



# Neurochemistry of the Avian Vocal Control Circuit: Hormonal Modulation and Sex Differences

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## 1 INTRODUCTION: SONG BEHAVIOR

Most avian species use vocalizations of one sort or another to communicate with each other. These vocalizations range from the very simple to the complex. Calls generally refer to simpler sounds used to maintain contact among flock members or to signal alarm. Song usually refers to a more complex vocalization used to attract mates and/or to defend ones territory.<sup>1</sup> Some authors limit the term song to the complex vocalizations produced by members of the order Passeriformes or 'perching bird' order.<sup>2</sup> This is particularly the case for the 'true' songbirds, members of the suborder Passeres (also referred to as 'oscines'), that constitute nearly one half of the over 9000 living avian species.<sup>3</sup> It appears that song is learned by all members of this suborder studied to date.<sup>4</sup> Song is generally a male typical vocalization though species differences in the degree of the sexual dimorphism do exist (e.g. Ref. 5). The neural circuit that mediates the acquisition and production of bird song has emerged as an important model system for study of several important issues in behavioral neuroendocrinology, including: (1) sex differences in brain and behavior, (2) sexual differentiation of the brain, (3) seasonal variation in brain and behavior, (4) the neural basis of sensitive period learning, and (5) neurogenesis in adulthood. Several excellent reviews have appeared over the last few years on these aspects of this remarkable circuit.<sup>6-11</sup> In this review we would like, first, to describe briefly the circuit and discuss the organizational and activational roles played by steroid hormones in the song control system. Sec-

ond, we would like to concentrate on the neurochemistry of the song circuit. This is a potentially rich area of investigation; however, it is still in its early stages. We would like to review the significance of comparative neurochemical studies of this circuit and sex differences in neurotransmission, concentrating on findings that have come out of our laboratory in the past 5 years.

## 2 DESCRIPTION OF THE NEURAL CIRCUIT MEDIATING VOCAL LEARNING AND PRODUCTION IN SONGBIRDS

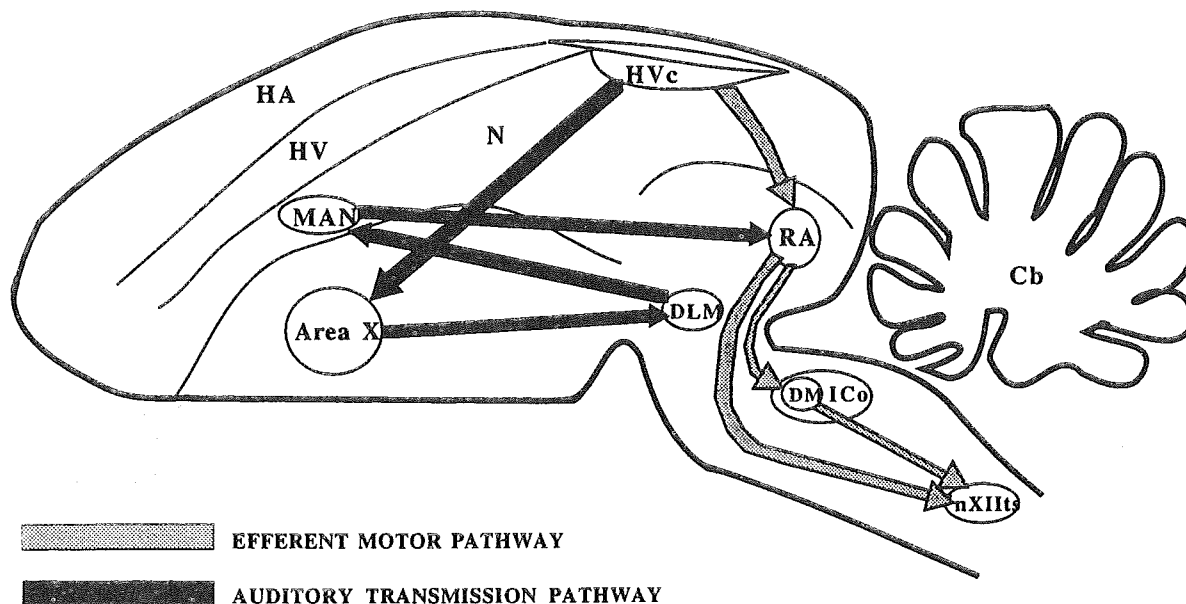
The complex of brain nuclei that mediates the acquisition and production of bird song was first described by Nottebohm and colleagues<sup>12,13</sup> working with canaries (*Serinus canaria*). Through a series of lesion and tract tracing studies, these investigators identified a motor pathway that ultimately innervates the vocal production organ, the syrinx, in canaries and other birds. Song is produced when air is forced through the two separate sides of the syrinx.<sup>14,15</sup> The motor pathway, needed for song production, consists of the caudal part of the ventral hyperstriatum (HVc) which is now considered as part of the neostriatum.<sup>13</sup> Because of its incorrect naming HVc is sometimes called the 'high vocal center'. HVc in turn projects to the robust nucleus of the archistriatum (RA), which collaterally projects to a dorsomedial subdivision (DM) of the intercollicular nucleus (ICo) and the tracheosyringeal division of the hypoglossal nucleus (nXIIIts). DM of ICo also sends an independent projection to nXIIIts. nXIIIts innervates the syrinx via the tracheosyringeal nerve.

