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SEX DIFFERENCES IN THE VOLUME OF AVIAN SONG CONTROL NUCLEI: COMPARATIVE STUDIES AND THE ISSUE OF BRAIN NUCLEUS DELINEATION

GREGORY F. BALL, JOSEPH M. CASTO, and DANIEL J. BERNARD
Department of Psychology, Behavioral Neuroendocrinology Group, Johns Hopkins University, Baltimore, Maryland, USA

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SUMMARY

Two goals of research on neural sex differences are to establish the behavioral function of such sex differences and to identify precisely what features differ between males and females. Comparative studies of sex differences in the volume of brain nuclei within the songbird vocal control circuit provide one way to address these goals. Informative comparisons can be either inter-specific or intra-specific. Inter-specific comparisons of species within the songbird suborder allow one to establish how species variation in the degree to which there is a sex difference in nuclear volume relates to species variation in the degree to which there is a sex difference in vocal behavior. Intra-specific comparisons of sex differences in nuclear volume involve the comparison of a variety of histochemical methods to define nuclei and describe a nucleus within a species. Sex differences in nuclear volume have now been measured for at least some song control nuclei in 10 different passerine species. In species with more complex male than female song, the volume of key song control nuclei is on average larger in males than in females. However, future studies will require more refined measures of vocal behavior and perceptual abilities to make more precise correlations between brain and behavior. In European starlings (*Sturnus vulgaris*), the volume of the vocal control nucleus, area X was found to be on average 1.95 times bigger in males than in females based on Nissl stained sections. Variation in neurotransmitter receptor density as determined by quantitative receptor autoradiography can also be used to define clearly the boundaries of a nucleus. When the boundaries of area X in male and female starlings were defined based on variation in muscarinic cholinergic and α_2-adrenergic receptor densities, volumetric estimates were obtained that are nearly identical to those obtained with the use of Nissl stains. Intra-specific comparisons of this sort extend our knowledge concerning the neurochemical basis of sex differences in nuclear volume. The wide application of this method would greatly increase our understanding of neural sex differences.

Keywords—Vocalization; Sexual differentiation; α-$_2$ Adrenergic receptors; Bird; European starling.

INTRODUCTION

IN THE 1980s it became generally accepted that substantial anatomical sex differences are present in the vertebrate central nervous system (Arnold & Gorski, 1984). Such differences in the brain have now been described in a wide variety of vertebrate taxa.

Address correspondence and reprint requests to: Dr. G.F. Ball, Department of Psychology, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA.

For example, significant sex differences have been described in the central nervous system of certain species of sound producing teleost fish (Bass, 1992); in a species of frog, *Xenopus laevis* (Kelley, 1992); in reptiles such as lizard species in the genus *Cnemidophorus* (Crews et al., 1990; Wade & Crews, 1992); in bird species in the orders Galliformes and Passeriformes (e.g., Arnold et al., 1986; see Panzica, 1988 for a review) and in species representing at least four mammalian orders: ungulates, carnivores, rodents, and primates (Baum et al., 1990; Breedlove, 1992; DeVries, 1990; Goy & McEwen, 1980; Van Eerdenburg & Swaab, 1991).

The discovery of these sex differences was inspired, initially, by studies designed to discover the neural bases of behavioral sexual dimorphisms (Arnold & Gorski, 1984; Breedlove, 1992; Goy & McEwen, 1980; Kelley, 1988). However, after the somewhat surprising discovery of marked neural sex differences in a few species, the nervous systems of many species were investigated with the goal of finding neural sex differences independently of detailed knowledge about sex differences in a species' behavior. Therefore, at present, in some cases the relationship between sex differences in the nervous system and sex differences in behavior and physiology is relatively well understood, while in other cases it is not. For example, the preoptic medial nucleus (POM) in Japanese quail (*Coturnix japonica*) is significantly larger in volume in males than in females and the function of this nucleus clearly relates to a sexually dimorphic behavior: copulatory behavior (Balthazart & Foidart, 1993). In the case of the sexually dimorphic nucleus of the preoptic area in the rodent brain described by Gorski et al. (1978), a substantial neural sex difference in volume has been clearly identified but its relevance to behavior or physiology is still something of a mystery (reviewed by Breedlove, 1992).

It is well known that neural sex differences are profoundly regulated by sex steroid hormones (Arnold & Gorski, 1984). Developmental studies in a variety of species have already established that in some cases early "organizational" actions of sex steroid hormones set up enduring sex differences in neuroanatomy, while in other cases sex differences in circulating levels of hormones in adults are the critical factor, so that by reversing the sex difference in circulating hormone levels one can reverse the sex difference in the nervous system (Arnold & Gorski, 1984; Kelley, 1988).

Now that it is clear that neural sex differences are widespread, and we have some knowledge about their regulation by gonadal steroids, important issues for future studies include: 1) the precise characterization of what is different in the brains of males and females; 2) the establishment of how these neural sex differences relate to sex differences in behavior and in physiology; and finally 3) an increased understanding as to how these differences develop, including the identification at the molecular level of what sex steroid hormones are doing to organize neural sex differences (Breedlove, 1992; McCarthy, 1994).

