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Androgen Receptor Immunoreactivity in Forebrain Axons and Dendrites in the Rat

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As members of the steroid receptor superfamily, androgen receptors (ARs) have been traditionally identified as transcription factors. In the presence of ligand, ARs reside in the nucleus, where, upon ligand binding, the receptors dimerize and bind to specific response elements in the promoter region of hormone-responsive genes. However, in this report, we describe the discovery that ARs are also present in axons and dendrites within the mammalian central nervous system. AR expression in axons was identified in the rat brain at the light microscopic level using two different antibodies directed against the N terminus of the AR protein and nickel intensified 3′-3′-diaminobenzidine, and also using fluorescence methods and confocal microscopy. This distribution was confirmed at the ultrastructural level. In addition, AR immunoreactivity was identified in small dendrites at the ultrastructural level. AR-immunoreactive axons were observed primarily in the cerebral cortex and were rare in regions where nuclear AR expression is abundant. The observation that ARs are present in axons and dendrites highlights the possibility that androgens play an important and novel extranuclear role in neuronal function. (*Endocrinology* 144: 3632–3638, 2003)

ANDROGENS AND ANDROGEN receptors (ARs) have powerful effects on the function of the central and peripheral nervous system and play a crucial role in maintaining masculine reproductive behaviors and neuroendocrine regulation (1). At the cellular level, androgens exert survival and trophic effects on healthy developing (2–5) and adult neurons (6), prevent or promote cell death in response to neurotoxic insults (7, 8), and promote the regenerative capacity of damaged adult neurons (9, 10).

Testosterone, the most prominent circulating form of androgen in males, can be metabolized in the nervous system into dihydrotestosterone (DHT) or estradiol. Traditionally, therefore, androgens have been considered to exert their effects via two classical steroid receptors, the AR and estrogen receptor (ER) (11). Both receptor types belong to the ligand-activated nuclear receptor family (12). Members of this family have been defined by their ability to bind to specific DNA sequences in promoter regions of hormone-responsive genes. Upon binding to these hormone response elements in the DNA, the receptors are capable of exerting powerful modulatory effects on transcription of specific genes. Thus, the steroid receptors have been identified as having a nuclear function. In addition to the classically defined nuclear action of steroid receptors, membrane-associated receptors for gonadal steroid hormones have been postulated for years, based primarily on the rapid actions of steroids that could not be attributed to transcriptional regulation (13–17). Considerable evidence has accumulated to support the existence of these nonnuclear receptors (18–26) and thus, ideas about the mechanisms of gonadal steroid receptor action have been broadened to include extranuclear sites.

In this report, we describe the observation that AR immunoreactivity is present in axons and dendrites in the rat forebrain. The distribution of AR-immunoreactive (AR-ir) axons within the forebrain, as detected at the light microscopic level, is restricted primarily, although not exclusively, to the cerebral cortex. This unique intracellular distribution of AR points to several intriguing putative mechanisms for AR action that remain unexplored.

Abbreviations: AR, Androgen receptor; AR-ir, AR immunoreactive; DAB, 3′-3′-diaminobenzidine; DHT, dihydrotestosterone; ER, estrogen receptor; PB, phosphate buffer.

Materials and Methods

Animals and tissue processing

Adult male Wistar rats from the in-house breeding colony at the Instituto Cajal were used, as well as adult male Sprague Dawley rats (Zivic-Miller, Pittsburgh, PA) housed at Loyola University Chicago. Animals were kept on a 12-h light, 12-h dark cycle, with *ad libitum* access to food and water. One group of animals (n = 3) was gonadectomized under sodium pentobarbital anesthesia (40 mg/kg body weight) one week before the animals were killed. Other animals (n = 10 for light and confocal microscopic work; n = 3 for electron microscopic studies) were gonadally intact at the time the animals were killed.

Experiments were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals, guidelines established by the European Union (86/609/EEC), and the Institutional Committee on Animal Care and Use at Loyola University.