In addition to the motor pathway, another related interconnected pathway has also been identified that appears to mediate the processing of auditory information necessary for song learning.<sup>16,17</sup> This pathway is referred to as either the 'auditory pathway'<sup>6</sup> or the 'recursive loop'.<sup>7</sup> Auditory information apparently enters the circuit via a projection from the primary telencephalic auditory projection area 'Field L' to 'shelf' regions adjacent to HVc and RA.<sup>18,19</sup> More recent data suggest that HVc receives a direct projection from Field L.<sup>20</sup> Auditory information progresses from HVc in the following fashion. HVc projects to a subdivision of the parolfactory lobe named 'area X'. The parolfactory lobe is generally thought to be the avian homologue of the caudate nucleus of the basal ganglia.<sup>21</sup> Area X projects to the medial portion of the dorsolateral nucleus of the thalamus (DLM) and this projects to the lateral part of the magnocellular nucleus of the anterior neostriatum (IMAN). IMAN projects to RA thus completing the circuit. HVc also receives projections from a small thalamic nucleus, Uva, and from the telencephalic nucleus interfascicularis (Nif). Nif appears to be involved in mediating temporal aspects of song<sup>22</sup> and Uva seems to modulate auditory input to HVc and direct it toward motor activities only.<sup>23</sup>

These two pathways have been described in the most detail in two species of songbird that readily breed in captivity, canaries and zebra finches (*Taeniopygia guttata*). There is evidence that a similar circuit is present in all members of the suborder Passeres, who constitute the 'true' songbirds.<sup>24,25</sup> A schematic representation of a generic 'song system' is presented in Fig. 1.

Lesions to two telencephalic nuclei in the motor pathway, HVc and RA, interfere with the production of singing in adults.<sup>12</sup> Lesions to MAN and area X in adult zebra finches do not disrupt the production of song; however, it has been shown in this species that lesions to either of these nuclei during the sensitive period for song learning disrupt a bird's ability to learn song.<sup>26-28</sup> In canaries, which add songs to their repertoires throughout their lives, an intact MAN appears to be necessary for this adult plasticity in song.<sup>29</sup>

It should be noted that most of this circuit appears to be a neural specialization that has evolved specifically in members of the sub-order Passeres for the learning and production of complex vocalizations.<sup>24,25</sup> The mesencephalic ICo and nXIIts of the brainstem appear to be the more 'primitive' parts of the circuit and are clearly recognizable in the brains of all birds outside the songbird suborder.<sup>24,25</sup> The telencephalic nuclei



**Fig. 1.** A schematic representation of a generic song 'system' in the sagittal plane. Dorsal is towards the top of the page, ventral is towards the bottom, rostral is towards the left and caudal is towards the right. The connections within the two major pathways described in the text are highlighted either with stippled arrows (for the efferent motor pathway) or solid black arrows (for the auditory transmission pathway). Abbreviations are as follows: Cb: Cerebellum; DLM: medial portion of the dorsolateral nucleus of the anterior thalamus; DM: dorso-medial sub-division of the ICo; HA: hyperstriatum accessorium; HV: hyperstriatum ventrale; HVc: hyperstriatum ventrale, pars caudale (sometimes referred to as the High Vocal Center); IC: nucleus intercollicularis; N: neostriatum; nXIIts: nucleus nervi hypoglossi, tracheosyringeal division; MAN: nucleus magnocellularis neostriatalis anterioris; RA: nucleus robustus archistriatalis.

such as HVC and RA are not recognizable in non-songbirds. This is true even among members of the order Passeriformes who are not members of the sub-order Passeres, such as various North American flycatchers. It has been found that these 'sub-oscine' flycatchers: (1) do not learn their vocalizations, (2) do not need auditory feedback either during ontogeny or in adulthood to produce their vocalizations, (3) do not clearly possess any parts of this circuit except ICo and nXIIIts, and (4) do not possess telencephalic nuclei that contain receptors for sex steroids (see below; Refs 25, 30–32). More passerine species that are not 'true' songbirds need to be studied to confirm this, but it seems to be the case that an important suite of neural and behavioral specializations are associated with evolution of song in the oscines.

