Neuroendocrinology of Song Behavior and Avian Brain Plasticity: Multiple Sites of Action of Sex Steroid Hormones

Gregory F. Ball,* Lauren V. Riters,† and Jacques Balthazart‡

*Department of Psychological and Brain Sciences, Johns Hopkins University, Baltimore, Maryland 21218; †Department of Zoology, University of Wisconsin—Madison, Madison, Wisconsin 53706; and ‡Research Group in Behavioral Neuroendocrinology, Center for Cellular and Molecular Neurobiology, University of Liège, Liège B-4020, Belgium

Seasonal changes in the brain of songbirds are one of the most dramatic examples of naturally occurring neuroplasticity that have been described in any vertebrate species. In males of temperate-zone songbird species, the volumes of several telencephalic nuclei that control song behavior are significantly larger in the spring than in the fall. These increases in volume are correlated with high rates of singing and high concentrations of testosterone in the plasma. Several song nuclei express either androgen receptors or estrogen receptors, therefore it is possible that testosterone acting via estrogenic or androgenic metabolites regulates song behavior by seasonally modulating the morphology of these song control nuclei. However, the causal links among these variables have not been established. Dissociations among high concentrations of testosterone, enlarged song nuclei, and high rates of singing behavior have been observed. Singing behavior itself can promote cellular changes associated with increases in the volume of the song control nuclei. Also, testosterone may stimulate song behavior by acting in brain regions outside of the song control system such as in the preoptic area or in catecholamine cell groups in the brainstem. Thus testosterone effects on neuroplasticity in the song system may be indirect in that behavioral activity stimulated by testosterone acting in sites that promote male sexual behavior could in turn promote morphological changes in the song system. Key Words: song system; catecholamines; afferent inputs; testosterone; preoptic area; seasonal plasticity; birds. © 2002 Elsevier Science (USA)

INTRODUCTION

It is only recently that scientists have recognized the existence of significant naturally occurring brain plasticity in adult homeothermic vertebrates. Most neuroscientists focus on laboratory species, which historically has limited the opportunity for the study of natural variation in the brain. The absence of substantial behavioral recovery after brain damage produced by accidental trauma or neurodegenerative diseases was, for many years, taken as evidence for the absence of marked plasticity in the adult central nervous system of
many vertebrate species, including humans. One major discovery that challenged this view was made by neuroethologists investigating the brain mechanisms underlying seasonal changes in behavior. It was discovered in the brain of songbirds (e.g., canaries, Serinus canaria) that the volume of telencephalic brain nuclei specifically involved in the control of vocal behavior changes with season (152).

This observation of substantial seasonal variation in the boundaries of brain areas as defined in Nissl-stained material raised a host of questions about the cellular basis of the phenomenon and which factors might regulate these changes. It was apparent from some of the initial investigations of volumetric changes and associated cellular changes in the song control system that testosterone (T) promotes various types of neuroplasticity in this system (81, 151). A quite plausible scenario for the regulation of these seasonal changes developed involving changes in photoperiod and other environmental cues that in turn regulate seasonal changes in reproductive physiology, such as plasma concentrations of T, that can then regulate changes in the morphology of the vocal control system, which results in seasonal changes in song behavior (23, 201). However, this view is based primarily on correlational data and the actual causal links among these variables have not been established. Other possible interactions among variables such as season, T concentrations, brain variation and song behavior need to be considered. For example, an emerging concept in the study of neuroplasticity is the importance of activity-dependent brain changes (205). It is intuitive to think of changes in the brain driving changes in behavior; it is less obvious but equally compelling to think that a change in an organism’s behavior can lead to long-lasting changes in neuronal organization. One way that T promotes changes in the volume of the song control system is via brain-derived neurotrophic factor (BDNF; 168). Interestingly, singing activity can also promote BDNF release (128). Thus correlations among high steroid concentrations, song behavior, and increased nuclear volume could result from bidirectional interactions.

There has been a renewed interest in the study of steroid hormone effects on brain and behavior because the influence of steroids in this regard is more wide ranging than previously thought (124). The study of steroid hormone action in songbirds is of general interest because of the dramatic induction of neuroplasticity by steroids in birds and because they act on multiple brain sites to activate a complex learned behavior that is expressed differently in males than in females in many species (9–11). Thus many fundamental issues in the study of how steroids regulate song behavior can be addressed in this system. In this chapter we (1) review some of the major findings about sex steroid action in relation to the regulation of the song control circuit and song behavior among species in the songbird suborder (suborder Passeres), (2) discuss the possibility that sex steroids are acting at multiple sites to modulate different aspects of song behavior, and (3) analyze the possibility that the steroid regulation of neuroplasticity in the song system might occur at different brain sites and have different cellular bases as compared to its effects on song behavior.
SEX STEROID HORMONES AND SONG BEHAVIOR

Basic Correlations among Gonadal Cycles and Song in Temperate-Zone Songbirds

The link between sex steroid hormone action and song behavior was first made based on field studies correlating seasonal changes in physiology with changes in song behavior. These descriptive studies have been reviewed in some detail (23, 201) but some of the major features of these findings are reviewed here. First, many male temperate-zone songbirds sing at high rates in the spring as compared to other seasons (e.g., 78, 183; see 76 for a review). In these species seasonal differences in male song are positively correlated with dramatic seasonal increases and decreases in aspects of reproductive physiology such as gonadal size and hormone secretion in the plasma (216). However, among these temperate-zone birds there is interspecific variability in the degree to which maximal rates of singing are observed outside the breeding period. For example robins (Erithacus rubecula) living in northern Europe sing at relatively high rates throughout the year (108), pausing only in July (78), while most other songbirds living in the same region do not. Birds that sing outside the breeding season do exhibit seasonal cycles in gonad size and endocrine secretions that are similar to other temperate-zone species. Studies of select species that exhibit territorial song production in the autumn such as song sparrows in the western United States, mockingbirds, and the European robin clearly suggest that song behavior in the fall can be elicited by the appropriate stimulus in the absence of gonadal T (129, 180, 217, 218). Interestingly, in the case of the song sparrow there is evidence that this autumnal singing does involve estrogen acting in the brain even though the gonad is inactive (194). The source of this neuroactive estrogen in this case does not appear to be from T of gonadal origin that is then locally metabolized (194). Overall, these data indicate that there is not a tight correlation between endocrine activity and song rate in all temperate zone species.

However, seasonal changes in reproductive physiology in these species may relate to changes in other aspects of song such as repertoire size or stereotypy. For example, seasonal changes in song repertoires have been observed in European starlings (Sturnus vulgaris; 86) and canaries (154), where the number of song types and other measures of song complexity may change. Additionally, seasonal changes in other measures of song such as stereotypy have been described in white-crowned sparrows (Zonotrichia leucophrys; 184) and song sparrows (Melospiza melodia; 186).

A careful consideration of behavioral data from temperate-zone songbirds suggests that although song output is positively correlated with various measures of reproductive physiology, including hormone concentration in the plasma, there is not necessarily a strong causal relationship between the two as is the case for sex steroids and certain reproductive behaviors such as lordosis in rats (167), the bow coo display in male ring doves, (125) or male-typical copulatory behaviors in Japanese quail (37). Experimental studies on the effects of exogenous hormone administration or castration with hormone
replacement on song have been performed on a relatively small number of species but these studies confirm this view of the relationship between steroids and song behavior. Administering exogenous T can clearly increase song rate (e.g., 109, 160). Several different studies of zebra finches have shown that castration greatly reduces but does not eliminate male-typical song (e.g., 6, 103), whereas in red-winged blackbirds castration was reported to eliminate adult song production (105). In the case of song sparrows in the western United States, castrated males were able to maintain fall territories and sang at high rates in response to territorial challenge in a manner that was indistinguishable from intact controls (217). The hormonal control of song behavior therefore appears to be a clear case of a hormone-enhanced, rather than hormone-dependent, behavior. Species-typical stimulus factors (the presence of a conspecific male and/or female as well as a nest site or a favorable environment) promote song production in males. The presence of gonadal steroids in the plasma can increase the probability and intensity of these behavioral responses to the appropriate stimulus but this presence is not essential for behavioral activation (217). One should therefore not be surprised by reports of substantial song production being observed in association with low steroid hormone concentrations in some cases. The stimulus factors releasing song can be so strong in some cases that high gonadal steroid concentrations are not necessary for song production to be observed.

