Use of PG-21 Immunocytochemistry to Detect Androgen Receptors in the Songbird Brain

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Introduction
The neural circuit that regulates song behavior in songbirds has emerged as a leading model for studying the effects of gonadal steroid hormones on neural and behavioral plasticity. Several of the brain regions that control song behavior concentrate androgens and/or estrogens. Investigations of the distribution and regulation of androgen receptors have been limited by the lack of a reliable immunocytochemical method to detect androgen receptors in the songbird brain. We describe a protocol by which the PG-21 polyclonal antibody to the rat androgen receptor can be used to label androgen receptor-containing cells in the songbird brain. By treating songbirds of several species with testosterone 90 min before sacrifice and by using relatively low concentrations (0.5–0.75 μg/ml) of PG-21 antibody to reduce nonspecific background staining, we were able to obtain strong specific labeling of cell nuclei in androgen-sensitive brain regions. This technique will facilitate the study of the role of androgens in mediating neural plasticity in the avian brain. Testosterone pretreatment may also facilitate the use of this antibody to label androgen receptors in tissues from a wide array of nonmammalian species. (J Histochem Cytochem 44:1075–1080, 1996)

Key Words: Androgen receptor; Immunocytochemistry; Songbird; Song control nuclei.

Technical Note

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The avian song control system is an excellent model in which to study the effects of gonadal steroid hormones on neural and behavioral plasticity. Several of the brain regions that control song behavior concentrate androgens and/or estrogens. Investigations of the distribution and regulation of androgen receptors have been limited by the lack of a reliable immunocytochemical method to detect androgen receptors in the songbird brain. We describe a protocol by which the PG-21 polyclonal antibody to the rat androgen receptor can be used to label androgen receptor-containing cells in the songbird brain. By treating songbirds of several species with testosterone 90 min before sacrifice and by using relatively...
the avian song system. ICC has the advantage of being more rapid than autoradiography while offering equivalent or a greater sensitivity (Ben-Hur et al., 1993; Prins et al., 1992; Quarmby et al., 1990). Application of ICC to study ARs in the avian song control system, however, has been hampered by the lack of an antibody that selectively labels ARs in songbird brains. Balthazart et al. (1992) used a rabbit polyclonal antibody raised against a 21-amino-acid sequence beginning nine amino acids from the N-terminus of the rat AR (AR32; Quarmby et al., 1990) to label AR+ cells in the brains of zebra finches, canaries, and Japanese quail (Coturnix japonica). They observed labeled cells in IC0 and hypothalamic nuclei of all three species, and in HVC, MAN, and RA of the zebra finch and canary. Labeling in these areas was eliminated when the AR32 antibody was preabsorbed with the synthetic peptide used for immunization.

Using the AR32 antibody and the protocol of Balthazart et al. (1992), however, we have been unable to selectively label cells in brain regions of zebra finches and canaries known from autoradiographic studies to contain AR (GT Smith and EA Brenowitz, unpublished observation). Rather, we found that this antibody produced unacceptably high levels of nonspecific background labeling over a wide range of dilutions and incubation times. Given these results, we explored the use of a polyclonal rabbit antibody (PG-21) that is raised against amino acids 1-21 of the rat AR (Prins et al., 1991). This antibody has been successfully used to label ARs in a variety of tissues in mammals and fish (Freeman et al., 1995; Leibersberg and Nottebohm, 1979). A similar pattern of staining was observed in all four species studied. The staining pattern was clearly smaller than thionin-stained cell bodies in adjacent sections. This was particularly apparent in lateral MAN, RA, and nXIIIs, which contain very large neurons.

Materials and Methods

These experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals. We labeled AR+ cells in the brains of male Gambel’s white-crowned sparrows (Zonotrichia leucophrys gambelii), house sparrows (Passer domesticus), song sparrows (Melospiza melodia), and canaries using the protocol described below.