Vocal learning and the production of complex vocalizations are not strictly limited to the order Passeriformes. Vocal learning has also been described in members of the order Psittaciformes (parrots, macaws etc) (e.g. ref. 33) and in certain members of the order Apodiformes (in particular hummingbirds).<sup>34</sup> For psittacines, a neural circuit mediating this vocal behavior, that may be analogous to the songbird system, has also been proposed based on studies of budgerigars (*Melopsittacus undulatus*).<sup>35</sup> However, this circuit seems to be very different from the songbird circuit based on both neurochemical and neuroanatomical criteria.<sup>24,36,37</sup>

### 3 SEXUAL DIMORPHISM OF BRAIN AND BEHAVIOR IN SONGBIRDS

Singing behavior has been described as sexually dimorphic in most species of songbirds studied to date. In general, when a behavioral difference has been described one of two situations has been observed: (1) either only males produce the complex song vocalizations, while females produce simpler call-like vocalizations; or (2) both sexes produce song but males will sing at a higher rate than females. The greater use of song by males is thought to be related to the effects of both intra-sexual (i.e. song is used to repel competing males from the territory) and intersexual selection (i.e. females choose males based on their song).<sup>1,38,39</sup> This sex difference in behavior is reflected by prominent morphological sexual dimorphisms within the song circuit. In canaries and zebra finches, HVC, RA, area X, and nXIIIts have all been found to be dimorphic in volume.<sup>40</sup> In the

most extreme case, RA has been reported to be 5.53 times larger in male zebra finches than in females. In duetting species from the tropics in which both males and females sing in roughly equal amounts such dimorphisms are not apparent.<sup>5,41</sup> Thus, there is a reasonable correlation between sexual dimorphisms in behavior and dimorphisms in the volume of the brain regions mediating the behavior.<sup>5,25,42</sup> More subtle sexual dimorphisms have also been described. For example, in HVC, RA and MAN there are many more neurons in males than in females (see Ref. 8 for review). In these three regions as well as in DM and nXIIIts cell bodies are larger in males than in females.<sup>43–45</sup>

### 4 DISTRIBUTION OF SEX STEROID HORMONE RECEPTORS IN THE SONGBIRD BRAIN

The distribution of receptors for the sex steroid hormones seems to be remarkably consistent among the major vertebrate taxonomic groups.<sup>46,47</sup> Morrell and Pfaff<sup>47</sup> have argued that there are four areas of the brain that contain such receptors: (1) the preoptic area, (2) various nuclei in the tuberal hypothalamus, (3) certain limbic forebrain areas such as the amygdala and septum, and (4) certain parts of the mesencephalon. Exceptions to this evolutionarily conservative pattern are rare. Therefore, one of the most striking discoveries that resulted from studies of the neural substrate for bird song was that several of the nuclei in the song circuit contain receptors for sex steroid hormones, such as the androgens and the estrogens. For example, five nuclei in the circuit, HVC, RA, MAN, ICo, and nXIIIts, have been shown to contain androgen receptors in males. This was first demonstrated using autoradiographic procedures in several songbird species including zebra finches<sup>48,49</sup> and chaffinches (*Fringilla coelebs*).<sup>50</sup> This pattern of receptor distribution was confirmed in zebra finches with the use of an *in vitro* binding assay.<sup>51</sup> More recently, an antibody to the androgen receptor was used to localize these receptors by immunocytochemical procedures in zebra finches and canaries and the results are in good agreement with the findings from autoradiographic and binding assay methods.<sup>52</sup>

Furthermore, in certain cases, there is a sex difference in the number of receptors, with males having a higher percentage of labeled cells than females.<sup>53</sup> Sex differences in androgen uptake were

studied systematically in zebra finches where it was demonstrated that in HVC and MAN males have a higher percentage of labelled cells than females.<sup>49,53</sup> Again this sex difference seems to be related to sex differences in behavior. Autoradiographic studies of androgen receptors in duetting bay wrens (*Thryothorus nigricapillus*), that exhibit high rates of singing by both males and females and do not show a marked dimorphism in the volume of the song control regions, confirmed the presence of androgen receptors in all five nuclei listed above and failed to find a sex difference in the pattern of androgen accumulation.<sup>54</sup> However, it should be noted that in canaries, a species in which there are marked sex differences in song and the song system, the proportion of androgen target cells in HVC, RA and IMAN does not differ between males and females.<sup>55</sup> This difference between canaries and zebra finches may account for the ability of T administration to induce song in female canaries but not in female zebra finches (see below).

Studies of the distribution of estrogen receptors in the songbird brain have produced results that are less consistent among the different methods employed than studies of androgen receptors.<sup>24</sup> In both male and female bay wrens, Brenowitz and Arnold<sup>56</sup> detected estrogen receptors in HVC and ICo with the use of autoradiography. Gahr *et al.*<sup>57</sup> visualized estrogen receptors by immunocytochemistry and found the receptors to be present in the HVC and ICo of male canaries. However, Gahr *et al.*<sup>57</sup> failed to find such receptors in the HVC of male zebra finches, though they were present on the ventral border of the nucleus. An autoradiographic study by K. Nordeen *et al.*<sup>58</sup> also failed to detect an appreciable number of estrogen accumulating cells in the zebra finch HVC or in any of the other forebrain nuclei involved in the control of song. Walters *et al.*<sup>59</sup> did detect estrogen receptors with *in vitro* binding assay methods in HVC, RA, MAN, area X and ICo of zebra finches. This study is the only report in any songbird species suggesting that area X contains sex steroid hormone receptors. There is no clear way at present to resolve the inconsistencies among the studies on zebra finches, however, procedural differences related to tissue preparation and sensitivity of the detection method are likely to be involved. It appears that the only forebrain song control area that definitely contains high, consistently detectable, levels of estro-