Although T does not appear to be necessary for the initiation of song production in all cases, it does appear to influence aspects of song quality such as stereotypy. For example, castration prevents the onset of crystallized (i.e., stereotyped) adult song in 1-year-old song and swamp sparrows singing for the first time in the spring (134). Upon receiving T the song rapidly crystallizes (134). Similarly, in both white-crowned sparrows and in song sparrows, fall song in the presence of low concentrations of T is less stereotypic than spring song produced in the presence of high concentrations of testosterone (184, 186). Overall the data indicate that song can be produced (though at a low rate) in the presence of low concentrations of T but that the stereotypic quality of the song is also regulated by the presence of T.

Finally, some steroid-hormone-replacement studies indicate that both androgenic and estrogenic metabolites of T are needed to completely restore high rates of singing (e.g., 103, 105). In zebra finches it has been suggested that estrogenic metabolites selectively promote female-directed song, which suggests that there might be different actions of the two primary metabolites of testosterone on song behavior (210).

THE SONG SYSTEM

The Vocal Control System of Songbirds

Songbirds (members of the suborder passeres or oscines) are notable because they learn and produce complex vocalizations (22). Species in this taxa have
evolved in association with their unusual vocal abilities a suite of neural specializations that includes an interconnected circuit of telencephalic, diencephalic, mesencephalic, and myencephalic nuclei that regulate the learning, production, and perception of song (70, 133, 213). This circuit was originally discovered based on tract-tracing studies that identified step by step the afferent motor inputs to the vocal organ, the syrinx (149). Additional studies used stereotaxic brain lesions and chemical neuroanatomy to further refine the knowledge of the anatomical organization and function of this specialized brain circuit.

The vocal control system of oscines or “song system” has been described in the most detail in zebra finches and canaries, but less complete evidence from a variety of species suggests that a typical song system is present in most if not all oscine songbirds. The circuit can be divided into at least two parts (see Fig. 1). There is a caudal pathway consisting of the nucleus HVc (sometimes referred to as the “higher vocal center”) and the robust nucleus of the archistriatum (RA) of the telencephalon as well as the intercollicular complex (ICo) in the mesencephalon.

The HVc (originally misnamed as hyperstriatum ventrale, pars caudale; see 71) is a telencephalic nucleus that appears to be unique to species in the songbird suborder (17, 70, 120, 150). It is a key part of the song system.
involved in the learning, production, and perception of song (70, 150, 159). The HVC projects to the nucleus robustus archistriatalis (RA), which in turn projects to the ICo, in particular the dorsomedial (DM) portion of this complex. Both the RA and DM project to several medullary components of this circuit, including the tracheosyringeal part of the nucleus of the XIIth cranial nerve (nXIIIts) that innervates the vocal production organ the syrinx as well as to nucleus retroambigualis (RAm) and the rostral ventral respiratory group of neurons (rVRG) that coordinate respiratory activity with song production (see Fig. 1). This pathway (HVC→RA→ICo→nXIIIts) is involved in the motor production of song: Lesions to nuclei in the pathway block song production (149, 182) and both immediate early gene induction studies (111, 116) and electrophysiological studies (219) indicate that neurons in these nuclei are active in association with song production. The second major pathway is an anterior forebrain pathway that includes HVC, area X of the parolfactory lobe (homolog of the caudate/putamen), and the lateral part of the magnocellular nucleus of the anterior neostriatum (IMAN); all of these are in the telencephalon. Another nucleus in this circuit is in the diencephalon, the medial part of the dorsolateral thalamic nucleus (DLM). This pathway is organized as follows: HVC→X→DLM→IMAN→RA (Fig. 1). The IMAN also projects to area X.

Thus there are two projection pathways that go from HVC to RA. The caudal pathway, described above, is clearly essential for song production based on lesion studies (149, 182), immediate early gene induction (111, 116), and electrophysiological recordings (219). The anterior forebrain pathway, which consists of a series of more indirect projections between HVC and RA, plays a role in song learning [see (63) for a review] and in the maintenance of stereotypic adult song (48), but lesions to nuclei within this pathway do not block adult song production (60, 178, 192). Nuclei such as area X exhibit immediate-early gene induction in singing male zebra finches that is context dependent (112). Males singing song that is directed at a female exhibit almost no induction of the immediate-early gene ZENK in area X while ZENK induction is high in males singing in isolation or in the presence of other males (112). Similar findings have been reported for electrophysiological activity in that multiunit activity in area X is much lower when the birds are engaging in song directed at a female than when they are singing in social isolation (106, 107). These findings suggest that this anterior forebrain circuit may modulate song output in adulthood, but not be directly responsible for the motor output.

**Neurochemical Characterization of the Song Control Nuclei**

The song-control nuclei of the oscine brain represent distinct morphological entities characterized by dense clusters of neurons that can be unequivocally delineated in brain sections stained by standard histology techniques (i.e., Nissl stains) such a cresyl violet or toluidine blue. These forebrain nuclei are not observed in nonsongbird species (120). Interestingly, in the course of evolution, these anatomical specializations have been accompanied by distinct
neurochemical specializations (13, 17). Several of the telencephalic song control nuclei contain steroid receptors and the presence of these receptors at a high level of expression in the telencephalon constitutes a unique feature that distinguishes songbirds among other vertebrates (see below). The forebrain song nuclei also express or receive projections that express at high concentrations specific neurotransmitter or neuropeptides. These song control nuclei can therefore often be characterized by the presence dense clusters of fibers containing neurotransmitters (e.g., catecholamines) or neuropeptides (e.g., Met-enkephalin or vasoactive intestinal polypeptide). In several cases, fibers containing specific transmitters or peptides or enzymes catalyzing their synthesis are selectively excluded from the song control nuclei while they are present in substantial densities in the surrounding areas. For example, this is the case for tyrosine hydroxylase-containing fibers that are specifically present in lower densities in the HVc in female canaries compared to surrounding areas (5). The relevant transmitter and peptide receptors have localized distributions that often match the boundaries of song control nuclei. The $\alpha_2$-adrenergic receptors, for example, specifically outline the boundaries of HVc, RA, and area X in the starling brain (17, 50, 173). In contrast, the nucleus HVc in zebra finches is specifically devoid of glutamate NMDA receptors (1).

In a number of more detailed studies, the volumes of the song-control nuclei as identified in Nissl-stained material and in sections stained for an enzyme, neurotransmitter, or receptor were reconstructed from serial sections in both males and females. In almost all cases, similar volumes were obtained by the two methods so that the sex differences or seasonal changes in volumes that were present in Nissl-stained sections were also observed in sections processed by immunohistochemistry or quantitative autoradiography (18, 19, 49). In one study, a discrepancy was observed between the seasonal variations in the volume of HVc as identified in Nissl-stained sections or in sections processed for the visualization of estrogen receptors (immunocytochemistry) and of neurons retrogradely labeled from area X (92). Gahr (95) therefore questioned the idea that neurochemical markers could be used to study seasonal variations or sex differences in the volume of song-control nuclei. This discrepancy, however, appears to be the exception rather than the rule (see 21, 64, 72 for discussion). It seems that, in general, sex differences (or seasonal variations) in volume described by neurochemical criteria match closely sex differences described in Nissl-stained material. The chemical specialization of the song control nuclei has only been partly investigated but the largest amount of information on the topic concerns the catecholamines and their synthesizing enzymes and receptors.

