Expression of androgen receptor in the brain of a sub-oscine bird with an elaborate courtship display

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Abstract

Sex steroids control vertebrate behavior by modulating neural circuits specialized for sex steroid sensitivity. In birds, receptors for androgens (AR) and estrogens (ERα) show conserved expression in neural circuits controlling copulatory and vocal behaviors. Male golden-collared manakins have become a model for evaluating hormonal control of complex physical courtship displays. These birds perform visually and acoustically elaborate displays involving considerable neuromuscular coordination. Androgens activate manakin courtship and AR are expressed widely in spinal circuits and peripheral muscles utilized in courtship. Using in situ hybridization, we report here the distributions of AR and ERα mRNA in the brains of golden-collared manakins. Overall patterns of AR and ERα mRNA expression resemble what has been observed in non-vocal learning species. Notably, however, we detected a large area of AR expression in the arcopallium, a forebrain region that contains a crucial premotor song nucleus in vocal learning species. These results support the idea that AR signaling both centrally and peripherally is responsible for the activation of male manakin courtship, and the arcopallium is likely a premotor site for AR-mediated displays

Keywords

Androgen receptor; Estrogen receptor; Bird; Avian; Courtship

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1. Introduction

The evolution of neuronal steroid sensitivity enables sex steroids to exert control over vertebrate behavior. Initially, studies exploring brain–steroid interactions focused on conserved hypothalamic circuits that control copulatory behaviors, with studies of birds playing a prominent role [3]. It is now recognized that sex steroids have diverse neural functions, so brain–steroid studies have expanded considerably. In birds, for example, steroids impact learning and memory [45], sensory processing [43,53], parental care [49], aggression [36], and socio-sexual behaviors involving vocal and visual communication [19].

Many animals also perform impressive physically complex courtship displays, but little is known about the extent to which steroids control these diverse behaviors. Among birds, the Manakins (Family *Pipridae*) stand out. Most manakins are polygynic; males breed in leks where they perform physically and acoustically elaborate courtship displays with unique mechanical sonations produced by rapid and/or powerful movements of the wings [41].

Our laboratories explore neuroendocrine control of courtship behavior in male golden-collared manakins (*Manacus vitellinus; GC-manakins*). In mid-January, adult male GC-manakins arrive on their traditional leks and remain on or near their courtship arenas for the duration of their 6–8 month-long breeding season. On the lek, adjacent males interact by producing loud roll-snaps and performing courtship displays to females. Courtship involves rapid movements within the arena punctuated by single wing-snaps produced in mid-air jumps between saplings [9,21]. Our studies indicate that androgens activate courtship behaviors, including wing- and roll-snaps [47]. Compared to females and non-breeding males, testosterone (T) is elevated in displaying males [12,18]. Exogenous T treatments activate courtship in non-breeding males and females [12] whereas treatment with AR antagonists [18,22] blunts performance of courtship in wild adult breeding males. Elevated expression of AR in spinal cord and peripheral muscles [17,23] might have evolved in manakins to facilitate androgenic control of courtship. Given the numerous coordinated neuromuscular systems required for male courtship, we were curious to examine AR expression in manakin brain.

Steroid receptor expression in the manakin brain is also of interest in an evolutionary contex. The Order Passeriformes includes two taxa: oscines (also called songbirds) and suboscines. The latter do not learn song and accordingly lack a neural song system. Across many species of oscines, nuclei comprising the well characterized neural circuitry subserving the learning and performance of song can express AR and ERα at high levels [8,25,38,39]. To date, AR and ERα have never been detected in the forebrain of avian species that do not belong to the oscine group such as suboscines, budgerigars, doves, owls, gulls, quails, and fowls (often referred to as non-oscines) [24]. The GC-manakin, like all other studied non-oscines, shows no anatomical evidence for a neural song system [46]. Thus, a second focus of this study was to compare distributions of AR and ERα we observe in manakins to those of other bird species.