gen receptors is HVC<sup>32</sup> and in the case of zebra finches these receptors may well be primarily adjacent to the nucleus but not in it. Gahr *et al.*<sup>32</sup> have recently published a survey of the distribution of estrogen receptors as determined by immunohistochemistry for 26 avian species in 6 orders. Forebrain estrogen receptors in nuclei such as HVC are only observed in oscine species; sub-oscines and non-passerine orders do not exhibit any signs of such forebrain receptors. It is important to note that no steroid sensitive forebrain areas were observed in either budgerigars or in the one hummingbird species investigated, Anna's hummingbird (*Calypte anna*). Both of these species are non-songbirds that are known to exhibit at least some forms of vocal learning. These data are consistent with the notion that there is a suite of forebrain specializations in oscines that is not observed in other avian taxa, even when these taxa exhibit behavioral phenomena reminiscent of what has been observed in oscines.

## 5 SONG INDUCTION AND THE MODULATION OF THE SONG CIRCUIT BY HORMONES

Pröve<sup>60</sup> and Arnold<sup>61</sup> first systematically demonstrated that testosterone is involved in the induction of song and the related courtship displays in male zebra finches. Testosterone has now been shown to be effective in stimulating song production in a variety of wild and domestic songbird species (e.g. the song sparrow, see Ref. 62). Song induction by testosterone can be mimicked to a certain degree by treatments with 17 $\beta$ -estradiol (E<sub>2</sub>) or 5 $\alpha$ -dihydrotestosterone (DHT) alone; however, the behavior is best reinstated in castrates when the two hormones are administered in combination.<sup>63</sup> Because it is known that these metabolites are produced locally in the brain of songbirds<sup>64</sup> it is assumed that the metabolism of T is required for behavioral induction. Direct evidence, obtained with the peripheral application of inhibitors of metabolism, has confirmed this interpretation.<sup>65</sup> The administration of both androgenic and estrogenic metabolites of T is required for the full reinstatement of singing after castration in both zebra finches and red-winged blackbirds (*Agelaius phoeniceus*).<sup>63,66</sup> This suggests that these hormones act synergistically to activate singing behavior and other courtship displays

shown by male songbirds. The exact site in the brain where this synergism occurs and the precise role that these different steroid target sites play in the activation of song has yet to be determined.

Besides its role in the behavioral induction of song, testosterone also has substantial effects on the morphology and neurochemistry of the song system in adult birds. The first suggestion that this might be the case was the discovery of seasonal changes in the volume of HVC and RA in male canaries.<sup>67</sup> These nuclei appear 40–50% smaller in the Fall than in the Spring. Such changes in nuclear volume also occur in females.<sup>68</sup> These changes have been related to the substantial decline in circulating levels of gonadal sex steroids that occurs in canaries and other songbird species from the Spring to the Fall.<sup>69,70</sup> Seasonal changes in synaptic morphology in the song system have also been described. DeVoogd *et al.*<sup>68</sup> found that there was a decline in the number of transmitter vesicles in RA in female canaries in Fall as compared to the Spring. These seasonal changes were originally related to the fact that canaries unlike most other songbirds continue to acquire new songs throughout their lives.<sup>71</sup> It was thought that the increase in size was needed for the storage of memories of new songs. However, seasonal changes in volume of song control nuclei have been described in species that do not add new songs to their repertoires (e.g. see Ref. 72), thus suggesting that seasonal variation in volume may be independent of repertoire modification.

The possible role that testosterone plays in the induction of these morphological changes in adult birds has been tested more directly by hormone manipulation studies (reviewed in Ref. 8). Most of these studies have used female canaries as experimental subjects. Testosterone can induce singing in adult female ovariectomized canaries, though the quality of song produced is not as diverse as that normally uttered by males.<sup>73–75</sup> There are also morphological changes associated with this hormone treatment. For example, RA increases in volume by more than 50% and the dendritic fields of RA neurons increase by approximately 50% and increase in radius by about 25%<sup>68,75,76</sup> (reviewed in Ref. 8). These morphological effects may also require the synergistic action of androgenic and estrogenic metabolites of testosterone because administering DHT alone or E<sub>2</sub> alone is not as effective in inducing these changes as administering T.<sup>77</sup>