Immunocytochemistry

Animals were anesthetized with sodium pentobarbital (50 mg) then perfused via the left cardiac ventricle with 0.9% NaCl followed by 200–300 ml of 4% paraformaldehyde in 0.1 M PBS. Brains were postfixed

for 4 h or overnight at 4 C. Brains were then transferred to PBS and sectioned on a vibrating microtome the next day, or alternatively, sunk in 30% sucrose-PBS, then frozen in dry ice and sectioned on a sliding microtome. Sections were cut at 40 micrometers and stored in ethyleneglycol based cryoprotectant at −20 C until processing for immunocytochemistry.

For light microscopic detection of AR immunoreactivity, two different polyclonal antibodies made in rabbit were used, PG-21 (3 μg/ml, gift from Dr. Gail Prins, University of Illinois, Chicago, IL) and N-20 (1 μg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Primary antibodies were diluted in 3% normal goat serum, 0.3% Triton X-100, PBS. Free-floating sections were incubated overnight at 4 C in primary antibody, then at room temperature for 2 h in secondary antibody (biotinylated goat antirabbit, from Vector Laboratories, Burlingame, CA; diluted 1:250 in PBS), then 1 h in ABC reagent (Immunopure ABC peroxidase staining kit, made according to kit instructions, in PBS; Pierce, Rockford, IL), with extensive rinses between incubations. After several rinses in sodium acetate buffer (0.1 M, pH 7.2), sections were transferred to a solution containing 50 mM nickel ammonium sulfate, 40 mg% 3′-3′-diaminobenzidine (DAB) and 0.0075% hydrogen peroxide in sodium acetate buffer. Sections were transferred to fresh sodium acetate buffer to stop the reaction, then rinsed in several changes of PBS and mounted onto chrom-alum/gelatin subbed slides. Slides were dehydrated and coverslipped, then examined using a Leica (Wetzlar, Germany) DMR microscope. Sections were examined from the olfactory bulb through the caudal brainstem to identify potential sites of AR-ir fibers. NeuroLucida software and a MicroBrightField imaging system (Burlington, VT) were used to produce a parasagittal map through the forebrain and brainstem to illustrate the distribution of AR-ir fibers. The Paxinos and Watson atlas was used to assist in cytoarchitectonic analyses (27).

For fluorescence immunocytochemistry, sections were washed as above, incubated in PG-21 overnight, and then incubated in a Cy-2 conjugated goat antirabbit secondary antibody (diluted 1:100 in rinsing solution; Jackson ImmunoResearch Laboratories, West Grove, PA). The tissue was then examined using a confocal scanning laser microscope (Zeiss LSM 510 with argon 458/488 and HeNe 543 lasers; Carl Zeiss, Jena, Germany).

For electron microscopy, animals were perfused with saline followed by a fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB). Brains were then immersed in the fixative for 1 h, rinsed in PB, and coronally sectioned on a vibrating microtome at 50 μm. The areas of interest were dissected free, collected into Eppendorf vials containing 10% sucrose in PB, and allowed to sink in the sucrose solution. To enhance antibody penetration, the vials were immersed in liquid nitrogen for 15 sec and then allowed to melt at room temperature. This procedure was repeated three times. Sections were then processed for immunocytochemistry following the protocol described above for light microscopy and the PG-21 antibody, except that Triton was omitted. The development of the peroxidase activity was performed with DAB, intensified or not with nickel. Sections were postfixed in 1% osmium tetroxide in 0.1 M PB with 5% glucose, dehydrated in an acetone gradient and embedded in Epon 812. Ultrathin sections (50 nm) were cut on an ultramicrotome, mounted onto grids and were either lightly counterstained with uranyl acetate and lead citrate or unstained. Sections were observed with a Joel 1200EXII electron microscope and photographed. The criteria used to identify a structure as an axon were that it was myelinated (to identify the shaft of an axon) or it contained presynaptic vesicles. Dendrites were identified by their contact with presynaptic terminals and the presence of postsynaptic densities.