Catecholaminergic Inputs to the Song-Control Nuclei

Several studies suggest that the catecholaminergic neurotransmitters nor-epinephrine (NE), and dopamine (DA) could play a major functional role in the control of song. A prominent catecholaminergic innervation of the song control
FIG. 2. The song control nucleus HVC is innervated by catecholaminergic fibers that originate in neurons located mostly in the mesencephalic central gray and the area ventralis of Tsai. (A) Photomicrograph of the HVC in a male canary stained by immunocytochemistry for tyrosine hydroxylase (TH), the rate-limiting step in catecholamine synthesis. The nucleus is outlined by a high density of TH-immunoreactive (TH-ir) cells by comparison with the surrounding neostriatum. (B) TH-ir cells located in the rostral portion of the mesencephalic central gray. (C) TH-ir cells of the mesencephalic central gray (stained by fluorescein in green) containing retrogradely transported red latex fluorospheres (RLF) that had been injected into the HVC. These fluorospheres demonstrate that the TH-ir neuron indicated by the white arrow actually projects to the HVC. (D) TH-ir cells located in the area ventralis of Tsai. (E) TH-ir cells of the area ventralis of Tsai (stained by fluorescein in green) containing retrogradely transported RLF that had been injected into the HVC. (F and G). Schematic drawings of coronal sections through the canary brain illustrating the distribution of TH-ir perikarya (open green circles) and of double-labeled cells (TH and RLF; larger red circles) after injection of the retrograde tracer in the HVC. The RLF-containing cells in nucleus Uva are represented by blue crosses (B). Sections labeled F through G are presented in a rostral to caudal order and illustrate the origins of the catecholaminergic inputs to HVC located in the mesencephalic central gray and area ventralis of Tsai. Abbreviations: CP, commissura posterior; FA, tractus frontoarchistriatalis; Gc, mesencephalic central gray (= area A11); HP, hippocampus; HVC, hyperstriatum ventrale, pars caudale, or higher vocal center; ICo, nucleus intercollicularis; IM, nucleus isthmi, pars magnocellularis; IP, interpeduncular nucleus; IPc, nucleus isthmi, pars parvocellularis; LAD, lamina archistriatalis dorsalis; MLd, nucleus mesencephalicus
system has been demonstrated in zebra finches by the presence of fibers immunoreactive for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH) (62, 190) and/or dopamine β-hydroxylase (137). Similarly, immunocytochemical studies of TH in male canaries have found that the boundaries of HVC and RA can be defined by a higher density of fibers immunoreactive for tyrosine hydroxylase compared to the surrounding neostriatum (5) (see Fig. 2A). Interestingly, this high density of tyrosine hydroxylase-immunoreactive fibers is not observed in the HVC and RA of female canaries, who sing less than males. In females these nuclei either have the same density of immunoreactive fibers as the surrounding neo- and archistriatum respectively or, in other functions, are outlined by the nearly complete absence of immunoreactivity (5).

High concentrations of NE and DA have accordingly been measured in the song control nuclei of zebra finches (39, 40, 176). High densities of noradrenergic receptors of the α2 and β1/β2 subtypes are also present in several song-control nuclei (HVC, RA, and area X) of European starlings (17, 18, 173) and high densities of dopamine D1 receptors have been described in area X of the lobus parolfactorius in the same species (75). In these cases boundaries of these song-control nuclei can be defined that are consistent with Nissl-defined boundaries, based on the high receptor densities compared to the surrounding brain area. The origins of the catecholaminergic innervation of the song control nuclei have been investigated to some extent by tract tracing in canaries. These studies indicate that, in both males and females, HVC and RA receive dopaminergic inputs from the mesencephalic central gray (GCT; homologous to the mammalian A11 cell group) and from the area ventralis of Tsai (AVT) and noradrenergic inputs from the complex of the locus coeruleus (4; Appeltants, Ball, and Balthazart, unpublished data) (see Figs. 2B–2G), whereas area X appears to receive catecholaminergic inputs originating mostly or exclusively in AVT (127).

Other Transmitters and Neuropeptides

Many other proteins associated with neurotransmission are also expressed in a unique way in the songbird song system. Arnold and collaborators originally demonstrated that several song-control nuclei can be identified in zebra finch brain sections by the presence of cholinergic markers such as the enzyme acetylcholinesterase or the acetylcholine receptors of the muscarinic subtype as well as by the neuropeptide enkephalin (174, 175). Ball et al. (12) similarly showed that several neuropeptides (vasoactive intestinal polypeptide, Met-
enkaphalin, cholecystokinin, and substance P) are distributed in a unique manner in the song-control nuclei of two songbird species, the song sparrow and the European starling. They also confirmed that muscarinic cholinergic receptors are specifically located within and outline the boundaries of song-control nuclei in European starlings (15). Neuropeptides such as VIP or Met-enkephalin are also specific markers for HVc and RA in zebra finches and the volumes defined by these peptidergic innervations match closely the volumes defined by more traditional histological methods (19).

Neurochemistry has also been used to demonstrate the existence of a sex difference in the nucleus interfacialis (Nif) that cannot be observed by traditional histological methods. Nif specifically projects to HVc and is considered as part of the motor circuit of the song-control system. Nif was originally identified by retrograde tract tracing from HVc (153), but it is not readily visible in Nissl-stained sections. It is, however, characterized by an accumulation of Met-enkephalin- and VIP-immunoreactive fibers and the volume occupied by these immunoreactive fibers is larger in males than in females (19).

Several of these neurochemical markers of song-control nuclei are neuropeptides (e.g., VIP and Met-enkephalin) that are not contained in neurons located within HVc or RA but are present in fibers originating from neurons whose location has not been identified. Given that the song nuclei defined by these fibers are different in males and females these projections and the cell groups from which they originate may also be sexually dimorphic. Studies combining retrograde tract tracing with immunocytochemistry should be carried out to identify these sexually dimorphic cell groups.

The distribution of vasotocin-immunoreactive (VT-ir) fibers has been studied in detail by immunocytochemistry in the brain of the canary (118), zebra finch (206, 208), and Junco (Junco hyemalis; 165). VT-ir fiber endings are observed in broad areas of the telencephalon but in particular a large supply of VT-ir fibers is present around the RA (118, 165), where they correspond to the presence of vasotocin binding sites (207). The mesencephalic song-control nucleus ICo is also labeled by VT-ir fibers in both oscines and nonoscine birds. In other cases, song-control nuclei can be distinguished from the surrounding tissue by a lower density of neurotransmitter receptors. This is, for example, the case for the HVc in zebra finches that is outlined from the surrounding neostriatum by a lower density of NMDA glutamatergic receptors (1, 2).

**ANDROGEN AND ESTROGEN RECEPTORS IN THE SONG SYSTEM**

One valuable aspect of studying behaviors regulated by steroid hormones is that one can use anatomical studies of the distribution of steroid receptors to guide one toward the identification of brain circuits that control behavior. As described above, song-control nuclei can often be delineated by a number of neurochemical markers exactly as they are in Nissl-stained sections. This was first discovered to be the case for androgen receptors that can be used for
example to identify HVc and RA in the zebra finch telencephalon (7). The presence of androgen receptors in these regions of the telencephalon of songbirds represents a specialization that distinguishes songbirds from all other vertebrates (114, 140). During the past 2 decades, a substantial amount of information has accumulated concerning the distribution of androgen and estrogen receptors in the oscine song-control nuclei. This information is briefly summarized below.

Androgen Receptors (AR)

The distribution of androgen receptors was originally studied in the avian brain by in vivo autoradiography with the use of tritiated testosterone as a ligand. However, because this steroid is readily converted in many parts of the brain to a variety of other steroids, including estrogens (26), the binding sites described in these studies are likely to include estrogen as well as androgen receptors. In a few cases, autoradiography was also carried out with tritiated 5α-dihydrotestosterone. Given that this compound cannot be aromatized into an estrogen its localization provides in theory a more specific visualization of the androgen receptors. Unfortunately, 5α-dihydrotestosterone (5α-DHT) is rapidly metabolized in birds (80) and this markedly limits its use as a ligand for in vivo autoradiography. More recently immunocytochemistry and in situ hybridization have also been used in a selected number of cases to confirm that the binding sites identified by autoradiography correspond to true receptors as identified by their protein or the corresponding messenger RNA.

In general, it appears that the distribution of AR in birds is restricted to the septal-preoptic area and to various nuclei in the hypothalamus and in the midbrain (for review see 13, 25, 68). This corresponds to the common pattern that has been previously described in all vertebrate classes (139, 166, 196). In addition to the receptors located in these brain areas that seem to be present in all bird (and vertebrate) species, members of the suborder passerines (i.e., songbirds) possess androgen-sensitive brain areas that are a part of the telencephalic network of sexually dimorphic nuclei that mediate the production and acquisition of male song (7, 119, 148, 150, 158). The HVc, RA, and MAN appear to contain androgen receptors based on both autoradiographic (7, 93, 130, 221; for review see 13, 68) and binding assay (104) methods. Additional autoradiographic studies utilizing the nonaromatizable androgen [3H]dihydrotestosterone as a ligand (8, 145, 191, 211) have confirmed that these telencephalic binding sites identified by testosterone autoradiography are specific for androgens.

More recently, antibodies to the androgen receptor have become available and have been used to confirm the presence of the androgen receptor protein in the songbird brain (30, 185, 193). The androgen receptor has also been cloned and sequenced in canaries (143, 96) and starlings (55) and probes based on this information have been used to localize the androgen receptor mRNA in the canary brain (90, 138, 144) and starling brain (55, 131, 132).
ization studies largely confirmed the AR distribution identified by in vivo autoradiography or immunocytochemistry. Molecular biology tools also brought further support to the specificity of the previously identified signals. Northern blot analysis of mRNA extracted from canary forebrain indicated the presence of a single low-abundance band between 8 and 10 kb in length corresponding to the rat or human AR (143). Sequencing of the canary AR also confirmed the high degree of homology of this protein between birds and mammals.