## 6 SEXUAL DIFFERENTIATION OF BRAIN AND BEHAVIOR IN SONGBIRDS

The discovery of substantial and long lasting morphological changes in response to steroid treatment in adult canaries was, in part, the reason why some investigators (e.g. see Refs 9 and 78) have questioned the utility of the classical distinction between the activational (referring primarily to behavioral induction in adults) and organizational steroid effects (referring primarily to ontogenetic actions that induce sex differences in morphology) as originally formulated by Phoenix *et al.*<sup>79</sup> Arnold<sup>9</sup> has pointed out that what appear to be organizational effects can occur in adulthood and therefore it is more accurate to call these effects permanent (instead of organizational) and transient (instead of activational). Although canaries show a remarkable amount of plasticity in response to steroid hormone treatment in adulthood, in other species such as zebra finches, adult sex differences in brain and behavior are still present even when males and females experience identical hormonal conditions. It has therefore always been assumed that these differences are permanently organized by the actions of steroid hormones early in life as is the case for sex differences in brain and behavior in many other species (see Refs 80–82 for reviews).

The administration of E<sub>2</sub> to nestling female zebra finches masculinizes the song control nuclei<sup>43</sup>. The sizes of HVC and RA and the number of neurons in these nuclei are substantially greater in females who have been treated with E<sub>2</sub> as nestlings as compared with those who have not.<sup>43,83,84</sup> Also nuclei such as MAN and area X (which can not even be readily discerned in untreated female zebra finches) are distinct in these birds.<sup>84,85</sup> This early E<sub>2</sub> treatment also affects sex differences in cell numbers, projections between nuclei and the number of steroid receptor containing cells in the song control nuclei (see Ref. 8 for review). These findings suggest that the female is the 'neutral' sex in zebra finches as far as song control is concerned (i.e. they have to be masculinized by exogenous E<sub>2</sub> to show male-like song behavior and brain morphology).

There are, however, several inconsistencies and unsolved problems associated with our understanding of the sexual differentiation of brain and behavior in zebra finches. For example, the three published studies that measured E<sub>2</sub> during ontogeny in zebra finches are inconsistent. One finds

higher levels of  $E_2$  in males than in females<sup>86</sup> and the other two do not find such a difference.<sup>87,88</sup> Also, the dose of  $E_2$  that is required to induce masculinization appears to be out of the physiological range, and half the usually administered dose is not effective.<sup>87</sup> Gonadectomy of zebra finch nestlings at age 1 week does not alter sexual differentiation,<sup>89</sup> though this could well be the result of extra-gonadal sources of steroids.<sup>87,88,90</sup>

Other attempts to suppress the action of  $E_2$  during ontogeny have also failed to clarify this problem. The administration of several antiestrogens to nestling zebra finches paradoxically results in a hypermasculinization of the song system.<sup>91,92</sup> As noted by Mathews *et al.*,<sup>91</sup> this paradoxical effect may have resulted from the fact that some antiestrogens can act as weak estrogens at times. Mathews and Arnold<sup>93,94</sup> have also presented data suggesting that tamoxifen does act as an estrogen in the zebra finch song system and that it does not block the accumulation of estradiol in the zebra finch brain. Other interpretations of these paradoxical effects include the idea that the antiestrogen works temporarily to block negative feedback from endogenous estrogen thus increasing the stimulation by the pituitary of endogenous levels. When the antiestrogen ceases acting or if the administered dose does not fully block all available sites then the brain would receive a high amount of endogenous estrogen (see Refs 92 and 95 for a discussion). It is difficult to resolve fully the reason for this effect with the data that are available at present. However, these studies, and the others reviewed above, make it clear that one important experimental approach that would help resolve this problem is to study neural and behavioral development in anhormonal zebra finches (specifically estrogen-free zebra finches). Recent studies by Schlinger and Arnold<sup>88</sup> suggest that in male zebra finches the source of circulating estrogenic metabolites comes from aromatization in the brain itself of a steroid substrate provided by the adrenal gland, indicating how difficult it may be to induce such an anhormonal state.

## 7 TOPOGRAPHY AND CHEMICAL NEUROANATOMY OF THE SONG CONTROL NUCLEI AND THEIR ENDOCRINE MODULATION

As noted by Arnold,<sup>9</sup> one of the many advantages of the song control circuit that makes it an excellent model system for neuroendocrine investiga-

tions is that the nuclei in the circuit are discrete and cytoarchitecturally distinct; therefore they can be easily defined with the use of standard histological methods (e.g. Nissl stains and myelin stains). However, a variety of studies have shown that the nuclei in the song control system can be sub-divided based on hodological criteria (i.e. their connectivity) and, in a limited number of cases, based on neurochemical criteria. For example, Vicario and Nottebohm have shown that nXIIIts contains discrete subdivisions based on the muscles in the syrinx that it innervates<sup>96</sup> and that RA can be subdivided based on the fact that the dorsal part innervates the ICo complex and the more ventral parts of RA consist of horizontal bands of cells that selectively project to the different parts of nXIIIts.<sup>97</sup> The nucleus uvaefornis (Uva) can be sub-divided into two parts based on the presence (dorsal cap part) or absence (ventral region) of cells immunoreactive for corticotropin-releasing factor.<sup>98</sup> HVC projecting neurons are present in the entire nucleus, but Nif projecting neurons are present in the ventral body of the nucleus only.<sup>98</sup> In other cases, investigations of transmitter systems that innervate the various nuclei of the song circuit, or descriptions of the pattern of hormone uptake in a given nucleus, have revealed definitions of the boundaries of the different nuclei that are similar to that ascertained with the use of standard histological methods that stain for Nissl substance (i.e. basophilic acidic protein) or myelin (e.g. see Ref. 99; see Ref. 24 for a review).