In control experiments, sections were processed through all steps of the immunocytochemistry except that a) the primary or secondary antibody was eliminated or b) the primary antibody (used at a concentration of 1 μg/ml) was preadsorbed overnight with the immunizing peptide [5-fold excess by weight, as per instructions of Upstate Biotechnologies (Rochester, NY), calculated as 5 μg immunizing peptide per microgram protein in the antiserum].

Results

Nuclear staining for AR

With methods to detect AR immunoreactivity using conventional light microscopy, strong nuclear staining was observed with both the PG-21 and N-20 antibodies in numerous forebrain regions in all gonadally intact animals (Fig. 1). The staining intensity within neurons was similar or slightly greater with the PG-21 antibody than with the N-20, but the distribution of the nuclear labeling was identical. Strong nuclear signal was also observed with the confocal microscope in the tissues reacted with PG-21 and a fluorochrome-tagged secondary antibody (not shown). Preadsorption of the primary antiserum, or elimination of the primary antiserum, eliminated nuclear staining (Fig. 1). In addition, a basic local alignment search tool (BLAST) analysis for peptide sequences homologous to those used as antigens to produce the PG-21 and N-20 anti-AR antisera yielded no hits, suggesting no overlap with other known amino acid sequences.

Regional distribution of AR expression in cell nuclei

The regional distribution of nuclear labeling corresponded entirely to previous reports on the distribution of AR expression in the rodent brain, which has been reported elsewhere (28–33). Briefly, intensely labeled AR-ir nuclei were detected within the bed nucleus of the stria terminalis and the medial preoptic nucleus, the medial amygdala, and, in the

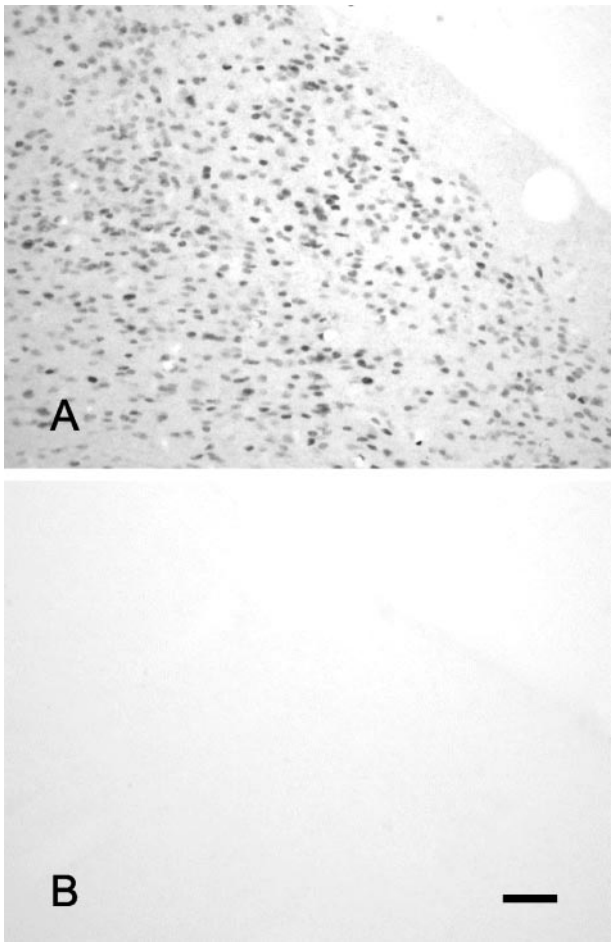

FIG. 1. Photomicrographs of nuclear AR stain in the medial amygdala (A) and the lack of AR staining in an adjacent section incubated in primary antiserum that had been preadsorbed with antigen (B). *Scale bar*, 50 μm.

hypothalamus, in the arcuate, ventromedial, ventral premammillary, paraventricular, and periventricular nuclei. Numerous moderately labeled AR-ir cells were also detected in CA1 and CA3 regions of the hippocampal formation and central and cortical nuclei of the amygdala. Abundant but less intensely stained AR-ir nuclei were also found in many regions of the cerebral cortex, with different cortical regions displaying a region-specific laminar distribution of AR expression.