In this paper, our discussion concentrates on how comparative studies of the songbird vocal control circuit can help us clarify the first and second issues. These comparative studies alone can never fully answer causal questions because they only produce correlational data, however, they can help guide subsequent experimental investigations. Within this circuit, large sex differences in the volume of specific brain nuclei are known to be associated with sex differences in vocal behavior (Arnold et al., 1986). In particular, we will review how comparative studies of sex differences in the volume of brain nuclei within this vocal control circuit may shed light on general principles relevant to our understanding of neural sex differences. These comparative studies involve both inter-specific comparisons and intra-specific comparisons. For inter-specific comparisons we

try to answer the question "How does species variation in the degree to which there is a sex difference in nuclear volume relate to species variation in the degree to which there is a sex difference in behavior?" The comparison of species variation of this sort is one type of information that can help us better characterize the function of a neural sex difference. Intra-specific comparisons of neural sex differences involve the comparison of a variety of histochemical methods to define nuclei and describe the brain. With these comparisons we try to answer the question "If one uses different methods to define the boundaries of a sexually dimorphic brain nucleus within a given species does one always detect a similar sex difference in nuclear volume?" The use of different neurochemical markers to characterize the same nucleus within a species provides one with different "views" of the brain and one can ask if similar sex differences are consistently observed with all these different views of the brain. These types of studies will help illustrate more precisely what is different between male and female brains that are known to contain volumetric sex differences.

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

The neural circuit underlying the acquisition and production of birdsong was first described by Nottebohm and colleagues (Nottebohm et al., 1976, 1982) working with canaries (*Serinus canaria*). These investigators identified a motor pathway that ultimately innervates the vocal production organ, the syrinx, in canaries and other birds. Song is produced when the muscles associated with the two separate sides of the syrinx are activated leading to a change in the configuration of syrinx and the internal tympaniform membranes (Nowicki & Marler, 1988; Vicario, 1991a, 1991b). Song production requires a close coordination with respiration (Wild, 1993a, 1993b) and recent studies in zebra finches (*Taeniopygia guttata*) have identified projections from telencephalic vocal control regions to brainstem structures in the lateral medulla involved in respiration, such as the nucleus ambiguus or areas in close associated with it (Vicario, 1993; Wild, 1993a, 1993b).

Based on detailed studies in both canaries and zebra finches (Bottjer et al., 1989; Nottebohm et al. 1976, 1982), the motor pathway that mediates song production is generally thought to involve a telencephalic nucleus first incorrectly named the caudal part of the ventral hyperstriatum (HVc) and now often referred to as the "high vocal center." HVc 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 (nXIIts). DM of ICo also sends an independent projection to nXIIts. nXIIts innervates the syrinx via the tracheosyringeal nerve. Vicario (1993) has shown that in zebra finches, a dorsal subregion of RA also projects to the ventrolateral medulla. DM also projects to this medullary area (Vicario, 1993). It is this pathway that Wild (1993a, 1993b) has shown seems ultimately to provide a link between the song system and areas controlling respiration.

In addition to this pathway, another related interconnected pathway has also been identified that connects HVc to RA (Bottjer et al., 1989; Okuhata & Saito, 1987). HVc projects to a subdivision of the parolfactory lobe named "area X." The parolfactory lobe is thought to be a component of the avian brain complex homologous to the basal ganglia, perhaps the caudate nucleus (Parent, 1986). 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 (lMAN). lMAN projects to RA thus completing the circuit. HVc also receives projections from the medial part of the magnocellular nucleus of the anterior neostriatum (mMAN), a small thalamic nucleus, Uva, and from the telencephalic nucleus interfacialis (Nif). Nif and Uva appear to be involved primarily in mediating temporal aspects of song (McCasland, 1987; Williams & Vicario, 1993), though the precise role played by each nucleus requires further investigation.

Many of the nuclei in this circuit contain neurons that are responsive to sound. In many cases these cells have been shown to have highly complex response properties (see Doupe, 1993 for a review). For example, studies in adult zebra finches have identified cells within several nuclei of the song circuit that respond most strongly to sounds derived from the bird's own song (Doupe & Konishi, 1991; Margoliash & Fortune, 1992). 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 (Katz & Gurney, 1981; Kelley & Nottebohm, 1979). More recent data suggest that HVc receives a direct projection from Field L (Fortune & Margoliash, 1992).

As stated above, the vocal control circuit including these two interconnected pathways has been best described in two species of songbirds that readily breed in captivity, canaries and zebra finches. However, there is evidence that a similar circuit is present in all members of the suborder Passeres, who constitute the "true" songbirds (Ball, 1990; Brenowitz, 1991a). Approximately, 45% of the over 9,000 living species of birds belong to this suborder. A schematic representation of the major projections within a generic "song system" is presented in Fig. 1.

Lesions to two telencephalic nuclei in the more direct pathway, HVc and RA, interfere with the production of singing in adult canaries (Nottebohm et al., 1976). Lesions to lMAN and area X in adult zebra finches do not disrupt the production of song by adults; 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 (Bottjer et al., 1984; Morrison & Nottebohm, 1993; Scharff & Nottebohm, 1991; Sohrabji et al., 1990). In canaries, who add songs to their repertoires throughout their lives, an intact lMAN appears to be necessary for this adult plasticity in song (Nottebohm et al., 1990; Suter et al., 1990).