We injected most birds IM with 10 mg/kg testosterone (T; Steraloids, Wilton, NH) suspended in peanut oil. Birds were deeply anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and were decapitated 90 min after T injection. Brains were removed, rapidly frozen on dry ice, and stored at -75°C. To collect tissues for positive controls, a male Long-Evans rat was sacrificed by an IP overdose of pentobarbital (Nembutal). The prostate and seminal vesicles were quickly removed, frozen on dry ice, and stored at -20°C.

Brains were sectioned at 20 μm on a cryostat. Sections were thaw-mounted onto gelatin-subbed slides. Sections of the syringeal muscles of the birds and/or rat prostate or seminal vesicles were placed on the same slides as positive controls. After air-drying on slides at room temperature (RT) for 10-15 min, sections were outlined with a PAP pen (Kyoto International; Elk Grove Village, IL) and postfixed for 1 min in 4% phosphate-buffered paraformaldehyde (pH 7.4) at 4°C. Slides were rinsed three times for 5 min each in 0.1 M PBS, pH 7.3. In some cases, sections were incubated 15 min in each solution of an endogenous avidin and biotin blocking kit (Vector Laboratories; Biogenex, CA). This step did not noticeably affect staining. Slides were incubated for 1 hr at 4°C in 4% normal goat serum (NGS; Sigma Chemical, St Louis, MO).

Slides were blotted and incubated at 4°C for 24-36 hr in PG-21 antibody diluted to a concentration of 0.3-0.75 μg/ml in 1% NGS containing 0.02% sodium azide. The PG-21 antibody was generated, purified, and characterized by methods described elsewhere (Prins et al., 1991). Using concentrations of primary antibody greater than 0.75 μg/ml produced unacceptable levels of nonspecific background staining. In some cases, slides were incubated in primary antibody that had been used once before or with a 1:1 mixture of new and used antibody. Incubating in used primary antibody did not adversely affect the staining. To control for nonspecific binding of primary or secondary antibodies, slides containing sections of song nuclei and hypothalamus were incubated in one of the following solutions (4°C, 24-36 hr) instead of the primary antibody: (a) 1% NGS without primary; (b) 1% NGS containing 0.5 μg/ml rabbit IgG (Vector Laboratories); or (c) primary antibody (0.5-0.75 μg/ml PG-21 in 1% NGS) preabsorbed for 30 min before incubation with a 10-fold molar excess of the AR-21 peptide used to generate the PG-21 antibody. Negative control sections were run in parallel with tissue to be immunostained.

Slides were rinsed three times for 10 min each in 0.1 M PBS and incubated for 1 hr at 4°C in biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in 1% NGS. To control for nonspecific binding of the avidin-biotin complex, some negative control slides were incubated in 1% NGS containing no secondary antibody. After being rinsed three times for 10 min each in PBS, slides were incubated for 45 min at 4°C and 15 min at RT in either standard peroxidase ABC kit reagent (Vector Laboratories) or peroxidase Elite ABC kit reagent diluted to the standard kit concentration (1 drop each of Reagents A and B per 5 ml PBS). Using the Elite kit at the manufacturer’s recommended concentration increased background staining.

Slides were rinsed for 5 min in PBS and five times for 5 min each in 0.1 M Tris-HCl (pH 7.6). Sections were then incubated 15-30 min in 0.1 M Tris-HCl containing 0.04% 3,3-diaminobenzidine (Sigma), 0.003% hydrogen peroxide, and 0.3% nickel ammonium sulfate. Sections were rinsed twice for 5 min each in Tris-HCl buffer, once for 5 min in distilled water, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped in DPX (BDH Laboratory Supply; Poole, UK). Slides containing sections adjacent to those stained by PG-21 ICC were stained with thionin to identify brain regions containing cells stained by PG-21 ICC.