Cellular and regional distribution of AR expression in neurites

Abundant AR-ir fibers (Fig. 2) were observed in gonadally intact animals using the PG-21 antibody and methods intended for either light microscopic detection or confocal microscopy. These AR-ir neurites had the appearance of axons, with punctate swellings along their course that appeared to belong to synaptic boutons. AR-ir fibers could frequently be followed up to 100 μm, and often further. None of the fibers could be traced to a cell soma. A thorough survey of the forebrain and brainstem did not reveal any cell bodies with long AR-ir fibers emerging from them. From here on, these neurites, as observed at the light microscopic level, will be referred to as axons.

AR-ir presumptive axons were most abundant in discrete regions of the cerebral cortex (Fig. 3). These axons were frequent in layer I, coursing parallel to the pial surface of the brain. AR-ir axons were also abundant in other cortical layers, especially in layers II–III, perpendicular to the cortical surface, and layer VI, in no predominant orientation. There were regional, as well as laminar, differences in AR-ir axon distribution in the cerebral cortex; more were observed in the piriform, entorhinal, perirhinal, and cingulate regions of the cortex than in the parietal cortex, and the frequency was higher in caudal than in rostral regions. Small numbers of AR-ir axons were also observed in the medial septum and vertical limb of the diagonal band of Broca and the hippocampal formation. In the amygdala, immediately lateral to the cortical nuclei, numerous long AR-ir axons were observed running both parallel and at an angle to the ventral surface of the brain. AR-ir axons were frequently observed in the cingulum and external capsule, remaining within the white matter or continuing on into deep layers of the cerebral cortex. AR-ir axons were also observed in the corpus callosum and the anterior commissure in some specimens. Very few AR-ir axons were found in regions where intensely stained nuclei were observed, such as the preoptic area, bed nucleus of the stria terminalis, and hypothalamus. There were no marked regional differences in the appearance of AR-ir axons, although the length that they could be followed was greater in the cerebral cortex and amygdala than in other regions. In addition, there were no apparent qualitative differences in the distribution of AR-ir axons in Wistar and Sprague Dawley rats.