A concordant pattern of distribution for AR has generally been described by these three independent experimental approaches in the preoptic area–hypothalamus–limbic system and in the mesencephalic IC of songbirds and nonsongbirds. In particular, large numbers of AR-expressing neurons are present in the medial preoptic nucleus (POM) of the POA (songbirds: 7, 13, 30, 68, 93, 130, 185, 193, 221; other avian species: 30, 38, 43, 115, 211). Androgen receptor-immunoreactive (AR-ir) cells were observed by immunocytochemistry in the POA of zebra finches and canaries but [3H]DHT uptake was never reported in the POA of any songbird species. [3H]Testosterone uptake was, however, reported in the POA of zebra finches (7), bramblings (130), and chaffinches (221). As discussed above, this discrepancy could be explained by the rapid metabolism of [3H]DHT in birds.

Songbirds, in addition, express AR in three telencephalic song control nuclei, HVc, RA, and MAN. These nuclei accumulate radioactive testosterone (e.g., 7, 130, 148, 150, 221) or 5α-dihydrotestosterone (8, 145, 146, 191) in autoradiographic studies. The HVc and RA were also found to bind the nonaromatizable androgen methyltrienolone during in vitro binding studies in zebra finches (104; MAN was not investigated in this study) and they contain AR-ir cells (e.g., 30, 185, 193).

In canaries, weakly labeled AR-ir cells were also observed in a position adjacent to RA in a “hooklike” structure that runs laterally and then ventrally (30). This area has been previously identified in zebra finches as a region receiving projections from the lateral MAN (61) and labeled by the a high density of α2-adrenergic receptors in starlings (13, 17). This area should thus be considered as part of the song system.

In the song-control motor output pathway, RA provides inputs to several medullary nuclei, the nucleus hypoglossus pars tracheosyringealis (syringeal motonucleus or nXIIts), the nucleus retroambigualis (RAm), and the rostroventral respiratory group (rVRG), that are thought to be present in all avian species (169, 213, 214). Interestingly, in songbirds these nuclei are also defined by the presence of androgen receptors (97). In contrast, no report has, to our knowledge, mentioned the presence of androgen receptors in these nuclei in nonsongbirds and there is actually evidence indicating that these receptors are not present in a diversity of species (98). One exception to this rule has, however, been reported recently. Males of certain hummingbird species such as Anna's hummingbirds (Calypte anna) learn their song during postnatal development. Interestingly, these birds also possess a neural circuitry controlling the learned vocalization that is largely similar (presumably analogous but
not homologous since singing is supposed to have emerged independently in oscines and hummingbirds) to the song-control system of oscines. Several of these “vocal” nuclei in the Anna’s hummingbird, including nXIIts and the equivalents of IMAN and HVc, also express dense populations of immunoreactive androgen receptors (98). The presence of AR in telencephalic and medullary song-control nuclei may thus be a characteristic of species that learn their vocalizations, not only of oscines. The apparent lack of AR in the corresponding nuclei in a vocal-learning member of the parrot order, the budgerigar (98, 195), would, however, contradict this generalization.

In situ hybridization was also used recently to analyze the distribution of AR in the canary brainstem (131, 132). Significant levels of AR mRNA were found in several catecholaminergic nuclei, including the locus coeruleus, the substantia nigra, and the area ventralis of Tsai (homologous to the ventral tegmental area). AR mRNA had already been previously identified in the area ventralis of Tsai of canaries (96).

**Estrogen Receptors of the Alpha Subtype (ERα)**

Many behavioral effects of testosterone, in birds like in mammals, are produced at the cellular level by the action of estrogens produced by local aromatization of androgens. In songbirds in particular, estrogens play a critical role in the activation of various aspects of the sexual behavior repertoire, including singing (103, 105, 209). The distribution of estrogen receptors has been investigated in a few avian species, including songbirds, by the same methods as used in the case of AR (in vivo autoradiography; in vitro binding; immunocytochemistry; and, in a few cases, in situ hybridization).

Until recently, a single estrogen receptor was known to exist. In the late 1990s, a new estrogen receptor was cloned in mammals (121, 141, 204). This second form of ER was called estrogen receptor-β (ERβ) to distinguish it from the classic receptor, which was then renamed ERα. ERβ has now been identified and cloned in two avian species, including one songbird, the European starling, and its distribution in the brain has been studied by in situ hybridization (24, 55, 88). These studies are summarized in a separate section below. We review here work that was performed before the second receptor was known and that therefore concerns the first identified form of receptor (ERα).

Similar to what has been observed for AR, estrogen receptor binding sites appear to be restricted to the hypothalamic and limbic structures and to the mesencephalic intercollicular nucleus in nonsongbirds and songbirds (e.g., 115, 135, 136, 211), but additional binding sites are found in telencephalic song nuclei of oscines (e.g., 67, 147).

An initial immunocytochemical study utilizing the monoclonal antibody H222SP raised against estrogen receptors purified from a human mammary tumor confirmed this distribution of estrogen receptors in the brain of two songbird species, the zebra finch and the canary (91). The same technique was subsequently used to analyze by immunocytochemistry the distribution of ERα
in many avian species (n = 26) belonging to a large number of avian orders, namely anseriformes, galliformes, columbiformes, psittaciformes, apodiformes, and passeriformes (17 species) (94). These data confirm that the distribution of ER-immunoreactive cells in the diencephalic and limbic structures is very similar across all species that have been studied. All songbirds, however, also display significant numbers of ER-expressing cells in three structures of the nonlimbic forebrain: the caudal neostriatum, including in some species HVc; the dorsorostral area surrounding RA; and an area in the rostral forebrain, dorsal to the lamina hyperstriatica and rostral to the nucleus MAN. A fourth forebrain area, the hyperstriatum accessorium, also contained many ER-immunoreactive cells in songbirds but a few positive cells were also found in this location in the budgerigar (94).

Because the activation of many aspects of reproductive behavior, including song, is obtained by a synergistic action of androgens and estrogens (103, 105, 209), Gahr (93) explored whether both AR and ER are colocalized in the same neurons. By combining immunocytochemistry for ER and autoradiography using tritiated 5α-dihydrotestosterone for AR visualization he showed that in the canary, many cells in the intercollicular nucleus ICo simultaneously contain both AR and ER. In other brain nuclei, such as HVc, both types of receptors are present in a large number of cells but their colocalization in the same cell is actually a very rare event. The functional synergism between androgens and estrogens can thus be mediated by the action of both types of steroids on the same neuron as well as by their action on organized neural circuits containing AR and ER in different cells (intra- vs intercellular interactions), but more studies of this type are needed to assess how general these findings are.

Recently, ER was also cloned in zebra finches and canaries and the distribution of the corresponding mRNA was analyzed in the brain of these species by in situ hybridization. These studies confirmed with a few exceptions, results previously established by autoradiographic and immunocytochemical methods (e.g., presence of ER in the neo- and archistriatal areas adjacent to HVc and RA (see 110). In addition, Northern blot analysis indicated the existence of a single transcript for ER in the zebra finch telencephalon at 4.1 kb but other transcripts with higher molecular weights were present in the ovary and oviduct (110). In canaries also the distribution of ER mRNA emerging from in situ hybridization studies coincides well with previous immunocytochemical results and with results obtained in zebra finches. There is, however, one noticeable difference between these two species: The ER mRNA and protein are present within the HVc in canaries but not in zebra finches, where they are located in the surrounding neostriatum but not in the HVc proper (90, 96, 138). Dense ER mRNA labeling overlapping with tyrosine hydroxylase immunoreactivity was also recently detected in the locus coeruleus and the area ventralis of Tsai of male canaries (131, 132). The presence of estrogen (and androgen) receptors within these cell groups opens the possibility that sex steroid hormones may affect song production by modulating the catecholaminergic sys-
tem at the site of synthesis by acting both as androgens or as estrogens (see below).

**Estrogen Receptors of the Beta Subtype (ERβ)**

The more recent identification of a second type of estrogen receptor (see above) raised new issues about the action of estrogens (121, 122). For example, it could be speculated that effects of estrogen on singing behavior in species that do not express ERα in HVC (e.g., the zebra finch or starling) is mediated, at least in part, through the binding of the steroid to ERβ present in this nucleus.