When concerned with endocrine modulation of the song control circuit it is potentially revealing to assess the volume of these nuclei based on hodological and neurochemical criteria that may or may not subdivide the nuclei. Endocrine induced changes in the volume of these nuclei based on these independent criteria may provide much greater insight into the functional significance of any endocrine modulation and may even significantly alter the interpretation of such endocrine induced changes as compared to an interpretation based on general staining procedures alone. This is well illustrated by the study of Gahr<sup>99</sup> where he asked whether seasonal changes can be discerned in the volume of the canary HVC when one defines the boundaries of this nucleus based either on immunohistochemical staining for the estrogen receptor or based on the cells that send long projections to area X. When using these criteria a seasonal change in volume was not apparent though he clearly observed a seasonal change in

volume when staining alternate sections by a Nissl procedure, as was previously reported by Nottebohm.<sup>67</sup> Thus the use of neurochemical markers is essential for a complete functional analysis of the endocrine modulation of this circuit. As discussed below several such potential markers have now been identified.

### 7.1 Immunohistochemical localization of neuropeptides in the song control circuit

A variety of neuropeptides have been localized in the songbird brain with the use of immunohistochemistry (see Ref. 24 for review). Of the many peptides investigated, two neuropeptides in particular have been found to specifically label MAN and HVc in the brain of zebra finches, European starlings (*Sturnus vulgaris*) and song sparrows (*Melospiza melodia*).<sup>100,101</sup> Immunoreactive fibers for both methionine-enkephalin (ENK) and vasoactive intestinal peptide (VIP) were found to define the boundaries of these nuclei. The boundaries defined in this way seem to correspond well with the boundaries defined by Nissl staining methods. Perikarya located within these nuclei were, in general, not found to be immunoreactive except in the case of ENK in MAN and HVc. In both cases, pre-treatment with colchicine revealed a few immunoreactive cells (this was also the case for RA) but their distribution was scattered and did not define well the boundaries of the nuclei. These results are described in detail in Ball *et al.*<sup>101</sup> This pattern of peptide immunoreactivity associated with these telencephalic regions appears to be a unique feature of the songbird brain, just as is the pattern of steroid uptake. When one examines the distribution of these peptides in non-songbird species the pattern of immunoreactivity in the hypothalamus and most other brain areas is roughly similar among the various species. However, no immunoreactivity for VIP or ENK is observed in the neostriatum of non-songbirds as is observed in the songbird neostriatum in association with MAN and HVc (reviewed in Ref. 24). Deviche and Güntürkün<sup>102</sup> recently compared the distribution of two opioid peptides, leucine-enkephalin and dynorphin B in the ICo-DM complex of a songbird species, the dark-eyed junco (*Junco hyemalis*) and a non-songbird, the domestic pigeon (*Columba livia*), a member of the order Columbiformes. As discussed above, this vocal control nucleus is a 'primitive' part of the song control circuit in that it appears to be common to all bird species. Unlike the pattern of immunoreactivity

observed in the forebrain, the pattern of immunoreactivity in the ICo-DM complex appears to be the same in these two distantly related taxa. ICo contains a high level of immunoreactivity but the DM sub-region is defined by comparatively low amounts of immunoreactive material in comparison to the surrounding ICo. Thus when one examines the neurochemistry of mid-brain areas important for vocal behavior one is less apt to observe features that appear to be oscine specializations.

### 7.2 The localization of neurotransmitter receptors in the song control circuit with the use of quantitative autoradiography

The neurochemical specializations associated with nuclei in the song control circuit are also apparent when one investigates the density of different neurotransmitter subtypes with the use of in-vitro quantitative autoradiography. As reviewed by Ball,<sup>24</sup> when one maps the distribution of various receptor subtypes in the Japanese quail and in songbird species, such as European starlings, song sparrows and zebra finches, the overall pattern of receptor density is quite similar among the various species with the exception of the nuclei in the song control circuit. For example, we labeled  $\alpha_2$ -adrenergic receptors with the use of the specific agonist [<sup>3</sup>H] *p*-amino clonidine (PAC) as the ligand and found a high density of receptors in MAN, HVc, area X, and RA as compared to the surrounding structures (i.e. the neostriatum, the parolfactory lobe and the archistriatum respectively). This high receptor density is particularly associated with these specific nuclei (reviewed in Ref. 24). This ligand for the  $\alpha_2$ -adrenergic receptor apparently defines an RA and an HVc nucleus that is substantially larger in males than in females. This is consistent with the significant sex difference in the volume of RA and HVc in starlings as measured from Nissl stained tissue.<sup>103</sup>