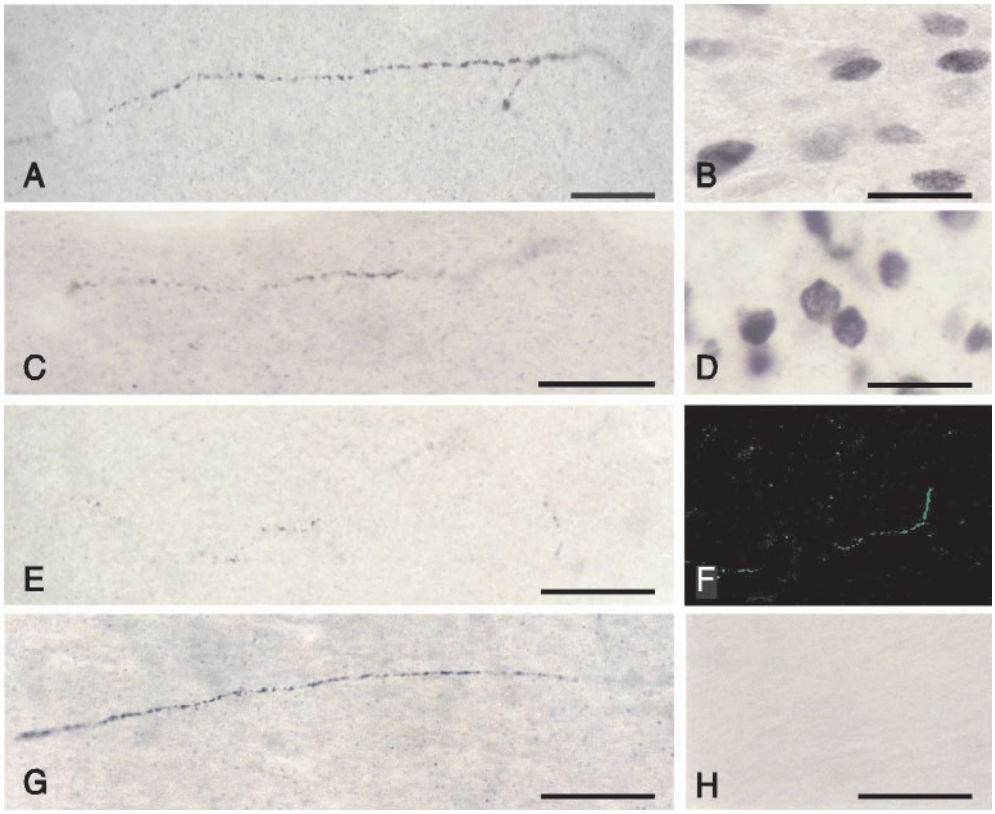

FIG. 2. A, C, E, and F, AR-positive neurites in the cerebral cortex and (G) in the corpus callosum. B and D, AR-positive nuclei in the medial amygdala (B) and premammillary nucleus (D). F, Confocal image. H, Control section adjacent to that in G; preadsorption eliminated all staining. Primary antibodies: PG-21, A and B, E–H; N-20, C and D. All sections from intact male rats except E, which was from a gonadectomized male. *Scale bar*s, 20 μm.