A few years ago, the ERβ was cloned in Japanese quail (88, 123) and then European starlings (55). A specific riboprobe was then used to analyze the neuroanatomical distribution of the mRNA in the starling brain with a special focus on the brain areas containing the song-control nuclei, MAN, area X, HVC, and RA (55). Alternate sets of sections were hybridized with species-specific AR, ERα, and ERβ antisense riboprobes (see Fig. 3). As indicated above, cells expressing AR mRNA were present in several song-control nuclei, including the HVC and the medial and lateral portions of MAN and RA. The ERα mRNA was expressed in the caudomedial aspect of HVC (also known as paraHVC; 113) but not in any of the other song nuclei examined. ERβ mRNA was not expressed at detectable levels in any forebrain song-control nucleus. A low level of expression was observed in the caudomedial neostriatum in a pattern distinct from ERα. In a nonsongbird species, the Japanese quail (88) relatively high levels of ERβ mRNA were present in areas implicated in the mediation of sexual behavior. For example, in the POM, ERβ mRNA was abundant and overlapped partly with the expression of ERα. As was observed in quail, the highest levels of expression were apparent in the lateral part of the POM (55). A dense expression of ERβ mRNA was also observed in nucleus taeniae, the avian homolog of parts of the mammalian amygdala (198). These data therefore provide no evidence for an implication of ERβ in the control of singing at the level of the telencephalic song control nuclei. A role of ERβ in the activation of sexual behavior taking place in specific nuclei of the hypothalamic–limbic system (e.g., in the POM and nucleus taeniae) is possible but has not been formally tested to our knowledge.

**STEROID HORMONE EFFECTS ON THE SONG CONTROL SYSTEM**

Along with the seasonal changes in the gonads and T described above are seasonal changes in the volumes of several song-control nuclei and aspects of song behavior. These correlated changes could reflect direct actions of steroids on the song-control nuclei and on song expression, but alternative mechanisms have also been suggested. Available evidence is reviewed below.
Correlations between Seasonal Increases in the Volume of Song-Control Nuclei, Singing and Plasma Steroids

Since the discovery of seasonal anatomical changes in the song-control system of canaries by Fernando Nottebohm (152); studies in several songbird
species have revealed strong positive relationships between the volumes of song nuclei, typically HVc, RA, and Area X, and both seasonal fluctuations in T and aspects of song production (e.g., 154, 156, 172, 186, 201). The primary environmental factor influencing the volumes of song nuclei is day length. In the laboratory, male songbirds exposed to artificial photoperiods typical of spring have significantly larger song-control nuclei and elevated T concentrations compared to males housed under fall-like photoperiod conditions (50). Similarly, in males captured from the field, seasonal peaks in T, associated with long day lengths in spring in most temperate-zone songbirds, correspond to increases in the volumes of song-control nuclei (e.g., 73, 186, 187, 189, 193). A recent study of wild song sparrows carefully correlated the late winter and spring onset of testis growth and other measures of reproductive development with the growth of the song system in wild sparrows and found that increases in HVc volume clearly preceded the growth and development of the reproductive system (203). Thus the maximal concentrations of T associated with breeding are not needed for the growth of HVc to be observed (203).

In addition to day length, several additional environmental factors interact to influence the volumes of song-control nuclei, including social factors (201, 203) and interactions between T concentrations and day length (53). Not all song-control nuclei show the same pattern of volume change (e.g., 186), but in general HVc changes were followed, depending on the species and whether the bird is studied in captivity or captured from the wild, by changes in RA and area X (202 for general discussion of this sequence). Compared to laboratory-housed males, males captured in the field are exposed to a much fuller array of environmental stimuli. This likely accounts for differences sometimes observed in the volumes of song-control nuclei in males studied in the laboratory compared to males captured in the field. For example, in starlings exposed to a springlike photoperiod, only the volume of HVc was enlarged relative to males under fall-like photoperiod conditions (46, 50, 53). In contrast, in males captured in the field and housed in outdoor aviaries HVc, RA, and area X displayed significant seasonal changes in volume (173).

The changes in the volumes of song-control nuclei reflect changes in the number of neurons, the size of the neurons, or changes in dendritic fields within the nuclei. In RA seasonal changes in volume do not appear to reflect an increase or decrease in neuron number, but changes in the size of the neurons, spacing between neurons, and changes in dendritic arborization (186, 199). In contrast to RA, new neurons are incorporated into HVc seasonally (69, 100, 157, 188). Given that HVc is largest when T concentrations are highest (e.g., 117, 173; though see 203), and cell death in HVc is associated with low concentrations of T (117, 155, 156), T appears to regulate, possibly by influencing neurotrophic factors (168), the incorporation of new neurons into HVc and possibly associated changes in song behaviors (117, 156).

Although seasonal changes in T and the volumes of song nuclei have been reported for several songbird species, the precise behavioral function of seasonal changes in the song control system is not clear. Based on work in canaries, the original hypothesis was that seasonal changes in the volumes of
song nuclei reflected seasonal changes in song learning (e.g., 154). Male canaries learn new songs each year outside of the breeding season. During song learning, the songs that are produced are variable and the volumes of song control nuclei are small. However, with the increase in T concentrations during the breeding season, song becomes much more stable and stereotyped and the volumes of HVc and RA increase 99 and 76% respectively (152), suggesting that volume increases play a role in the storage of a learned song. However, more recent studies have called this possibility into question (54, 66, 187). For example, in song sparrows seasonal changes in the volumes of song control nuclei are observed even in species that do not learn to produce new songs in adulthood (69). An alternative to the hypothesis that changes in nuclei relate to song learning is that seasonal changes in song nuclei are related to seasonal changes in aspects of song performance such as the amount of song produced or changes in song structure (23, 201). As in canaries, in song sparrows the largest volumes of HVc and RA were observed at times of the year when male song is extremely stereotyped, whereas smaller volumes were associated with times during which song was more variable (187). Given that song sparrows do not learn new song each year, seasonal changes in the volume of nuclei cannot relate to changes in song learning, but seem instead to relate to changes in song structure. Like canaries and song sparrows, male starlings sing at relatively high rates for most of the year (87). Unlike song sparrows, starlings appear to be able to learn new songs throughout their lives (77) and unlike canaries song learning in starlings does not appear to be restricted to a particular season (59). However, in starlings the volumes of HVc, RA, and area X change considerably across an annual cycle (173), again suggesting that changes in volume do not correspond well to song learning. It is unknown whether starlings display seasonal changes in stereotypy; however, starlings do sing a longer song bout in spring compared to fall, indicating that some structural changes do occur seasonally in this species (172). Furthermore, during spring a strong relationship was identified in male starlings between the volumes of HVc and RA and song rate (52). Together these data build a strong case for the idea that changes in the volumes of song-control nuclei underlie seasonal changes in aspects of song production such as stereotypy or other aspects of song structure.

Steroid Effects on Catecholamine Activity

As mentioned above, in songbirds, catecholamines and their synthetic enzymes are present at high concentrations in brain nuclei that control song (5, 39, 40, 62, 137, 176, 190). The boundaries of several song control nuclei can be defined by high densities of $\alpha_2$-adrenergic (17, 18) or (75) dopaminergic receptors.

This prominent catecholaminergic innervation of the song control nuclei is clearly modulated by steroids. This is demonstrated by immunocytochemical studies of tyrosine hydroxylase in canaries [including sex differences detected
in HVc and RA (5) and increases in TH immunoreactivity following treatment with exogenous testosterone (Appeltants et al., unpublished data), by the presence of a steroid modulation of NE and DA baseline levels and turnover (39, 40), and by the observation that the density of $\alpha_2$-noradrenergic receptors in HVc and RA vary seasonally in starlings (see below).

This steroid modulation of catecholaminergic inputs represents one possible way that steroids may influence song learning (134, 190) and production (63, 103, 179). Indeed in other vertebrate model systems, functional interactions between steroids and catecholamines have been reported in many vertebrate species, and catecholamines are involved in the control of a variety of steroid-regulated reproductive behaviors (27, 41, 42, 57, 58). It has thus been speculated that steroids may influence song control nuclei and singing activity at least in part by a direct action at the level of catecholaminergic structures. However, the roles of NE and DA and of their specific receptors located within the song system are not well understood.