Results

With the above protocol, we observed labeled cells in the song nuclei HVC, RA, MAN (both medial and lateral portions), IC0, and nXIIIs (Figure 1). We also observed staining in the tuberal, preoptic, and magnocellular paraventricular nuclei of the hypothalamus, in the nucleus of the striaterminals, in syringeal muscles, and in rat prostate and seminal vesicles (not shown). Each of these brain regions and tissues is known to contain androgen receptors (Brenowitz and Arnold, 1992; Prins et al., 1991; Gaht, 1990; Nordeen et al., 1986; Leiberg and Nottebohm, 1979). A similar pattern of staining was observed in all four species studied. The staining pattern was replicated in our laboratory using three different lots of the PG-21 antibody (present data; and K. Soma, personal communication).

Staining was confined primarily to the nuclei of labeled cells (Figures 2A and 3A-C). The structures labeled by PG-21 antibody (present data; and K. Soma, personal communication).

PG-21 labeling was absent in the negative control sections (Figure 2B). We did not observe nuclear labeling in sections of song nuclei or hypothalamus when primary or secondary antibody was excluded or when the primary antibody was preabsorbed with the immunogenetic peptide. These observations, together with the lack
of nonspecific labeling in other regions of the brain, suggest that the PG-21 antibody bound selectively with ARs in these birds.

Breeding condition and T pretreatment influenced specific staining of ARs (Figure 3). Although we found PG-21-labeled cells in the brains of both breeding and nonbreeding males that received T injections before sacrifice (Figures 3A and 3C), we did not observe any specific staining above background in brain regions of four nonbreeding males (two white-crowned sparrows, one house sparrow, one canary) that were not treated with T before sacrifice (Figure 3D). These nonbreeding males had completely regressed testes (<2 mm diameter), indicating that their plasma T concentrations were low. Although we did not measure plasma T concentrations in these birds, other studies on canaries, white-crowned sparrows, and house sparrows found that males in a similar state of gonadal regression had plasma T concentrations near or below the detection limits of a radioimmunoassay (i.e., less than 0.78 ng/ml in canaries and approximately 0.1 ng/ml in white-crowned sparrows and house sparrows) (Hegner, 1986; Wingfield and Farner, 1978; Storey, 1976). Overnight incubation of sections from these males in 10% NGS containing 0.25 mg/ml T did not induce subsequent labeling of ARs. We did find labeled cells in the brains of breeding males that were not treated with T before sacrifice (Figure 3B). Staining in the brains of these males was fainter, however, than that observed in the brains of males treated with T.

Discussion

We were able to immunocytochemically label ARs in several song nuclei and hypothalamic regions of the songbird brain using the PG-21 antibody. Androgen accumulation has been observed with [3H]-DHT autoradiography in HVC, RA, nXIIIts, MAN, and ICo, as well as in the tuberal, preoptic, and paraventricular regions of the hypothalamus and in the nucleus of the striaterminals of canaries and zebra finches (Gaht, 1990; Nordeen et al., 1986). Using PG-21 ICC, we observed staining in all of these regions.

We observed AR+ cells in the brains of both breeding and nonbreeding male songbirds that received injections of T 90 min before sacrifice and in the brains of breeding males that did not receive T injections. We were unable, however, to stain AR+ cells in brains of nonbreeding males that did not receive T before sacrifice. Our inability to label ARs does not necessarily indicate, however, that males with low T lack ARs. Rather, low concentrations of ARs may be present in the brains of these birds but may be undetectable with the low concentrations of PG-21 needed to reduce nonspecific background labeling.

There are four possible explanations for the effectiveness of T in improving PG-21 staining of ARs: (a) T may rapidly upregulate the expression of AR genes; (b) T may influence the rates of synthesis or degradation of AR protein; (c) ARs that are occupied by
Figure 2. Preabsorption of PG-21 antibody with the peptide against which it was generated eliminated nuclear staining. Adjacent sections of medial MAN from a song sparrow injected with T 90 min before sacrifice. (A) PG-21-labeled cell nuclei in MAN. (B) Nuclear staining was eliminated in adjacent section of MAN by preabsorbing PG-21 antibody with a 10-fold molar excess of AR-21 peptide. Bar = 0.1 mm.