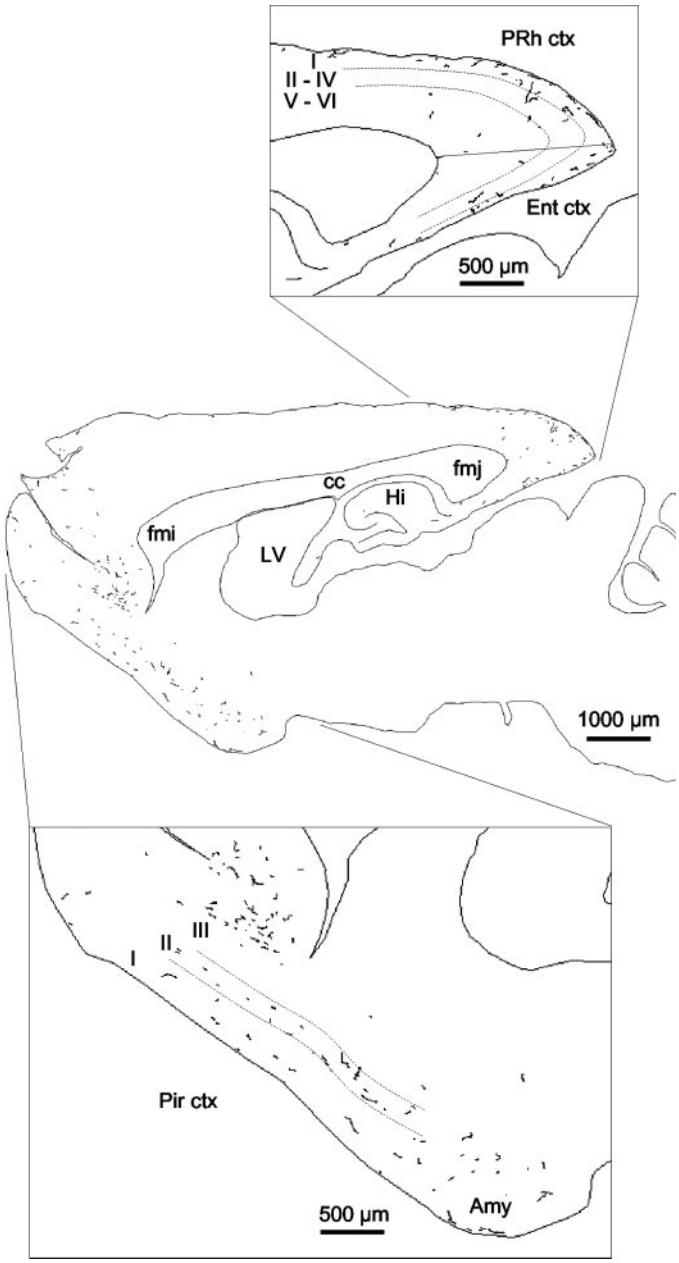

FIG. 3. Distribution of AR-ir axons in the forebrain and brainstem. This map was produced using NeuroLucida software (MicroBrightField Inc.) and illustrates a single 40-μm parasagittal section, 2.9 mm lateral to bregma (27). The entire section was scanned at $\times$40, and all axons observed were traced. An adjacent Nissl-stained section was used to delineate cortical layers. Amy, Amygdala; cc, corpus callosum; ctx, cortex; Ent, entorhinal cortex; fmi, forceps minor of the corpus callosum; fmj, forceps major of the corpus callosum. Hi, hippocampal formation; LV, lateral ventricle; Pir, piriform cortex; PRh, perirhinal cortex.

The PG-21 antibody produced intense and abundant signal in axons in gonadally intact animals. Within cortical regions, up to several hundred axons were observed per 40-μm section. However, AR-ir axons were extremely rare in the gonadectomized males (Fig. 2); AR-ir axons were only observed in one of the three gonadectomized males, and in this case only three axons were found throughout the forebrain, and these three were in the cerebral cortex. Similarly, AR-ir nuclei were virtually absent in the gonadectomized males, with a small number of cells with cytoplasmic label in the lateral septum and periventricular nuclei.

Tissue that had been incubated with the N-20 antibody also contained AR-ir axons. The frequency of detectable axons was much lower than observed with the PG-21 antibody and the length that the axons could be followed was shorter (Fig. 2).

Ultrastructural analyses confirmed the existence of AR-ir axons (Fig. 4), based on the stringent criteria used. In addition, ultrastructural analyses revealed a low level of AR expression in small dendrites and somata in pyramidal neurons in the neocortex and in the amygdala (Fig. 4). In some cases, the AR immunoreactivity was localized within presynaptic or postsynaptic sites. The frequency of types of synapses was not explored in these qualitative studies. Perimitochondrial labeling was also frequently observed (Fig. 4).

Elimination of the primary or secondary antibodies or preadsorption with the immunizing peptide abolished all staining (Figs. 1 and 2).

Discussion

The existence of AR immunoreactivity in axons and dendrites suggests a unique mechanism of action for androgens that has not been previously explored. The morphology of these fibers observed at the light microscopic level, with their long processes punctuated by varicosities with the appearance of synaptic boutons, is consistent with an axonal localization, as confirmed by the ultrastructural results.

Numerous AR-ir axons could be observed emanating from the cingulum and dorsal external capsule, and AR-ir axons were observed in the corpus callosum and anterior commissure of some specimens, although we did not observe any fiber tracts densely filled with immunoreactive fibers.

None of the AR-ir axons in the cerebral cortex could be followed directly back to a neuronal cell body, nor were AR-ir fibers observed emerging from cell bodies in any region of the forebrain or brainstem, suggesting that insufficient AR is present in the proximal axon to identify the cell body of origin. Numerous AR-ir axons were observed in layer I coursing parallel to the pial surface, arguing that the source of the AR-ir axons may be local, although the identification of AR-ir axons in the corpus callosum and external capsule, and of myelinated axons, would argue for a more distant site of origin.