The specific function of the catecholaminergic afferent inputs to the song system has, however, not been studied in great detail. In zebra finches, systemic injections of DSP-4, a specific noradrenergic neurotoxin that preferentially destroys catecholaminergic innervation of telencephalic regions (89, 220), decreases male courtship behavior, including female-directed singing (41, 42). This effect appears to result from attention deficits rather than from impairments of the motor aspects of song because in DSP-4 treated birds the latency to initiate singing was increased but once song begun it was quite normal.

In female canaries, the pharmacological depletion of central norepinephrine levels obtained by injection of DSP4 decreases the incidence of copulation solicitation displays that are produced in response to the playback of sexually stimulating male songs (Appeltants, Del Negro, and Balthazart, unpublished). This effect of DSP4 was observed in conditions where the sexually stimulating songs were partly masked by nonstimulating wild canary songs or heterospecific songs, but not when they were masked by simpler signals such as white noise. These data therefore suggest that central noradrenergic inputs (presumably to the song system) modulate sexual responsiveness of female canaries by affecting the auditory processing of the relevant information contained in sexually stimulating male songs.

Dave, Yu, and Margoliash (79) also demonstrated that NE acting in HVc regulates in a gate-like fashion the auditory responsiveness of efferent neurons to RA. Studies of the modulation of the neural activity in the anterior forebrain pathway (Area X, dorsolateral nucleus of the anterior thalamus, and nucleus magnocellularis of the anterior neostriatum) as a function of social context have also implicated ascending catecholaminergic projections (106, 112) as being responsible for the contextual regulation. These studies suggest that catecholamines have an important role in the control of certain aspects of song behavior but there are still major lacunae in our knowledge about the chemical neuroanatomy of this system in songbirds.
One possibility is that NE is involved in differences in song expressed by males within and outside of the context of reproduction. In a reproductive context, male song directed toward females is critical for mate attraction (76). Outside the context of reproduction, males often sing less and do not direct song toward females or use song for purposes of dominance or territory maintenance (215) (76). NE levels within some song nuclei are positively correlated with courtship singing in male zebra finches (41), and selective neurotoxic lesions of noradrenergic neurons produce specific behavioral deficits in male courtship song displays (41, 42), indicating a role for NE in song sung within a reproductive context. Song behavior is related to the activity of testosterone (T) and its metabolites, presumably acting within the song control system (9). Estrogenic T metabolites, known specifically to increase female-directed courtship song in male zebra finches (210), increase NE turnover in the song system (39, 40), suggesting that the role of NE in song relates to T activity.

In contrast to zebra finches, a species that does not restrict breeding to a particular season, many temperate-zone songbirds display seasonal variation in T concentrations, song behavior, and reproductive activities (23, 216). Changes in T are associated with changes in the context in which males will sing. During both the breeding and nonbreeding seasons, when T is high and low respectively, song can be observed during intermale aggression and in males singing alone; however, only during the breeding season is male song observed to play a direct role in mate attraction (23, 76, 86, 216).

The studies in zebra finches discussed above suggest that NE plays a role in the control of courtship song and that T (or its metabolite, estrogen) mediates this relationship. If this relationship exists, then in seasonally breeding birds, differences might be expected in NE or NE receptors in birds during the breeding season as compared to outside of the breeding season. In agreement with this idea, T regulates concentration of NE and the density its receptors in specific brain areas of at least one seasonally breeding bird (a nonsongbird, the Japanese quail (14, 27, 29). These data suggest specifically that seasonal changes in T might be associated with variation in noradrenergic transmission within the brains of seasonally breeding birds.

**Seasonal Changes in \( \alpha_2 \)-Adrenergic Receptor Densities**

In songbirds also, T can regulate the activity of catecholamines through the regulation of receptors. Consistent with this idea are studies of T and \( \alpha_2 \)-adrenergic receptors in songbirds. In seasonally breeding male starlings, seasonal changes in the density of \( \alpha_2 \)-adrenergic receptors were identified within HVC and RA (173) that correspond with changes in courtship song. These changes mirrored both seasonal changes in T concentrations and changes in HVC and RA volume. T-dependent seasonal decreases in NE within the song system might result in an up-regulation of \( \alpha_2 \)-adrenergic receptors in fall and a down-regulation of receptors in spring, a pattern that would be consistent with the seasonal changes in density observed in starlings.
\(\alpha_2\)-Adrenergic receptors were least dense in spring, when song is important for a male to attract a female. In contrast, during molt, in fall, and in winter, when males were likely not singing (during molt, 86) or when song is not directly sung to attract mates for immediate reproduction (86, 87), nuclei were smallest and \(\alpha_2\)-adrenergic receptors were densest. These data suggest that changes in \(\alpha_2\)-adrenergic receptors might reflect differential NE involvement in female-directed spring song compared to undirected fall song, with NE possibly regulating the attention a male directs toward a potential mate during female-directed song, as proposed previously in zebra finches (42).

A general concept emerging in the study of hormones and behavior is that steroids may regulate brain morphology in areas important for reproductive behaviors not only by acting directly on cells in the brain area of interest, but also by acting indirectly (i.e., transsynaptically) via cells at a distant brain site that then projects to the site of interest and modifies neural activity (31, 56). This mode of action is well illustrated by studies of sex steroid feedback on the gonadotropin-releasing hormone system (GnRH). Sex steroids are known to exert negative feedback on GnRH immunoreactive cells but these neurons do not contain detectable levels of receptors for sex steroid hormones. Rather, the effects of steroids on these neurons are mediated by changes in the activity of peptidergic (e.g., opioids) and catecholaminergic (e.g., norepinephrine) inputs to the GnRH cells (44, 212).

The presence of AR and/or ER\(\alpha\) in the neural network mediating the learning, perception, and production of song in oscine songbirds has often been taken as evidence indicating that steroids control singing behavior by binding directly to these receptors. However, steroid receptors are also found in the catecholaminergic cell groups (132) that are known to project to the song-control nuclei such as the locus coeruleus, the substantia nigra, and the area ventralis of Tsai (4, 74, 127) so that steroids could also affect song by modulating these catecholaminergic inputs. There would therefore be a direct action of steroids in the telencephalic song control nuclei and in an indirect action through the modulation of catecholaminergic inputs.

These indirect effects of steroids could themselves reflect two different modes of actions. Steroids are known to modify the release and thus turnover of NE and DA in song-control nuclei. This possibly results from a direct nongenomic action at the level of the catecholaminergic terminals. Alternatively, steroid hormones could modify the expression of catecholamine-synthesizing enzymes by acting genomically on catecholaminergic perikarya. This mode of action is namely suggested by the observation that treatment of castrated male canaries with testosterone increases the density of tyrosine hydroxylase-immunoreactive fibers in HVC and RA (Appeltants, Ball, and Balthazart, unpublished data). This change in immunoreactivity is likely to reflect changes in concentration of the enzyme that most likely are caused by increases in transcription of the corresponding protein. This effect thus has to take place at the level of the catecholaminergic perikarya that are at the origin of the tyrosine hydroxylase-immunoreactive fibers that innervate the song-control nuclei. The presence of steroid receptors in the nuclei that contain
these perikarya (131, 132) clearly supports this mode of indirect action for steroids.

Taken together, the available data suggest that seasonal variation and/or steroid-induced changes in catecholaminergic afferent input into the song-control system are an important factor regulating changes in the morphology of the vocal control system and in vocal learning or production.

**DISSOCIATIONS AMONG PLASMA TESTOSTERONE CONCENTRATIONS, SONG-CONTROL NUCLEUS MORPHOLOGY, AND SONG BEHAVIOR**

As reviewed above, there is now a substantial amount of data indicating that seasonal increases in testosterone concentrations do have dramatic effects on the volumes and cellular properties of key nuclei in the song-control system such as HVc, RA, and area X (see reviews in 23, 201). Four types of studies have confirmed the importance of the effects of testosterone on song-system morphology as follows: (1) castration with testosterone replacement in male songbirds, (2) photoperiodic manipulations that changed gonadal size and result in changes in the song system and/or behavior, (3) the collection of brains from birds at different seasons throughout the year, and (4) the administration of exogenous T to female songbirds that results in a male-typical song behavior as well as with an increase in the size of the song control nuclei (23, 201). Therefore strong positive correlations between high testosterone concentrations, song behaviors, and large volumes of telencephalic song-control nuclei have been identified.