2. Experimental procedures

2.1. Animals and tissue preparation

We collected seven male GC-manakins in February and March using mistnets in the canal zone of Panama. Manakins were killed by an overdose of isofluorane and immediately perfused through the heart with 30 ml 0.9% saline followed by ice cold 4% neutral buffered formaldehyde using a peristaltic pump. All procedures were approved by the UCLA Chancellor's Animal Care Committee and the AALAC committee of the Smithsonian Tropical Research Institute.

Brains were postfixed for 2 h in 4% formaldehyde, and then cryo-protected with 30% sucrose in PB. The brains were frozen and stored on dry ice, transported to our lab at UCLA, and stored at −80° until processed.

2.2. Subcloning of the manakin AR

Total RNA was isolated from adult male manakin brain (Trizol; Invitrogen). Two microgram of DNase *I*-treated RNA was reverse transcribed with random primers. A 741 bp cDNA was amplified HotStarTaq (Qiagen) with PCR primers based on the canary AR sequence (S: TGA CGT GTG GGA GCT GCA AA and AS: GGC CAT CCA CTG GAA TAA TAC TGA). The amplification proceeded at 95 °C for 15 min, then 40 cycles of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min, with a 5 min final extension at 72 °C. Gel-purified amplicons were ligated into the *SrfI* site of PCRScript per manual (Stratagene) and clones were sequenced to confirm identity. We selected two clones, pman200B and pman50B, as templates for downstream in vitro transcription reactions (below) because they were identical except that the cDNA had ligated into the plasmid in opposite orientations.

2.3. Synthesis of riboprobe

Antisense- and negative control sense-configured AR riboprobes were transcribed from two pmanAR clones after linearization with NotI (T7 RNA polymerase; Promega, Madison, WI). Riboprobes for ERα were synthesized by linearizing the plasmid containing the 2792-bp zebra finch ERα sequence (EJZER1, [30]) with MluI or EcoRI to obtain the antisense (T7) and sense (SP6 RNA polymerase) probes, respectively. ^{33}P labeled probes were prepared by in vitro transcription from ~100 ng of linearized plasmid, 10 μl ^{33}P-UTP (2000Ci/mmol; New England Nuclear, Boston, MA), and 1 μl of the appropriate RNA polymerase. Unincorporated nucleotides were removed with G-50 sephadex columns (Boehringer Manneheim, Indianapolis, IN).

For in situ hybridization, 20 μm tissue sections were processed as described in [34] with modifications: we did not include proteinase K digestion, and hybridization was performed at 55 °C with 60 °C high-stringency post-hybridization washes. Dried sections were exposed to film (Kodak BioMax) for 2–3 days to estimate the length of exposure needed for subsequent emulsion autoradiography. The slides were then dipped in emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY) at 42 °C, stored in light proof, desiccated boxes at 4 °C, and developed after 3–4 weeks (Eastman Kodak D-19; Fixer). Slides were examined

under light and darkfield microscopy to determine the presence and distribution of labeled cells.

3. Results

The neural distribution of AR, ERα, and their mRNAs in the brain has previously been described in details for several avian species [8,24,25,30,38]. Thus, here we focus only on the differences in AR and ERα mRNA expression between the GC-manakin and other studied bird species. In particular, we found novel expression of AR mRNA in a large field of labeled cells in the arcopallium, a pallial region that is the main output of the avian telencephalon and that in oscines contains the n. robustus arcopallii (RA) of the song system. To our knowledge, this is the first report of substantial AR mRNA expression in the forebrain of a non-oscine bird [24]. In the following description, we adhere to the revised avian brain nomenclature [42]. Although we studied only the distribution of the AR and ERα mRNAs, hereafter we will speak of AR and ER expression for brevity. Previous studies have shown that the mRNA localization matches well the distribution of the receptor protein [8,24,25,30,38].