In non-songbird species, such heterogeneity in receptor binding in brain regions such as the neostriatum is not apparent (reviewed in Ref. 24). Thus, when one starts to place several different aspects of the chemical neuroanatomy of the song control circuit in a comparative context it is apparent that the neurochemical specializations of this circuit extend well beyond the occurrence of steroid receptors. The pattern of receptor density of a given transmitter receptor sub-type in the songbird circuit is not always such that there is a higher receptor density in comparison to the sur-

rounding structure. In some cases, the song control nuclei are associated with a lower receptor density. For example, muscarinic cholinergic receptor density, as labeled by the binding of [<sup>3</sup>H]-N-methyl scopolamine (NMS), is higher in area X than in the surrounding parolfactory lobe.<sup>103-105</sup> However, the density of muscarinic cholinergic receptors in MAN and RA is lower than in the surrounding neostriatum and archistriatum, respectively. Along the same lines, the density of N-methyl-D-aspartate (NMDA) receptors in lMAN, HVc, and RA as defined by [<sup>3</sup>H] MK-801 binding is lower than in the surrounding structures in starlings and zebra finches.<sup>106,107</sup>

In summary, when one uses a broad number of neurochemical markers in various oscine and non-oscine avian species, it becomes apparent that each of the song control nuclei has a particular neurochemical 'signature'. This 'signature' is characterized by the total configuration of neurochemical differences (different in comparison to non-songbird species), involving both the distribution of hormone receptors as well as the distribution of a variety of transmitters. The functional significance of this variation is not well understood at present. The role that these different receptor sub-types may be playing in these different nuclei in the regulation of song learning and production has not yet been investigated with the application of neuropharmacological manipulations. However, it is clear that various neurochemical components have, over the course of evolution, become specialized in the different song control nuclei. One can take advantage of this variability and characterize the sexual dimorphism of these markers, their changes over ontogeny (in relation to sensitive periods for song learning) and their regulation by steroids in adulthood. By studying the modulation of these specialized transmitter systems, in regions of a neural circuit with well defined functions, one can gain insight into both the circuit and the transmitter systems themselves. One can also study these neurochemical signatures with the use of the comparative method. As reviewed above, vocal learning seems to have evolved independently in songbirds, hummingbirds and parrots. The neural specializations associated with the evolution of this learning also appear to be different in these different taxa. Detailed comparisons though have only just begun. Studies of the neurochemical basis of song learning in oscines can form the basis for detailed comparative investigations. Male/female comparisons within

species may also be useful in understanding the functional significance of transmitter system specialization in the song system.

### 7.3 Endocrine modulation of neurotransmitter receptors and steroid metabolizing enzymes in avian vocal control regions

As discussed above, the mesencephalic nucleus ICo seems to be a 'primitive' part of the avian vocal control circuit that is present in all species of birds including non-vocal learners such as the Japanese quail. This nucleus also contains receptors for both androgens and estrogens in all species studied to date, including quail.<sup>24</sup> Both  $\alpha_2$ -adrenergic and muscarinic cholinergic receptors are modulated by testosterone in the quail ICo.<sup>108</sup> These changes in receptor density were identified in discrete sub-regions of this nucleus with the use of in-vitro quantitative neurotransmitter receptor autoradiography.<sup>108</sup> With this technique one can detect small changes in the pattern of the receptor binding induced by steroids while maintaining a high degree of neuroanatomical resolution.

Steroid metabolizing enzymes, such as  $5\alpha$  and  $5\beta$  reductases that reduce T to DHT, and aromatase that converts T to  $E_2$  have been identified in the song control nuclei of zebra finches.<sup>64,109</sup> The activity of these enzymes is sexually dimorphic in many of the telencephalic song control areas which may contribute to the sexual dimorphism in the response to T (males sing in response to T and females do not). For example, the production of more estrogen in one nucleus might amplify the action of T, as has been described in other systems (e.g. Ref. 110). The specific role that the various metabolites of T are playing in these nuclei has not been investigated. Gonadectomy and steroid replacement methods have been used to study the modulation of the steroid metabolizing enzymes in the song control circuit of zebra finches.<sup>109</sup> These enzymes were modulated by T in some brain regions but not in others, suggesting that there is variation among the different steroid sensitive song control nuclei in the processing of T.