Figure 3. Effects of breeding condition and T pretreatment on PG-21 labeling. (A) PG-21-labeled cells in HVC of breeding male sparrow that was injected with T 90 min before sacrifice. (B) PG-21 staining of HVC cells in a breeding male not injected with T is less intense. (C) PG-21-labeled cells in HVC of nonbreeding male treated with T 90 min before sacrifice. (D) Lack of labeled cells in HVC of a nonbreeding male that was not treated with T. Bar = 0.1 mm.
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T or its metabolites may be concentrated in the nucleus, where they are more easily detected by ICC than if they are diffusely distributed in the cytoplasm; or (d) the PG-21 antibody may have a higher affinity for occupied than unoccupied ARs in avian tissue. These explanations are not mutually exclusive.

In some tissues, AR mRNA is rapidly upregulated by androgen treatment (Varriault and Serino, 1994; Gonzáles-Cadavid et al., 1993). It is unlikely, however, that T improves PG-21 labeling in the song nuclei by upregulating AR gene expression. In canaries, the mRNA for the AR in HVC is rapidly downregulated by androgen treatment (Nastiuk and Clayton, 1994).

A second possibility is that T may increase the amount of AR protein either by stabilizing the protein against degradation or by increasing the production of the protein. In LNCaP prostate carcinoma cells, androgen treatment increased the cellular content of AR protein twofold over 24 hr, despite a 35% reduction in AR mRNA levels (Krongrad et al., 1991). In cultured cells, androgens decrease the rates of AR protein degradation (Zhou et al., 1995; Kemppainen et al., 1992; Syms et al., 1985). This stabilization of AR protein depends on androgen binding AR but is independent of new protein synthesis, receptor dimerization, or DNA binding (Zhou et al., 1995). Androgens also increase the rate of synthesis of AR protein (Syms et al., 1985). It is therefore possible that T injection may increase PG-21 labeling in the song nuclei by decreasing degradation or increasing synthesis of AR protein.

Another possibility is that T increases the proportion of occupied ARs that are translocated to the nucleus, where they are more easily visualized by ICC. Androgen binding increases nuclear localization of AR protein in transfected COS cells (Kemppainen et al., 1992; Simental et al., 1991). In both the paraventricular nucleus of hamsters and in ventral and dorsal lobes of rat prostate, castration decreased nuclear and increased cytoplasmic staining of ARs, and T treatment rapidly reversed this effect (Prins and Birch, 1993; Wood and Newman, 1992). Cytoplasmic ARs may be difficult to detect by ICC, possibly because they are distributed more diffusely in the cytoplasm than in the nucleus (Wood and Newman, 1992).

Finally, it is also possible that the PG-21 antibody has a higher affinity for occupied than for unoccupied receptors. Estrogen receptor immunoreactivity in the rat hypothalamus is diminished by estradiol treatment when one estrogen receptor antibody, but not others, is used to label the receptor (Blaustein, 1993). Binding to ligand or DNA may alter the conformation of the receptor protein and change the structure of the epitopes recognized by particular antibodies. Such a mechanism may account for the enhancement by T of PG-21 staining in the avian brain.

In summary, the protocol we describe enables one to use PG-21 ICC to robustly label ARs in the songbird brain. This technique will facilitate studies of the distribution of ARs in the song nuclei and of the developmental, seasonal, and hormonal regulation of ARs in the songbird brain. The critical features of our protocol are T pretreatment to increase labeling of ARs and the use of low concentrations of the PG-21 antibody to reduce nonspecific staining. It is possible that modifications of our protocol may enable the use of this antibody to label ARs in other systems. For example, our protocol might be modified for use on nonmammalian tissue fixed by perfusion or processed free-floating, as has been done with mammalian brain tissue (Iqbal et al., 1995; Wood and Newman, 1992).

The technique we have presented may be of wide applicability in labeling ARs in tissues of other nomenclature species. Attempts to label ARs in other nomenclature species have also encountered difficulties in labeling ARs in nonbreeding males or in females (Gustavson et al. 1994). We suggest that pretreatment of animals with T and the use of our ICC protocol may facilitate the use of this antibody in a wide array of animals.

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