3.1. Androgen receptors (AR)

The distribution of AR in the GC-manakin forebrain resembled that previously described for non-oscine species, in that no vocal control nuclei containing AR mRNA were found in the forebrain [5,24,38]. However, there was one noticeable exception: we found intense AR expression in the nucleus taeniae amygdalae (TnA) and in the arcopallium (Fig. 1D, E, G and H). Rostrally, the field of AR expression extended from TnA to the arcopallium mediale and then laterally into the arcopallium dorsale, following the structure previously called lamina archistriatilis dorsalis (LAD, Fig. 1E). This field of AR expression extends caudally to occupy virtually the whole arcopallium intermedium (AI), extending from the medial end of the forebrain laterally to the LAD, and from the caudal portion of TnA to the caudal end of the forebrain. This same region in oscines contains the AR-sensitive nucleus robustus arcopallii (RA), but a distinct RA is not recognizable in the GC-manakin (Fig. 1G and H).

In the diencephalon, AR expression was abundant in the septum (not shown) and in the preoptic–hypothalamic area (Fig. 1A, C and D). In addition, we found a field of AR expression in the nucleus sub-rotundus (SR, Fig. 1A and C) and in a layer of cells which delimits the ventral edge of the nucleus rotundus (Fig. 1B). In the mesencephalon, AR was abundant in the nucleus intercollicularis (ICo, Fig. 1G and J). In the rhombencephalon, we found strong AR expression in the nucleus oculomotorius dorsalis, pars medialis (OMdm, Fig. 1K and I). Finally, we found AR in cerebellar Purkinje cells (PJ, Fig. 1D and F) as well as in the deep cerebellar nuclei (DCN, Fig. 1K and L). No labeling was found in any area using a sense probe.

3.2. Estrogen receptor (ERα)

We observed sparse expression within the manakin brain using in situ hybridization. Overall, the distribution of ERα in the manakin brain was similar to what has been described for other non-oscine species in which ERα are found mainly in the septal region, preoptic–

hypothalamic region, in the hippocampus, and in the amygdala (Fig. 1M–O) [8,25,30,38]. No labeling was found using a sense probe.

4. Discussion

The unique pattern of AR expression revealed by this study suggests that AR sensitivity in the manakin brain coevolved with the ability to perform acrobatic, androgen dependent courtship displays. This has implications both for the control of manakin courtship as well as for the evolution of steroid-dependent neural circuits underlying avian social behavior, topics to be discussed below.

4.1. Evolutionarily conserved premotor region?

We hypothesize that AR expression in the GC-manakin arcopallium is related to this species' ability to perform its unique courtship display. The arcopallium is known to have strong pre-motor functions, particularly in passerines [52], suggesting that androgen action at this brain center might help control the refined and complex movements of courtship. Our discovery that GC-manakins show strong expression of AR throughout this region is therefore noteworthy because the arcopallium of non-oscines typically shows little to no AR, except within adjacent amygdaloid regions [24]. The enhanced sensitivity to steroid hormones fits well with the evidence that the GC-manakin arcopallium is larger in males than in females, a relationship that mirrors the sex difference in courtship display [13]. The region of AR-expressing cells in the GC-manakin arcopallium overlaps with the lateral intermediate arcopallium (LAI), a region that in songbirds, parrots, hummingbirds and doves is specifically activated when birds are moving their limbs [16]. The LAI projects to pre-motor neurons of the brainstem reticular formation, a group of cells that controls wing and leg movements [15,50]. Future studies will determine if the arcopallium projects to spinal motoneurons that control the acrobatic male courtship display, including forelimb motoneurons and muscles that express AR abundantly [17,23].