It should be noted that most of this circuit appears to be a neural specialization that has evolved specifically in members of the suborder Passeres for the learning and production of complex vocalizations (Ball, 1990; Brenowitz, 1991a; Doupe, 1993). 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 (Ball, 1990; Brenowitz, 1991a). The telencephalic nuclei 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 suborder 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 nXIIts, and 4) do not posses telencephalic nuclei that contain receptors for sex steroids (Brenowitz, 1991a; DeVoogd, 1986; Gahr et al., 1993; Kroodsma & Konishi, 1991). More passerine species that are suboscines need to be studied to confirm this generalization, but it does seem to be the case that an important suite of neural and behavioral specializations are associated with the evolution of song in the oscines.

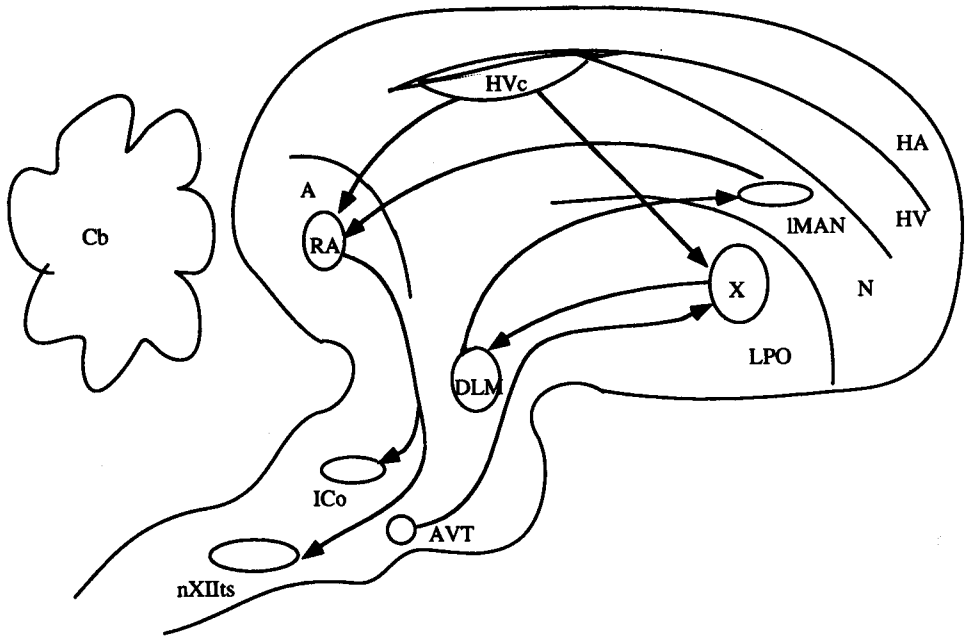

FIG. 1: A schematic of a "generic" songbird brain illustrating many of the nuclei in the network of brain areas that controls the acquisition and production of birdsong. An important portion of the motor pathway involved in song production consists of the HVc to RA to nXIIts projection. The HVc to X to DLM to MAN to RA pathway possesses auditory characteristics and is important for song learning. HVc = hyperstriatum ventrale, pars caudale or high vocal center; RA = robustus archistriatalis; nXIIts = tracheosyringeal division of the hypoglossal nucleus; lMAN = lateral part of the magnocellular nucleus of the anterior neostriatum; X = area X; DLM = medial nucleus of the dorsolateral thalamus; Cb = cerebellum; HA = hyperstriatum accessorium; HV = hyperstriatum ventrale; N = neostriatum; LPO = parolfactory lobe; AVT = area ventralis of Tsai; A = archistriatum; ICo = nucleus intercollicularis.

SEX DIFFERENCES IN VOCAL BEHAVIOR IN SONGBIRDS

Extreme sex differences in behavior of the sort that lead to the designation "sexually dimorphic" behavior most often occur among behaviors associated with courtship and reproduction (Goy & McEwen 1980; Kelley, 1988). In many species of birds copulatory behavior is sexually dimorphic in that mounting and cloacal contact movements are only observed in males (Balthazart, 1983). Vocal behavior of some sort is present in most avian species and is frequently elaborate and well developed (Nottebohm, 1975). Because vocalizations are dynamic and variable in time and space, precisely characterizing the degree of a sex difference in this behavior is difficult in many cases, even if one picks a relatively well defined measure such as repertoire size (Kroodsma, 1982). However, it does appear that vocal behavior is more apt to be different between the sexes among species within the songbird order as compared to species studied in other avian orders (Nottebohm, 1975). This is especially true if one concentrates on more complex vocalizations referred to as "songs" rather than simpler vocalizations often referred to as "calls." If one reserves the term "song" to refer to the complex vocalizations used to attract mates and defend a territory then it appears that singing behavior is substantially different between the sexes in most species of songbirds studied to date (Nottebohm, 1975). In

