juvenile monkeys the association areas of the frontal lobe contained among the highest levels of androgen binding (Clark et al., 1988). Further, while in rats AR-immunoreactivity tends to be fairly well localized to particular layers, in primates AR-immunoreactivity is more evenly distributed and is modest to dense in all major laminae, including layer I (e.g. Clancy et al., 1992; Finley and Kritzer, 1999). Finally, the double-labeling strategies used in this study showed that in rats outside of piriform and entorhinal areas, cortical AR-immunoreactivity is sequestered mainly in pyramidal neurons. In biopsied samples of human temporal cortex, however, analyses of adjacent
paraffin sections localized AR-IR nuclei to neurons of varied morphology, as well as to GFAP-immunoreactive astrocytes, microglia identified by the lectin *Ricinus communis* agglutinin-1, and to histologically identified oligodendrocytes (Puy *et al.*, 1995). Double-label immunocytochemistry in the prefrontal cortices of rhesus monkeys also identified as many as half of all cortical AR-IR profiles as GFAP-immunoreactive astrocytes (Finley and Kritzer, 1999). That this latter study had both histochemical procedures and immunochemical markers in common with the present study adds strength to conclusions that there may be qualitative differences in the regional, laminar and cellular distributions of androgen receptor-bearing cells — at least in association cortices, in rats versus primates. These differences should be borne in mind, especially when considering how experimental findings in rodents relate to observed sex differences and/or androgen stimulation of higher order cortical function and cortical dysfunction in disease in man (Stenn *et al.*, 1972; Christiansen and Knussman, 1987).

**AR in the Adult Rat Cerebrum: Comparison with ER**

Previous light microscopic studies examining the regional, laminar, and cellular distributions of immunoreactivity for the classical and beta isoforms of intracellular estrogen receptors (ER\(\alpha\), ER\(\beta\)) in the rat cerebrum revealed that these two receptor subtypes are sequestered in the nuclei of non-overlapping subpopulations of cortical neurons (Blurton-Jones and Tusznyski, 2002; Kritzer, 2002). Based on findings from the present study, there seems little possibility of anatomical overlap between nuclear estrogen receptors and AR-immunoreactivity as in most areas of the cortex their distributions are complementary. For example, while AR-immunoreactivity is least dense in areas surrounding the rhinal sulcus, those insular cortices are precisely where nuclear ER\(\alpha\)-immunoreactivity is most enriched (Kritzer, 2002). Further, for cortical regions and layers where ER\(\alpha\), ER\(\beta\) and AR-IR nuclei may be spatially overlapping, ER-immunoreactivity is found in the nuclei of either PV-immunonegative and -immunopositive non-pyramidal interneurons (Blurton-Jones and Tusznyski, 2002; Kritzer, 2002) while AR-immunoreactivity is usually associated with the nuclei of pyramidal cells. There may be exceptions to
colocalize ERα and ERβ to individual, identified cholinergic neurons in rat basal forebrain (Shughrue et al., 2000).

Other than the possibilities sited above, immunoreactivity at least for nuclear intracellular ER and AR seems to occupy anatomic and functionally distinct sets of circuit elements in both cerebral and hippocampal cortex (Milner et al., 2001). These differences in distribution, and in particular the alignment of AR with excitatory projection versus the alignment of ER with local inhibitory cortical cells, could be substrates for the separate transcriptional actions of estrogens versus androgens in stimulating the cerebral cortex, perhaps including their opposing influences on, for example, endpoints of cortical development (Zhang et al., 2000) and neurotransmitter physiology (Handa et al., 1997).

**AR in the Adult Rat Cerebrum: Connectional and Functional Implications**

The localization of AR-IR within the nuclei of PV-IR or CB-IR interneurons in the entorhinal and piriform cortex gives intracellular androgen receptors in these phylogenetically older regions functional connotations of local inhibition. The calcium binding protein signatures of these cells may also be telling of the synaptic relations maintained by AR-IR inhibitory neurons, as in other areas of rat cortex PV-immunoreactive interneurons are either basket or chandelier cells that synapse on the somata or axon initial segments of target neurons, and CB-immunoreactive neurons include double bouquet, neogliaform and Martinotti cells, whose terminals contact the dendritic domains of postsynaptic cells (Celio, 1990; Kubota et al., 1994; Gabott et al., 1997). In areas beyond the medial temporal lobes, however, AR-IR nuclei are sequestered perhaps exclusively in pyramidal neurons, thus linking androgen signaling to excitatory, projection rather than local inhibitory cortical neurons.

Although the layers where AR-IR pyramids are especially enriched (layers II/III, V/VI) house cells of origin of multiple afferent systems, studies combining retrograde tracing and immunocytochemistry indicated that few to no AR-IR cells project to the superior colliculus, thalamus, or basal ganglia. However, injections made within the cortex itself revealed that substantial proportions of potentially androgen-sensitive pyramids are involved in making corticocortical connections. Further, there seems to be a relatively stronger relationship between androgen receptors and associationally projecting neurons that interlink cortical regions within the same hemisphere. This was especially so for sensory and motor cortices where it was consistently found that significantly more retrogradely labeled cells in injected hemispheres cells were receptor-immunoreactive compared with the callosally projecting cells labeled by the same injections. This finding was surprising in view of the literature identifying sex differences in androgen stimulation of the morphometrics and/or axon density of the corpus callosum in rats (Berrebi et al., 1988; Fitch et al., 1991; Nunez and Juraska, 1998).

While the richness of colocalization seen between cortically projecting cells and AR-immunoreactivity was impressive, it is tempting to speculate that analyses of larger cortical injection sites, which tend to produce more densely labeled cortical fields (Kritzer et al., 1992; Kritzer and Goldman-Rakic, 1995) could uncover even higher degrees of overlap between retrograde labeling and androgen receptors. At the same time, however, it is clear that not all of the AR-IR cells of the cortex...
were accounted for by the projection systems evaluated in this study. Connectional signatures for the AR-IR neurons scattered within in layers IV and lower VI, for example, were not identified. These and perhaps other groups of AR-IR neurons could represent populations of androgen-sensitive cells that project to as yet unexplored brain areas, e.g. brainstem or spinal cord (Killackey et al., 1989). Nonetheless, substantial proportions of AR-IR neurons in the rat cerebral cortex do participate in making local, long lateral intrahemispheric, and to a lesser extent interhemispheric cortical connections. Because these connections have established roles in sculpting cortical receptive field properties (e.g. Gilbert, 1983; see Katz and Callaway, 1992), and in networking the functionally co-activated cortical regions engaged in sensory, motor, and higher order tasks (see Goldman-Rakic, 1987), androgen signaling pathways may be aligned with elements of circuit structure that have critical involvement in the cortical processing of sensory, motor, and higher order information at some of its most complex levels.

Notes
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