4.2. Motor behavior

We detected ample AR expression in the manakin ICo. This brain region participates in avian calling behavior [10] and is a conserved site of androgen action [2,48]. Manakins are highly vocal, producing cheepoo calls. Calling frequency increases in the presence of T and is reduced in birds treated with AR antagonists [18], so the ICo is a likely target of androgen action in controlling production of cheepoos. ICo might also participate in controlling wing-snapping behavior. There is emerging consensus that neural control of vocal systems overlap, perhaps functionally, with circuits that control some pectoral movements, including those that evolved in gesturing [7]. Midbrain structures that include ICo in birds are pre-motor elements in this vocal-gesturing pathway [7]. As wing snaps are acoustic signals, they may share with calls some elements of their neural control.

4.3. Skilled motor behavior

As manakin courtship is motorically complex, it is no surprise that AR were expressed at high levels in the cerebellum, a primary motor control region. Studies of passeriform species show that limb movements activate cerebellar immediate-early gene expression [16],

cerebellar lesions disturb motor performance [51], and cerebellar volume correlates with motor and cognitive functions associated with avian courtship [14]. AR were most abundant in Purkinje neurons and in deep cerebellar nuclei, regions that express steroidogenic enzymes in birds [35]. Future studies are needed to determine if neurosteroids participate in the activation of male manakin courtship.

4.4. Social interactions

We found AR expressing cells in the nucleus taeniae and the pre-optic area, which have been reported in other bird species. These brain areas are involved in the activation of courtship behaviors [4,29,31], and in the ability to defend one's territory from competing males [11].

A novel finding is the intense AR expression in a region of the visual sensory pathway, the n. rotundus. N. rotundus is part of the tectofugal (or collothalamic) pathway that projects from the retina to optic tectum to n. rotundus to the ectopallium [32]. In pigeons, lesions of the n. rotundus impair discriminations of color, pattern, and brightness [27,28]. This suggests that the expression of AR in these areas is related to visual discrimination of displays, which in manakins are involved in both intra- and inter-sexual selection [6,37]. We also found strong AR expression in the n. oculomotorius, as reported previously for the zebra finch [33]. AR expression in the n. oculomotorius may also allow androgens to facilitate visually guided motor reactions that enable the accuracy of the male's display performance.

4.5. Limited ERα expression

Our study confirmed that ERα expression in manakins resembles that described for other non-oscines: ERα are mainly found in the medial preoptic area [8,25,38] where they are probably involved in modulating courtship behavior [20] including vocal activity [44]. The manakin brain shows a relatively widespread distribution of aromatase and relatively high (compared to oscines) levels of activity [46]. We are currently examining if brain aromatization of androgen is required for the full activation of male manakin courtship; preliminary findings suggest only limited effects (Day and Schlinger, unpublished). ERα expression in the nucleus taeniae amygdalae is likely to be involved in the regulation of sexual and aggressive behaviors as shown for other species of birds [1,26]. Similarly, estrogen may function in the manakin hippocampus to promote spatial learning and memory [40].

In summary, AR expression in the manakin brain provides insight into the putative central targets whereby hormones control the complex courtship display of males. We have not studied the distribution of the receptor protein, however previous studies show concordant patterns of expression of mRNA and protein (reviewed in [24]), therefore we are confident that our study reflects actual receptor-mediated steroid sensitivity. In addition, these data offer a hint at how steroid-dependent neural systems have evolved to facilitate complex visuo–acoustic mating displays. This work enhances our understanding of how androgen-dependent circuits in birds contribute to different mating strategies and facilitate complex/coordinated male display behavior.

Acknowledgments

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HIGHLIGHTS

- Androgen controls elaborate courtship of male golden-collared manakins.

- Brain androgen receptor expression is unusual and includes the entire arcopallium.