TABLE I. MALE/FEMALE RATIOS OF THE VOLUME OF SELECTED SONG CONTROL REGIONS

Species	Brain nucleus		
	Area X	HVc	RA
Orange Bishop*	No data	Not visible in females	29/1
Zebra Finch†	Not visible in females	5.01/1	5.53/1
Canary†	3.82/1	4.28/1	2.88/1
Red-winged blackbird‡	6.2/1	3.2/1	4.7/1
White-crowned sparrow§	No data	3.71/1	2.44/1
Chat¶	2.78/1	2.93/1	2.34/1
European starlings#	1.9/1	1.64/1	1.66/1
Rufous-White Wren¶	1.68/1	2.16/1	1.7/1
Buff Breasted Wren¶	1.46/1	1.28/1	1.49/1
Bay Wren¶	1.13/1	1.5/1	1.1/1

*Based on Arai et al., 1989; †based on Nottebohm & Arnold, 1976; ‡based on Kirn et al., 1989 (spring values only); §based on Baker et al., 1984; ¶based on Table IV from Arnold et al., 1986; and #based on Bernard et al., 1993.

investigations have revealed correlations between the degree of behavioral sex difference and the degree of a sex difference in volume that fit the general principle described above. For the nucleus HVc the ratio of male to female volume is, on average, 3.71 for white-crowned sparrows, 3.2 for red-winged blackbirds and 1.64 in European starlings. In all three of these species, females have been observed singing, though not as often as males nor with as much complexity (i.e., fewer notes per song and smaller repertoires) as males. Therefore the degree of sex difference in behavior is "intermediate" to those species described above and the degree of the sex difference in nuclear volume of the song control nuclei is "intermediate."

However, when one tries to further interpret this general correlation between brain and behavior it becomes clear that several more precise questions need to be addressed. What are the relevant behavioral variables that one should focus upon when trying to establish these brain-behavior relationships? A measure of singing frequency and of song complexity are often invoked, but even when one concentrates on a fairly well defined trait such as repertoire size, problems of comparison arise (Kroodsma, 1982). Assigning a specific number to an estimate of the degree of behavioral dimorphism can clearly be a problem, in part because behavioral measures are well known to be influenced by many variables such as time of day, season of the year, environmental conditions (including weather for field studies), etc. Measuring female song seems to be more difficult in many species than measuring male song. Recent studies on white-crowned sparrows and European starlings have suggested that female song is more complex and occurs at a greater frequency than was thought previously (Baptista et al., 1993; Hausberger & Black, 1991). In the case of the white-crowned sparrow, female song was observed at a higher rate during the non-breeding season than during the breeding season (Baptista et al., 1993), a time when field researchers are typically less apt to be sampling song. This suggests that one must be very cautious when trying to quantify properly differences in male and female vocal behavior.

Another complication arises from the suggestion that the song control system is im-

portant for the perception of song as well as the production (e.g., Williams & Nottebohm, 1985). In particular it has been shown that when HVc is lesioned in female canaries they no longer show a preference for conspecific over heterospecific song (Brenowitz, 1991b). There is also evidence from both electrophysiological studies (Williams, 1985) and from behavioral investigations (Cynx & Nottebohm, 1992; Searcy & Brenowitz, 1988) that there are sex differences in songbird species in how song is perceived. It is thus very plausible to postulate that sex differences in the volume of nuclei within the vocal control system are related to sex differences in song perception as well as in song production (Brenowitz & Arnold, 1986b). However, a recent attempt to test this possibility by comparing the volume of the female's HVc in eastern and western marsh wrens (*Cistothorus palustris*) failed to find any support for this hypothesis (Brenowitz et al., 1994). In North America, male western marsh wrens have substantially larger song repertoires than male eastern marsh wrens. Brenowitz et al. (1993) therefore speculated that females in the west might have an HVc that is larger in volume than the HVc of females in the east. No such difference was detected. Despite these negative data it remains possible that inter-specific variability in the volume of the song control nuclei is related to variation in song perception as well as in production.

It is also difficult to assign numbers to estimates of brain differences that are not associated with high error rates, because the brain is itself more dynamic than was thought in the past. This is especially true for the songbird vocal control circuit where seasonal changes in the volume of nuclei such as RA and HVc are known to occur (e.g., Nottebohm, 1981; see DeVoogd, 1991 for a review). In the case of red-winged blackbirds, Kirn et al. (1989) found that the average male/female ratio for HVc was 3.2 under Spring photoperiodic conditions and 5.9 under Fall photoperiodic conditions; for RA the average ratio was 4.7 as compared to 6.3. Therefore more refined measurement methods need to be developed for both behavioral assessements of possible sex differences in vocal behavior and neural assessements of sex differences in nuclear volume.

One way to address the issue of a more refined measure of sex differences in nuclear volume is to use other indicators of nuclear boundaries besides the widely employed Nissl staining methods. By defining the borders of a nucleus with a neurochemical marker that is clearly linked to a transmitter system one may discover associations and dissociations within a species between the boundaries of a nucleus as defined with the Nissl stain and the boundaries of a nucleus as defined by some other marker. We have started such an approach in our work on sex differences in the European starling song system and this work is described in the next section.