### 7.4 Sex differences in neurotransmitter receptors in the avian vocal control circuit

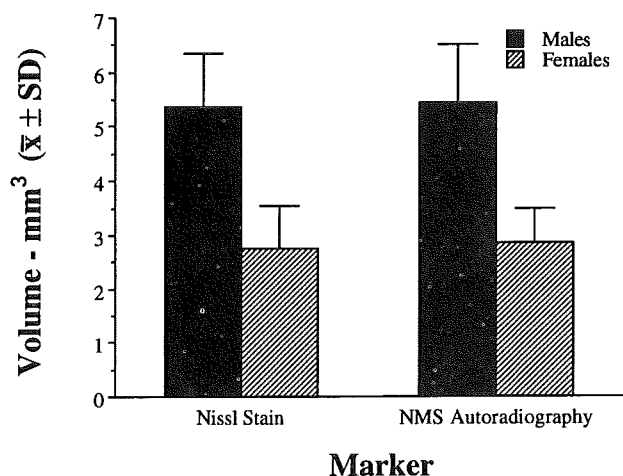
One of the most striking aspects of the song system is the apparent correlation between the degree of dimorphism in the volume of various



song nuclei and the extent of behavioral sex differences in song. This brain-behavior relationship provides researchers with the opportunity to investigate the specific neurochemical and neuroanatomical substrates of a highly specialized and learned behavior. That is, by comparing the song control nuclei in males and females, one can elucidate the components that are common to both sexes and those that are unique. Further exploration of the characteristics that distinguish the two sexes will contribute to the illumination of the necessary and sufficient neurochemical bases of song.

At this point, however, we know relatively little about the neurochemical features that distinguish males and females. Highlighting these sex differences in volume with standard histological techniques is an important first step in directing attention to possible sources of variation. However, a complete reliance on this approach is too limiting. Just as Gahr's<sup>99</sup> finding (see above) demonstrated the need to employ independent neurochemical (and hodological) markers to establish the bases of seasonal plasticity, so too should we employ these markers to understand more fully the foundations of sex differences in song.

Although the research in this area is still in its infancy, we have some data that demonstrate the valuable information that can be garnered by means of this approach. Bernard *et al.*<sup>103</sup> used a



**Fig. 2.** Histograms illustrating sex differences in the volume of area X in European starlings measured with the use of two different neurochemical markers. Alternate sections were either stained for Nissl substance or labelled for muscarinic cholinergic receptors. Area X is well defined by the high density of muscarinic cholinergic receptors in comparison to the surrounding parolfactory lobe. Volumetric reconstructions using these two different methods provided identical results. See text for further details.

Nissl stain and autoradiography for muscarinic cholinergic receptors to measure the volume of area X in male and female starlings. Both markers indicated that the nucleus is approximately twice as large in males as in females (see Fig. 2). Thus, in contrast to the lack of correspondence between a Nissl stain and immunocytochemistry for estrogen receptors in the HVC of Fall canaries,<sup>99</sup> in this case the two methods defined the dimorphism in the same way. In addition, measurement of the density of muscarinic receptors in area X revealed no sex difference (see also Ref. 105). Thus, while the two sexes do not differ in terms of receptor density, they do differ in overall volume of the nucleus. Therefore, male starlings must have more muscarinic receptors in area X than females. The functional significance of this sex difference is unknown at present; however, given the role of acetylcholine in the formation and maintenance of memories in other vertebrates (e.g. Ref. 111) and the putative role of area X in song learning,<sup>27,28</sup> it is possible that the sex difference in muscarinic receptor number may modulate the differential ability of acetylcholine to contribute to the acquisition and later production of song in males and females.<sup>103</sup>

In contrast to these studies of muscarinic cholinergic receptors in area X, studies of dopamine receptor sub-types in this nucleus in starlings have revealed a sex difference in receptor density relative to receptor density in the LPO. Lewis *et al.*<sup>112</sup> suggested that area X in zebra finches receives a dopaminergic projection from the area ventralis of Tsai (AVT). This is not surprising since area X is a sub-region of the avian homologue of the caudate, LPO, which is a well-known dopaminergic target. In an attempt to characterize the receptor sub-types mediating this projection, Casto and Ball<sup>113</sup> labeled D1 dopamine receptors in male and female European starlings with the use of the D1 specific antagonist [<sup>3</sup>H] SCH 23390. The nucleus was defined by a higher receptor density in area X than in the surrounding LPO in both males and females, and the form of this higher receptor density seemed to correspond to the shape of the nucleus as defined in Nissl stained material. However, this was not systematically measured as was done for NMS binding described above. Receptor density in dorso-lateral LPO was subtracted from area X receptor density for each individual, and these difference scores were then subjected to a *t*-test which revealed a significant sex difference ( $p < 0.05$ ).

Males had significantly higher D1 receptor density in area X relative to dorsolateral LPO than did females. This suggests that there is a sex difference in the dopaminergic modulation of the auditory pathway that may be necessary for song learning in starlings.

Application of these detailed autoradiographic investigations to other song control nuclei and to other receptor systems will certainly shed more light on the characteristics that distinguish males and females. By pinpointing these differences we will be able to target more effectively functional neuropharmacological studies of these neurochemical sex differences, and thereby elucidate the neural mechanisms underlying birdsong.

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