- Brain androgen sensitivity might be linked to elaborate motor patterns of courtship.
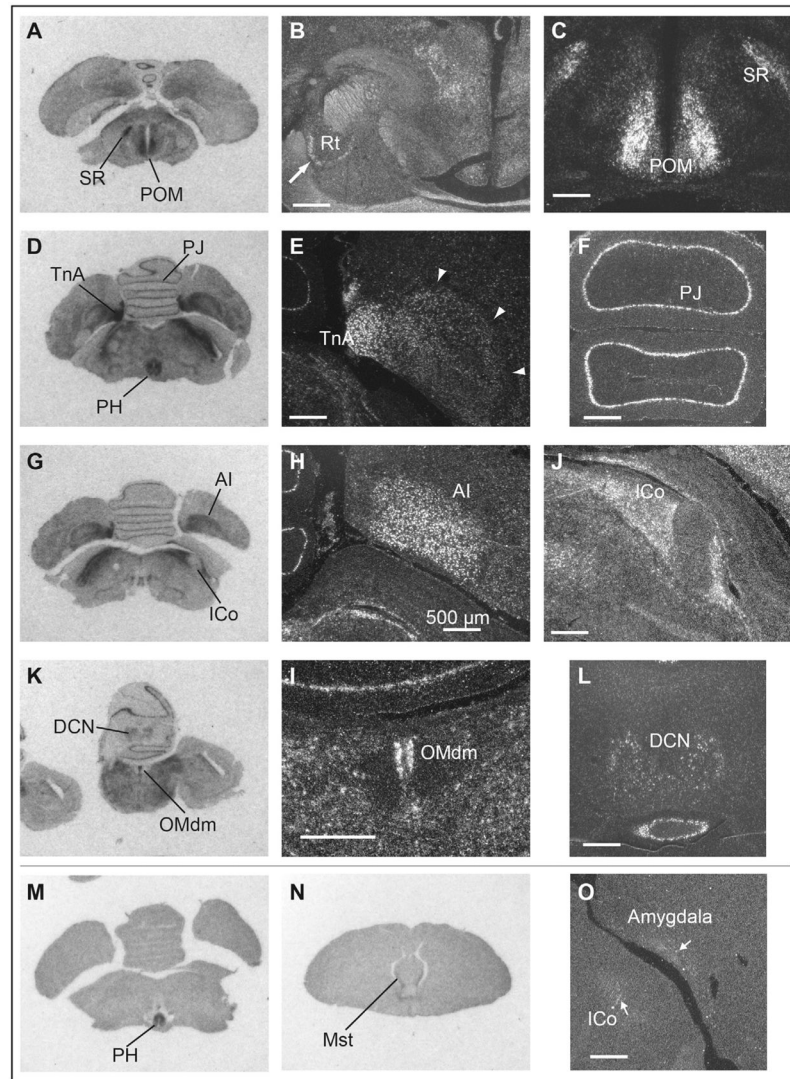
Fig. 1.
Expression of AR-mRNA (panels (A)–(L)) and ERα-mRNA (panels (M)–(O)) in coronal sections of golden-collared manakin brain after in situ hybridization. Panels (A), (D), (G), (K), (M) and (N) show whole-brain autoradiograms, whereas panels (B), (C), (E), (F), (H), (J), (I), (L), and (O) show darkfield photomicrographs of the AR-mRNA and ER-mRNA expression. AR expression is visible in the nucleus preopticus medialis (POM, (A) and (C)); the nucleus subrotundus (SR, (A) and (C)); a layer of cells surrounding the nucleus rotundus (Rt) indicated by the white arrow in (B); the nucleus taeniae amygdalae (TnA, (D) and (E); the white arrowheads in E show the border of the AR labeled area of the arcopallium which corresponds to the lamina archistriatilis dorsalis); the posterior hypothalamus (PH, (D)); the Purkinje cells (PJ, (D) and (F)); the nucleus intercollicularis (ICo, (G) and (J)); the arcopallium intermedium (AI, (G) and (H)), where AR expression delimits the entire region; the nucleus oculomotorius dorsalis, pars medialis (OMdm, (K) and (I)); and the deep nuclei of the cerebellum (DCN, (K) and (L)). Low levels of ERα expression was found in several

areas including the PH (M), medial striatum (MSt, (N)), and the ventral part of the amygdala (O). Scale bar is 500 μm.