However, as discussed previously, these correlations are not perfect and T may well not be exerting its effects on song behavior completely via its action on cells in the song-control system. It is also possible that within the song-control system the actions of T that promote song behavior could be separate at the cellular level from the actions of T that promote variation in the morphology of the song-control system. First testosterone is not equally effective in inducing changes in the song-control system in birds in different reproductive states (53). In European starlings administering T to photosensitive male birds (a reproductive state characteristic of late winter and early spring) promotes increases in the volume of HVc while the same treatment regimen in photorefractory starlings (a reproductive state characteristic of late summer and fall) results in no such change (53). Song behavior was not measured in this study but previous studies of song sparrows had clearly shown that administering T to photorefractory song sparrows does lead to an increase in song rate with a song quality comparable to that seen in T-treated photosensitive sparrows (16, 160). In dark-eyed juncos administering T to juvenile males on short days (indicative of fall) induced a dramatic increase in song with no concomitant change in HVc (though there appeared to be a small increase in RA (101). Administering T to female canaries or starlings promotes more malelike song behavior and increases the size of the song-control nuclei
(51, 151). However, the morphological effects of T are blunted in both species when it is administered under fall-like photoperiods (51, 82). Song behavior was not measured in the canary study (82), but in the case of the female starlings, administering T to the photorefractory females did indeed induce high rates of song behavior despite the fact that the growth of the song nuclei was attenuated (51). A recent field study of male canaries confirms the fact that there can be dissociations between seasonal changes in song behavior, T concentrations, and the size of song-control nuclei (126). Male canaries were studied in the wild on some of the islands in the Madeira complex and distinct seasonal changes in song behavior as assessed by repertoire complexity and song rate were discerned that were associated with the expected changes in T concentrations in the plasma. Despite these changes in T concentrations and song behavior the size of the song nuclei was stable (126). It is possible that these authors failed to detect seasonal changes that do occur in the wild due to sampling problems (they did not know the exact breeding state of the males collected during the breeding period, for example); however, these data still indicate that significant dissociations among T, song-control nuclei morphology, and song behavior do occur in this species.

There is also increasing evidence that the song system can grow in response to photoperiodic cues independent of the action of T. The discovery that the volumes of song control nuclei in tree sparrows and white-crowned sparrows can increase quite rapidly (within 1 week after photostimulation) suggests that these seasonal changes in the song control system may not be solely the result of T or at least of high concentrations of T (20, 202). Studies in several species of wild songbird such as tree sparrows, white-crowned sparrows, starlings, and juncos have shown that long photoperiods can stimulate the growth of the song system, even in castrated birds or in birds with low T concentrations (45, 54, 83, 187). The magnitude of these effects varies among species. In white-crowned sparrows the T-independent effects of photoperiod are quite small while in starlings and tree sparrows they appear to augment significantly the effects of T (45, 54). In juncos it was reported that the T-independent effects of photoperiod could completely explain seasonal changes in the volume of HVc (83)! A recent field study of gonadal development and song-system development in two populations of song sparrows illustrates the complexities of the relationship between T and song system development quite well. In western Washington state a coastal population of song sparrows initiates gonadal development 2 weeks prior to an inland montane population even though they experience the same photoperiod (203). The development of the song system was not different between these two populations and the volumes of the nuclei reached breeding season size well before plasma concentrations of T or testis size were maximal (203). This is consistent with the notion that the song system can regrow rapidly in the spring in response to increasing photoperiods and/or cues supplementary to photoperiod (see 200) and that these changes can occur prior to seasonal maxima in concentrations of T.
IS TESTOSTERONE ACTING OUTSIDE THE SONG-CONTROL SYSTEM TO ACTIVATE MALE SONG BEHAVIOR?

As reviewed above research on the neuroendocrine mechanisms regulating song demonstrates a clear role for the song control nuclei in specific aspects of song such as song perception (HVc; 68), song production (HVc and RA; 149), song learning (HVc, area X, IMAN; 63), auditory feedback needed for song maintenance (65), and the context in which song is sung (area X; 107, 112). Both the close relationship between T and song behavior and the presence of androgen and/or estrogen receptors within particular nuclei of the song system indicate that T and its metabolite E₂ act within the song system to regulate aspects of song such as learning, production, and perception. However, there are still major gaps in our understanding of the specific role of each nucleus in the control of specific functions and, in addition, little is known about how the brain regulates other aspects of song production such as the motivation to sing.

In starlings, as in many temperate-zone songbirds, song is motivated by different stimuli during different seasons. In fall, song is important for dominance or flock cohesion, behaviors not directly involved in immediate mate attraction. In contrast, in spring males use song to attract females and often respond dramatically to the introduction of a female with courtship behaviors, of which song is an important component (172). For many songbirds, song in spring is part of a sequence of male sexual behaviors that culminates in copulation. Brain areas outside of the song control system, such as those involved in the anticipation of copulatory behavior or sexual motivation in general, are also likely to play an important role in this type of singing behavior.

The Preoptic Region and Song Behavior

Growing evidence indicates that the POM, and possibly T, activity within this nucleus plays a key role in the regulation of male sexual motivation, including song, in male songbirds. A role for this nucleus in male sexual motivation in birds has been established in a seasonally breeding nonsongbird, the Japanese quail (36). In quail the POM is larger in males with high T (161–163) and the larger volume is associated with the expression of male sexual behavior (164, 197). Both androgen and estrogen receptor (both the α and β forms) proteins and the mRNA encoding these receptors have been located within the POM in male quail (24, 37, 164). The POM is also rich in aromatase, the enzyme converting T into estrogen (28, 33). Peripheral blockade of aromatase in castrated, T-treated male quail abolishes the expression of behaviors associated with sexual motivation (i.e., the amount of time a male quail spends near or looking at a female located behind a window; 35), suggesting that estrogen is the active metabolite involved in the expression of these behaviors. Lesions to the POM in quail are followed by a significant reduction in these same behaviors (36) and castrated male quail with T
implants located directly in the POM exhibit an elevation in measures of sexual arousal (170), suggesting that the POM is the site in which the aromatization of T is critical for the regulation of behaviors associated with male sexual arousal or motivation.

In songbirds as in quail, the POM is rich in the enzyme aromatase (zebra finches, 32; starlings, 172) (see Fig. 4), and cells within the starling POM express the mRNA for androgen receptors and for both forms of the estrogen receptor (55). In male zebra finches castration results in a significant reduction in courtship behaviors, including song (103). This deficit can be completely restored by treating males with aromatizable androgens, such as androstenedione (103, 210), whereas treatment with an aromatase antagonist completely blocks the effects of androstenedione, and estrogen treatment restores courtship behavior, including song, disrupted by the blockade of aromatase (209). Thus the aromatization of T appears to play an important role in the regulation of behaviors reflecting male sexual arousal (i.e., courtship, including song) in zebra finches as in quail.

Where in the brain aromatase is acting to support courtship behaviors is not known. Notably, the nuclei known to participate exclusively in song learning and production contain neither the aromatase protein (32, 177) nor cells expressing aromatase mRNA (181), indicating that courtship singing is regulated by aromatase activity outside of these brain areas, perhaps in the aromatase-rich POM. In support of this hypothesis, lesions to the POM in reproductively active male starlings interfere with both song expression in response to the presentation of females and the gathering of green nest materials (another courtship behavior 85, 102), suggesting that the POM plays a role in male courtship behaviors, including song (171; see Figs. 5A and 5B).

Together the findings from quail, zebra finches, and starlings suggest the attractive hypothesis that the role of the POM in song is exclusively related to its role in sexual motivation. Correlational evidence demonstrates that compared to fall, the volume of the POM in male starlings is largest in spring, when T concentrations are highest (172; see Figs. 5C–5E). This result indicates that there are seasonal changes in the POM that correspond to seasonal changes in the primary function of male song (i.e., the POM is largest when males use song largely to attract females and is smallest when the function of song is not related to immediate mate attraction). Additionally, the volume of the POM was positively correlated with song bout length in these males (17). Males sang longer songs in spring, when a longer song bout serves to attract mates and repel competitors (84, 99, 142), providing additional evidence for a role for the POM in song behaviors exclusively observed in spring. Finally, in fall aromatase is completely absent from the POM of starlings, whereas in spring this nucleus contain dense aromatase immunoreactive cells, a change that is likely steroid dependent as has been shown in quail (33, 34). The effects of POM lesions on courtship song and the dramatic seasonal changes in POM volume and aromatase immunoreactivity highlight the POM as at least one brain area in which the aromatization of T might act to regulate the expression of male sexual arousal, including courtship song.
The evidence so far suggests that the POM interacts with the song-control system to initiate song within the context of breeding. Song has generally been thought to be regulated by seasonal changes in T activity and neural plasticity.
within the song-control system (e.g., (156, 187); see 23, 201 for reviews). The close associations among seasonal changes in steroid concentrations, song activity, and the volume of song-control nuclei have been taken as evidence that androgens and estrogens acting directly on cells within nuclei of the song
control system are critical for the activation of song (9). However, the exact function of T in the song system is unknown. One possibility is that T in the song system might act to fine-tune song behavior [e.g., increase song stereotypy (e.g., 186) or song bout length in spring], and T activity within brain areas known to regulate male sexual arousal, such as the POM, might be involved in the motivation to sing in a reproductive context. Converging evidence supports a role for the POM in courtship song. In future work a more detailed analysis of the precise role of the POM in song is warranted. A body of correlational data suggest that the POM regulates courtship song; however, direct studies investigating POM involvement in song sung outside the context of courtship are necessary to more precisely define the role of the POM in song. Additionally, investigations into the neuroanatomical connections between the POM and song system are necessary to understand how this structure is positioned to influence song.