INTRA-SPECIFIC COMPARISONS OF SEX DIFFERENCES IN NUCLEAR VOLUMES WITH THE USE OF A VARIETY OF NEUROCHEMICAL MARKERS

Just as it is difficult to know what features of song to investigate when making inter-specific comparisons of the relationship of dimorphism in song behavior and song control nuclei volume, it is also difficult to know what criteria should be used when making comparisons of the volume of a song control nucleus between different sexes within the same species. Traditionally, standard histological techniques (i.e., Nissl stains) have been used to define the song nuclei. Like all methods, Nissl stains are limited in the information they provide. These stains label darkly those parts of a cell that are highly basophilic, such as free ribosomes and ribosomes bound to the rough endoplasmic reticula

(Raine, 1989). Because ribosomes are important organelles in the cascade of events involved in protein synthesis, it is likely that Nissl stains are good indicators of relative cellular activity (Raine, 1989). That is, cells that stain more darkly are presumably more active than cells that stain less darkly. In the case of the song system, many of the nuclei in the circuit are remarkably distinct when stained with Nissl stains and it is the darkness, and in some cases also the size and/or density of cells, within the various song nuclei relative to their surrounding structures that is often used to define the boundaries of the nuclei and hence their volumes. Using these methods, prominent sex differences in brain nuclear volume have been observed in the song system (see above). There are potential drawbacks of this approach, however. Because the pattern of Nissl staining is influenced by the activity of cells, the sex differences reported to date may reflect fundamental differences in the phenotype of cells in males and females in a given brain region, or may reflect differential activation of otherwise similar populations of cells. For example, HVc in female canaries may be as large as in males, but fewer cells may be actively involved in protein synthesis and, therefore, the nucleus would appear smaller in volume. Thus, to further understand the nature of sex differences in nuclear volume that have been described to date, a variety histochemical procedures should be employed. This approach allows one either to validate findings obtained with different methodologies or to modify those results by highlighting potential incongruities between different approaches.

The usefulness of this approach was first illustrated by the work of Gahr (1990). The volume of HVc was assessed in male canaries that were either in full breeding condition or had undergone testicular regression following the breeding season. As was previously demonstrated (Nottebohm, 1981), a seasonal change in the volume of HVc was detected with Nissl stained tissue. However, when HVc volume was defined using immunohistochemical staining for estrogen receptors, or back-filling via neurons projecting to area X no seasonal change in the volume of HVc was detected (see Kirn et al., 1991, for similar results). These studies suggest that the activity of a subset of HVc cells is what changes seasonally rather than the number of cells and that many aspects of an HVc cell's phenotype, such as whether it is hormone sensitive or sends projections to another brain region, do not change seasonally. It has often been assumed that these seasonal changes in brain nuclear volume are caused by seasonal changes in circulating levels of the androgen testosterone. However, Johnson and Bottjer (1992) compared the volume of HVc in castrated canaries on short days that were either testosterone treated, given anti-steroid compounds or not treated. They defined the boundaries of HVc in these birds in three different ways: based on a Nissl stain, the distribution of projection neurons from HVc to RA, or cells that contain androgen receptors as determined via autoradiography for [^{3}H] dihydrotestosterone. The volume of HVc as defined by the Nissl stain was larger in the testosterone treated males than in the group treated with antisteroid compounds. Furthermore the boundaries of HVc, as it was defined with these three different methods, seemed to be closely aligned. This study suggests that testosterone treatment can change many aspects of the phenotype of cells within a brain area such as HVc. Why this study found congruence among the three different markers of HVc and the two seasonal studies cited above did not find congruence will require further study, but this does suggest that seasonal changes in brain morphology can be influenced by factors in addition to testosterone. Furthermore, these studies illustrate the problems inherent in interpreting differences in the brain based on a single marker, and the benefits of using independent neurochemical markers in the functional analysis of brain dimorphisms.

Immunocytochemistry and quantitative receptor autoradiography have been used ef-

fectively in studies exploring the neurochemistry of the song system (e.g., Ball, 1990; Bottjer, 1992). Indeed, in many cases these techniques allow one to define clearly the boundaries of the song control nuclei. In many cases, the boundaries appear even more distinct than with Nissl stains, and therefore volume reconstructions can be performed. These methods provide us with two valuable tools in the study of the sex differences in the song system: 1) they allow us to reconstruct volumes, and 2) they allow us to ask more detailed questions regarding sex differences in the particular cellular attributes they highlight. In the following section we review some of our recent work on intra-species comparisons of area X in European starlings and zebra finches using in vitro receptor autoradiographic techniques in an attempt to illustrate the utility of this approach to the study of sexual dimorphisms in the brain. Immunocytochemical studies of a variety of neuropeptides (e.g., Ball et al., 1988; Ryan et al., 1981) and of the synthetic enzyme for the catecholamines tyrosine hydroxylase (Bottjer, 1992) have also provided neurochemical markers that specifically label the boundaries of sexually dimorphic song control nuclei by the high level of immunoreactivity in comparison to the surrounding structures.