The most abundant population of AR-ir axons that we have observed to date was located in the cerebral cortex as demonstrated in the parasagittal map (Fig. 3). The more typical, and expected, pattern of AR protein expression within nuclei was also observed in the cortex, but in contrast with nuclear staining in subcortical cell groups within the septum, preoptic area, hypothalamus and amygdala, the staining intensity within cortical cell nuclei was much lower. In most areas with intensely labeled AR-positive nuclei, sparse or no AR-positive axons were observed.

Although no AR-ir dendrites were observed at the light microscopic level, the ultrastructural analyses revealed a population of small dendrites that were AR positive. Given

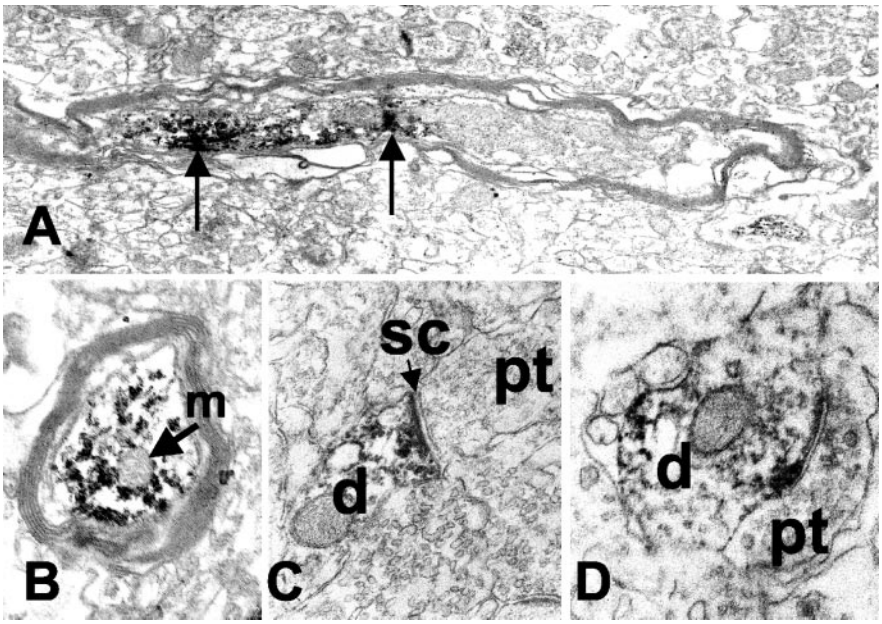

FIG. 4. Ultrastructural localization of ARs in axons and dendrites in the cerebral cortex. The dense granular clusters represent label. A, Longitudinal; and B, cross-sectional views. *Arrows* in A point to dense clusters of DAB-nickel deposits. The *arrow* in B points to a mitochondrion with perimitochondrial label. C and D, Small AR-positive dendrites. d, Dendrite; m, mitochondria; pt, presynaptic terminal; sc, synaptic cleft.

that the label in the dendrites was punctate and not homogeneously distributed throughout the dendrite, it is perhaps not surprising that the label was not observed at the light microscopic level. A similar finding has been reported for the ER-positive dendrites in the hippocampal formation, which can only be identified at the ultrastructural level (34).

Several different pieces of evidence, of both a biological and technical nature, point to the specificity of AR immunoreactivity in axons and dendrites in the rat. First among the biological evidence is that the distribution of the AR-ir axons is restricted to specific brain regions. Second, punctate AR immunostaining in fibers has been described in the lizard cortex (35, 36), suggesting some phylogenetic conservation of this expression. Third, AR-ir axons were abundant in gonadally intact male rats but extremely rare in gonadectomized animals, suggesting that the presence of AR protein in axons depends upon gonadal hormone exposure. Fourth, other steroid receptors have been identified in axons; ultrastructural studies have identified ER immunoreactivity in axon terminals in the ventrolateral nucleus of female guinea pigs (37, 38) and in the rat lateral habenula (39) and hippocampal formation (34, 40).