NEW VIEWS ON THE INTERRELATIONSHIPS AMONG HORMONES, BRAIN PLASTICITY, AND SONG BEHAVIOR IN SONGBIRDS

Based on this review, it is apparent that there are significant gaps in our knowledge about the actions of sex steroids on song behavior and the song system. General correlations have been made among the variations in plasma T concentrations, song system morphology, and song behavior but links among these at the cellular level are missing. Important and significant questions that remain include Where are T and its metabolites acting to promote song behavior and seasonal changes in song system morphology? Are these actions on brain and behavior at the same brain sites or different sites? What are the specific roles of estrogenic and androgenic metabolites in the activation of song behavior and/or in inducing seasonal changes in the song system? Do T and its metabolites regulate behavior via actions on the song system and/or other brain areas or do changes in the behavior come first and then promote changes in the song-control system?

Recent work has identified two brain sites outside of the song-control system where steroids could act to modify song production: the medial preoptic nucleus that regulates appetitive aspects of male sexual behavior in a variety of species and the catecholaminergic nuclei of the mesencephalon and pons that send afferent projections to the song-control nuclei. The anatomical connections between these brain areas and the neural circuit that controls of singing are still poorly understood. No direct projection between the POM and the song control nuclei has been described to this date. Given the large number of tract-tracing studies that have been previously carried out, it appears probable that such connections should have been detected if they exist. Available evidence suggests, however, one indirect link between these structures (see Fig. 6). It has been recently shown that in Japanese quail, the aromatase-immunoreactive cells of the POM massively project to the mesencephalic central gray (3). In addition in canaries, dopaminergic cells of the central gray are
known to be the major source of catecholaminergic inputs to HVC (4) and RA (Appeltants, Ball, and Balthazart, unpublished data). We are currently investigating whether these two groups of projections are simultaneously present in songbirds. If this was shown to be the case, one could then suggest that these projections represent the anatomical substrate that coordinates the actions of POM and of song control nuclei on song production. Projections from the POM to the mesencephalic central gray to the song system could not only confirm the role of the POM in the regulation of song production but also clarify a role for catecholaminergic inputs in the control of song.

Another important question to be answered is whether T action on the song-control nuclei is the prerequisite for its effects on behavior or whether causal relationships between these different variables could be exerted in other directions. It has always been assumed that steroids change the morphological and neurochemical organization of song-control nuclei, which in turn affects the expression of vocalizations (see in Fig. 7A). Data have, however, accumulated recently indicating that the volume of the song-control nuclei correlates with measures of song performance in some cases, which raises the intriguing idea that a large song-control nucleus could be the consequence rather than the cause of high rates of vocal production (Fig. 7B).
FIG. 7. Schematic representation of potential mechanistic models explaining the correlations between seasonal changes in plasma testosterone (T); morphological features, including size of the song control nuclei (SCN); and singing activity (song). The effects of T could be sequential on the two responses with SCN modulating song or vice versa or T effects on morphology and behavior could be relatively independent.
A potential mechanism that could underlie this causal interaction has been recently identified: singing activity was shown to promote the release of BDNF (128), a neurotrophin that promotes growth of HVc (168). It could also be the case that the morphological and behavioral effects of steroids are implemented independently at the cellular level and that correlations between these steroid-induced changes reflect only the changes in the plasma steroids or some secondary form of interaction largely unidentified at this time (Fig. 7C). This would explain the dissociation sometimes observed between seasonal or steroid-induced changes in song-control nuclei and measures of vocal behavior.

One major challenge of future studies will be to discriminate between these alternative hypotheses and identify the specific loci of steroid action that mediate different aspect of vocal behavior (learning, repertoire size, stereotypy, context, and amount of song production). To this date, most of the work trying to answer this question has been correlational in nature and it has been very difficult to dissect the web of multiple correlations between endocrine, morphological, neurochemical, and behavioral measures. Providing detailed answers to these questions will thus represent a complicated task in part because steroids obviously must act simultaneously at multiple loci to activate a full song. It is therefore difficult to conceive of experiments in which implants of steroids or steroid inhibitors can be placed stereotaxically in the different brain nuclei of castrated subjects to activate their vocal behavior.

Finally, although it is not the focus of this review, there is emerging evidence that hormones other than the sex steroids are also important in the seasonal regulation of the song control system (45, 46, 47). For example, Bentley et al. (45) performed an experiment that involved the manipulation of melatonin in castrated starlings. Castration eliminated any possible effects of fluctuating concentrations of gonadal steroids in the circulation. Photoperiod was manipulated to induce different reproductive states in the birds, and half of the birds in each group were implanted with melatonin. Reconstruction of the volumes of the song-control nuclei revealed that exogenous melatonin decreased the volumes of HVc and area X, regardless of reproductive state. Song nuclei in the starling such as HVc and RA exhibit high densities of melatonin receptors (46). This action of exogenous melatonin upon the volumes of song-control nuclei indicates that there may be a seasonal interaction between the effects of melatonin and the effects of testosterone on the song-control system. Melatonin appears to act in opposition to the stimulatory effects of testosterone (45, 47). In this way, as circulating testosterone concentrations slowly increase in the circulation during the late fall and winter; the long durations of melatonin secretion at that time of year keep the effects of testosterone in check. Only when day lengths increase during the spring does the nocturnal duration of melatonin secretion decrease rapidly. This, and the large increase in testosterone, may cause the volumes of song control nuclei to increase to a maximum during the breeding season. In other words, the balance between the effects of melatonin and testosterone may fine-tune the timing of the increase in volumes of the song-control nuclei (47). Other hormones fluctuate seasonally in addition of melatonin and the gonadal steroids. It is thus possible that some of
the dissociations between gonadal activity, song behavior and the size of the song control nuclei will be explained by other hormone systems that interact with the gonadal sex steroids.

ACKNOWLEDGMENTS

This research was supported by grants from the NINDS (35467) to G.F.B. and J.B., NIMH (MH65645) to L.v.R., and grants from the Belgian FRFC (2.4555.01), the French Community of Belgium (ARC 99/04-241), and the University of Liège (Fonds Spéciaux pour la Recherche) to J.B. The collaboration of J.B. and G.F.B. was supported by a NATO collaborative research grant (CRG973000).

REFERENCES


21. Ball GF, Balthazart J. How should brain nuclei be delineated? They don’t need to be! Trends Neurosci 1997; 20: 344.


40. Barclay SR, Harding CF. Differential modulation of monoamine levels and turnover rates by estrogen and/or androgen in hypothalamic and vocal control nuclei of male zebra finches. Brain Res 1990; 523: 251–262.


98. Gahr M. Neural song control system of hummingbirds: Comparison to swifts, vocal learning (songbirds) and nonlearning (suboscines) passerines, and vocal learning (budgerigars) and nonlearning (dove, owl, gull, quail, chicken) nonpasserines. J Comp Neurol 2000; 426: 182–196.


171. Riters LV, Ball GF. Seasonal changes in the densities of ?2-noradrenergic receptors are inversely related to changes in testosterone and the volumes of song control nuclei in male European starlings. J Comp Neurol 2002; 444: 63–74.


214. Wild JM, Li DF, Eagleston C. Projections of the dorsomedial nucleus of the intercollicular complex (DM) in relation to respiratory-vocal nuclei in the brainstem of pigeon (Columba livia) and zebra finch (Taeniopygia guttata). J Comp Neurol 1997; 377: 392–413.