DEFINING THE BOUNDARIES OF AREA X WITH THE USE OF QUANTITATIVE AUTORADIOGRAPHY FOR NEUROTRANSMITTER RECEPTORS

In a recent report (Bernard et al., 1993), we demonstrated that the volume of area X in male European starlings is approximately 1.95 times larger than in females as defined in Nissl-stained tissue. Area X in Nissl-stained material in starlings can be defined by the high density of large cells in comparison to the surrounding LPO. We also processed sections adjacent to those used for Nissl staining with in vitro receptor autoradiographic techniques for muscarinic cholinergic receptors. It had been shown previously (Ball et al., 1990) that the nonselective muscarinic antagonist, [^{3}H] *N*-methyl-scopolamine (NMS), defines the boundaries of area X based on its high binding in area X compared to the surrounding LPO. When the volume was reconstructed from the autoradiographic images, the same degree of differences in area X volume between males and females was observed. Thus, unlike the findings of Gahr (1990) for seasonal changes in HVc volume of canaries, an independent marker and a Nissl stain indicated the same volumetric sex difference. Recall that the use of neurochemical methodology allows one to ask more specific questions about the cellular characteristic under investigation. In the present case, we investigated whether or not there were any sex differences in muscarinic receptor density in area X. Males and female starlings did not, however, differ in this regard. Yet, given the larger volume of area X in males, coupled with the lack of a sex difference in receptor density, we concluded that there are more muscarinic cholinergic receptors in area X in male than in female starlings.

More recently, we attempted to extend these findings by exploring the sex difference in area X volume using another neurochemical marker. Overall, the methodology was similar to the study described above (Bernard et al., 1993), however, there were some noteworthy differences. First, previously alternate sections were processed for autoradiography with [^{3}H] NMS and Nissl staining. In the present case, serial sections were processed for Nissl substance, muscarinic cholinergic receptors, and α_2-adrenergic receptors. It had been shown previously (reviewed by Ball, 1990) that the α_2-adrenergic receptor agonist [^{3}H] *p*-amino clonidine (PAC) defines area X by a high degree of binding relative to the surrounding LPO. PAC has been shown to specifically label α_2-adrenergic

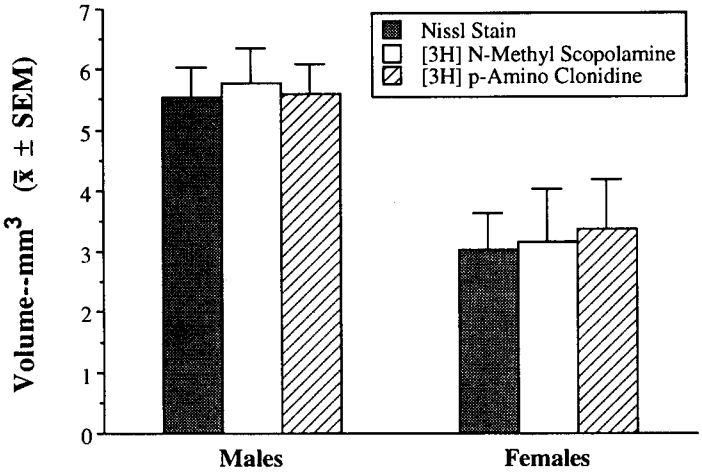

FIG. 2: Histograms illustrating the volume of area X in male (n = 4) and female (n = 2) photorefractory European starlings. These birds had regressed gonads and undetectable levels of testosterone. The volumes were calculated using three different markers of the boundaries of area X. One method utilizes standard Nissl staining methods and the other two utilize quantitative receptor autoradiography. The boundaries of area X can be delineated by the sharp variation in receptor density in area X in comparison with the surrounding LPO. [^{3}H] N-methyl scopolamine labels muscarinic cholinergic receptors, [^{3}H] p-amino clonidine labels α_2 -adrenergic receptors.

receptors in the avian brain (Ball et al., 1989) and the autoradiographic method described in detail in Ball et al. (1989) was used in the current investigation. Second, birds in the previous study were photosensitive birds caught in the wild when they were naturally experiencing a photoperiod close to 11L:13D and then housed on an 11L:13D photoperiod for approximately 6 weeks. Starlings who are photosensitive and experience gradual increases in daylength and are then kept on a photoperiod of 11L:13D for 6 weeks as these birds were, do not go photorefractory and attain a gonadal size about 50% of the maximum possible size (Dawson et al., 1985). In the present case, the animals were caught in the wild in the late winter when they were photosensitive and then housed on a photoperiod of 16L:8D for a period of 5 mo prior to sacrifice. Thus the birds were photorefractory, and their reproductive systems were quiescent (Dawson et al., 1985). It has been well established for many years that photorefractory starlings have regressed gonads, low to undetectable levels of gonadotropins, undetectable levels of androgens and low levels of hypothalamic gonadotropin releasing hormone (see reviews in Ball, 1993; Nicholls et al., 1988). This fact was confirmed with the animals used in this investigation. The average testis volume of the males was 8.08 mm^3 ± 2.0 (mean ± SD), characteristic of tiny regressed testes. Also the beaks of the males were black at the time their brains were collected. Beak color, in starlings, is a very sensitive indicator of the presence or absence of circulating levels of testosterone in males and females. When the beaks are black it is indicative of non-measurable levels of testosterone circulating in the plasma (e.g., Ball & Wingfield, 1987). The females also had black beaks and the mean size of their largest follicle was 0.66 mm ± 0.3 (mean ± SD). These factors are clearly indicative of photorefractory starlings.

The present study, therefore, was valuable on several fronts. First, a new marker was used to delineate the sex difference in area X of starlings. Second, we had the opportunity to replicate our previous findings. Third, because the animals in the previous study were

photosensitive and had elevated testosterone (T) titers and the animals in the presentcase were photorefractory and had low T titers, we also had the opportunity to see if volume and/or receptor density were influenced by the difference in steroidal milieu.