The technical evidence arguing in favor of the specificity of AR immunoreactivity in axons is principally that staining was abolished by elimination of the primary or secondary antibody or preadsorption of the primary antibody with the immunizing peptide. In addition, AR-ir axons were observed with different detection methods, that is, with either the ABC method and nickel-intensified DAB as the chromagen, or with a fluorochrome-labeled secondary antibody, and AR-ir axons were detected with two different AR antibodies, PG-21 and N-20. PG-21 and N-20 are similar in that both are polyclonal antisera made in rabbit, and both were directed against a synthetic peptide mapping at the amino terminus of the AR, specific for rat AR (41) and human AR, respectively. The number and intensity of stained axons was greater by far with PG-21 than with N-20, perhaps because of differences in the human and rat AR sequence in the amino terminus (42).

ARs may function in a ligand-dependent manner as essentially a traditional transcription factor, but in an extranuclear location, by binding to hormone response elements in mitochondrial genes. Mitochondrial genes have been identified that are regulated by testosterone (43, 44) and have functional androgen response elements (44). However, to date, we have observed peri-mitochondrial labeling at the ultrastructural level, but not intramitochondrial label, arguing against this possibility.

A second, perhaps more likely, function for ARs in axons may not involve the transcriptional regulatory function of the AR, but instead modulation of membrane or cytoplasmic physiology. In bone, both androgenic and estrogenic ligands are able to bind to ARs or ER α or β and protect osteoblasts and osteocytes from induced apoptosis (25); this effect depends on the presence of the ligand-binding domain and a nonnuclear distribution of receptor. Moreover, the antiapoptotic effects of the steroids appear to be mediated through Src-dependent phosphorylation of ERK and are not dependent on transcriptional activity. These authors and others (24, 26) have suggested that the classical steroid receptors may have rapid nongenotropic actions through interaction with signal transduction pathways, perhaps binding to caveolin or similar proteins, such as flotillin, which tether functionally related components of several signal transduction cascades to docking sites (*e.g.* caveolae). A recent study demonstrated that ARs, and in particular the ligand-binding domain of AR, coprecipitate with caveolin-1, and this interaction is ligand dependent (45). Thus, ARs located in the cytoplasm may also participate in intracellular signal transduction cascades. As previously mentioned, membrane-associated functions for steroid hormone receptors have also been postulated for many years, and there is now compelling evidence from transfection experiments that functional ERs can be expressed in the membrane and are capable of activating signal

transduction cascades (21). Whether ARs have a similar capacity to activate signal transduction cascades via actions in the membrane or cytoplasm is unknown, but is a possibility that must now be explored in light of the present findings.

Studies of AR dynamics have shown that unliganded ARs are present primarily in the cytoplasm, moving into the nuclear compartment within 15–60 min, and back to the cytoplasm for recycling (46, 47). This observation is consistent with the finding that treatment with androgens up to 1 h before the animals were killed enhances nuclear AR staining in the brain (48). It is unlikely that ARs in axons represent a pool of receptors ready to translocate to the nucleus, because the receptors would have to be actively transported first down the axon and then back to the cell soma; the time frame for this retrograde transport would seem to be inconsistent with such a role for axonal ARs.

A wealth of studies have now shown that gonadal hormones, particularly estrogen, induce profound synaptic plasticity in multiple brain regions in the adult (49–51) as well as the developing brain (52). The finding that ERs (34, 38–40) and ARs are located in both presynaptic and postsynaptic elements may have important implications for the mechanisms involved in steroid-induced plasticity in neuronal architecture, in that steroids may modify neuronal architecture not only via the classic transcriptional regulatory mechanisms in the nucleus, but also perhaps via transcriptional effects on mitochondrial genes or via direct protein:protein interactions within the presynaptic and postsynaptic terminals.

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