As illustrated in Fig. 2, defining area X in male and female starlings with a Nissl stain, or by the density of muscarinic cholinergic receptors or α_2-adrenergic receptors all indicated approximately the same the degree of dimorphism in area X between the males and females. In Figs. 3 and 4, one Nissl-stained section and two autoradiograms derived from two adjacent sections that were labeled for muscarinic cholinergic or α_2-adrenergic receptors, respectively, are presented for the region including area X in a male and a female starling. The shape and borders of area X are nearly identical in the three different brain sections for each sex.

The male to female ratio for the three different markers ranged from 1.66 to 1.82, which is very similar to our previous findings (Bernard et al., 1993). In fact, the volumes of area X in birds in the two studies are virtually indistinguishable. The fact that these birds were collected when they were photorefractory (a condition characteristic of the late summer and fall) and the birds in the previous study were collected when they were photosensitive and on a photoperiod of 11L:13D (characteristic of early spring) further suggests that there is no effect of photoperiodic condition on the volume of area X in male and female starlings. With respect to receptor density, again the present study confirmed our prior report of no sex difference in muscarinic receptor density in area X. In fact the densities were so similar to our previous findings that it seems unlikely that muscarinic receptor density in area X is influenced by differences in T titers (it should be noted, however, that the actual difference in T were not measured in this case, and a larger difference in circulating levels of T may have influenced receptor density and/or volume). Finally, the density of α_2-adrenergic receptors in area X did not differ between the sexes. Based on the sex difference in volume and the lack of a sex difference in α_2-adrenergic receptor density, area X in male starlings appears to have a greater number of α_2-adrenergic receptors than in female starlings, as was the case for muscarinic cholinergic receptors. However, with both these ligands, because we are measuring receptor variation with the use of autoradiography, changes in the density of the receptors could be the result of changes in actual receptor number or be the result of changes in receptor affinity. Saturation analyses are required to clarify this issue. Previous work in birds using PAC to investigate variation in α_2-adrenergic receptor density has suggested that variation in receptor density is associated with changes in the maximum number of binding cites rather than with a change in receptor affinity (Ball et al., 1989).

The results of these two investigations of sex differences in volume demonstrate that three independent markers delineate a significant sex difference in the volume of area X in European starlings. In addition, they indicate that even though the sexes differ in nucleus volume they do not appear to differ with respect to density of two different neurotransmitter receptors. There are, however, receptors for which there do appear to be significant sex differences in density as is discussed below.

In addition to cholinergic and adrenergic innervation of area X in songbirds, studies in zebra finches of this nucleus indicated that it also receives a dopaminergic projection from the Area ventralis of Tsai (Lewis et al., 1981). In light of this known dopaminergic projection, area X in male and female starlings and zebra finches has been defined based on the binding of [^{3}H] SCH 23390, a D_1 dopamine receptor antagonist (Casto & Ball, 1994; Casto, Balthazart, & Ball, unpublished results). Similar to [^{3}H] PAC binding and [^{3}H] NMS binding, area X, in starlings, is defined by a high density of [^{3}H] SCH 23390

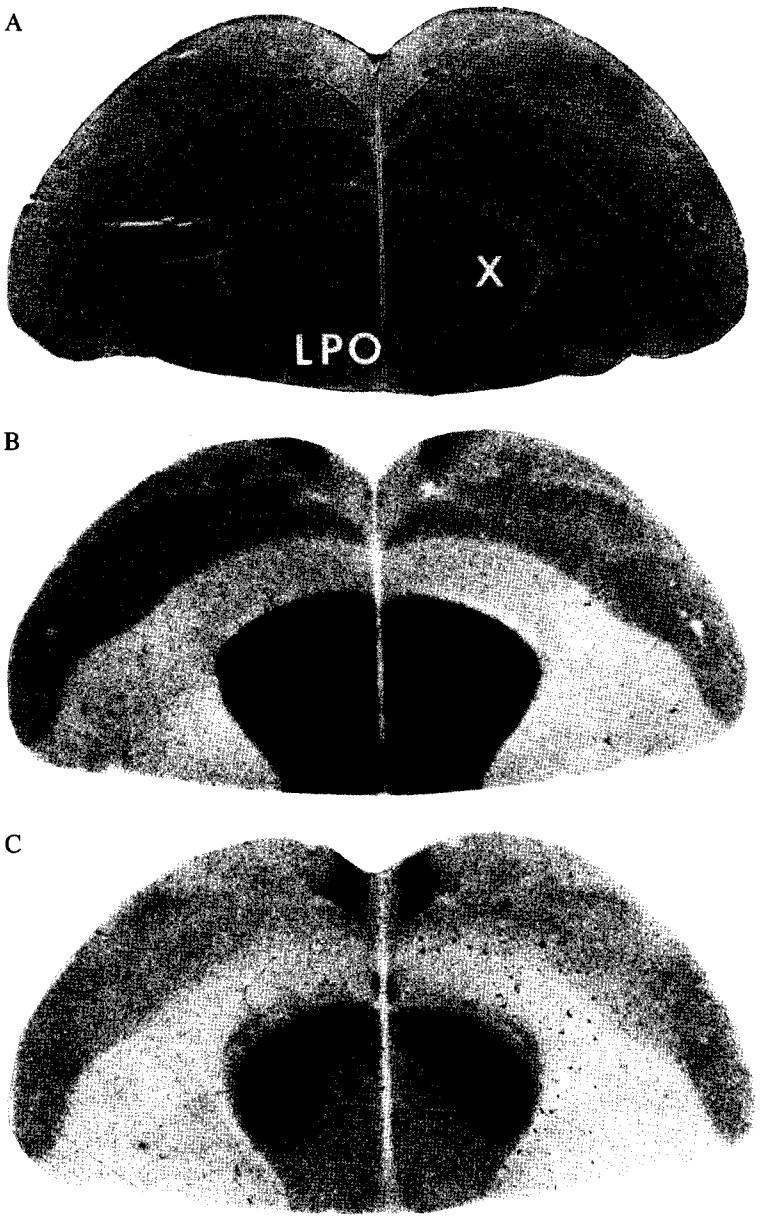

FIG. 3: A comparison of the definition of area X utilizing the three different markers in a male European starling. (A) is a Nissl-stained section, (B) and (C) are adjacent sections that were labeled via autoradiography for muscarinic cholinergic receptors (B) or α_2 -adrenergic receptors (C). The images presented in (B) and (C) are from the autoradiograms generated by the labeled adjacent sections. In (A), the boundaries of area X can be defined in the Nissl-stained section by an apparent higher density of large cells relative to the LPO. In (B) and (C), the boundaries of area X on the autoradiograms can be defined by the higher density of the respective receptor subtypes within area X as compared to the surrounding LPO.

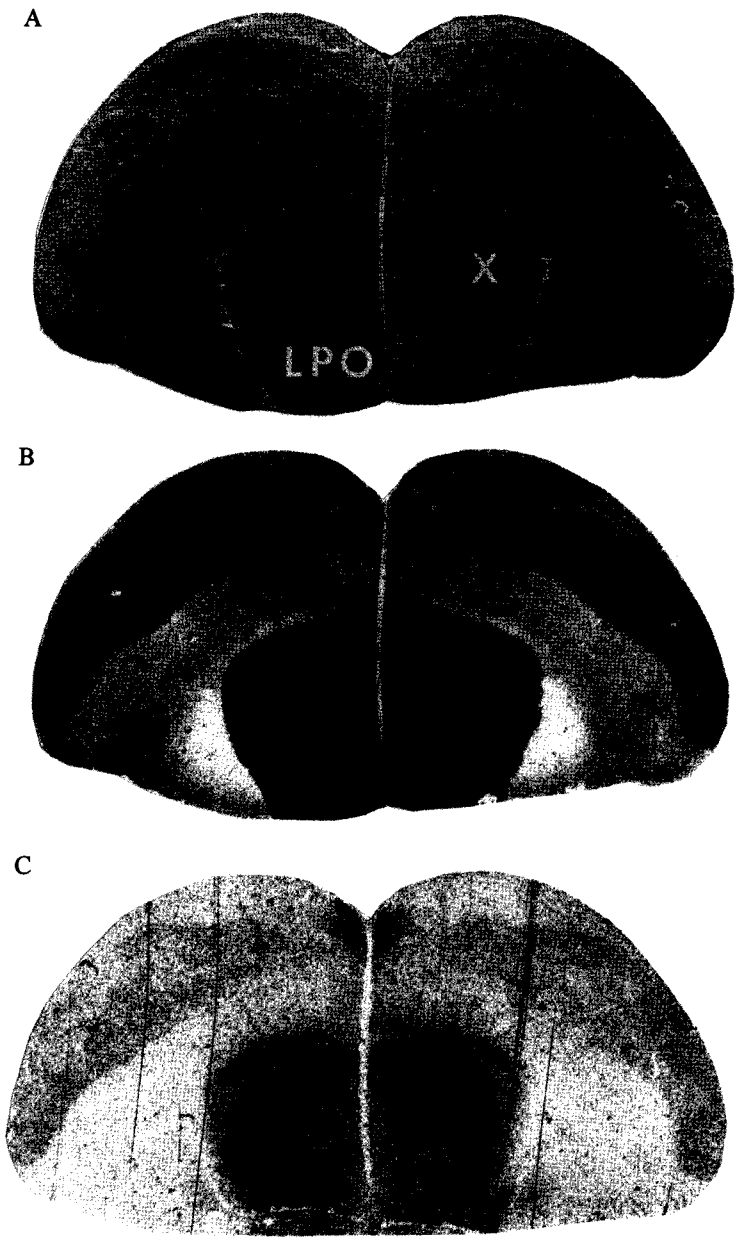

FIG. 4: A comparison of the definition of area X utilizing the three different markers in a female European starling. (A) is a Nissl-stained section, (B) and (C) are adjacent sections that were labeled via autoradiography for muscarinic cholinergic receptors (B) or α_2 -adrenergic receptors (C). The images presented in (B) and (C) are from the autoradiograms generated by the labeled adjacent sections. In (A), the boundaries of area X can be defined in the Nissl-stained section by an apparent higher density of large cells relative to the LPO. In (B) and (C), the boundaries of area X on the autoradiograms can be defined by the higher density of the respective receptor subtypes within area X as compared to the surrounding LPO.

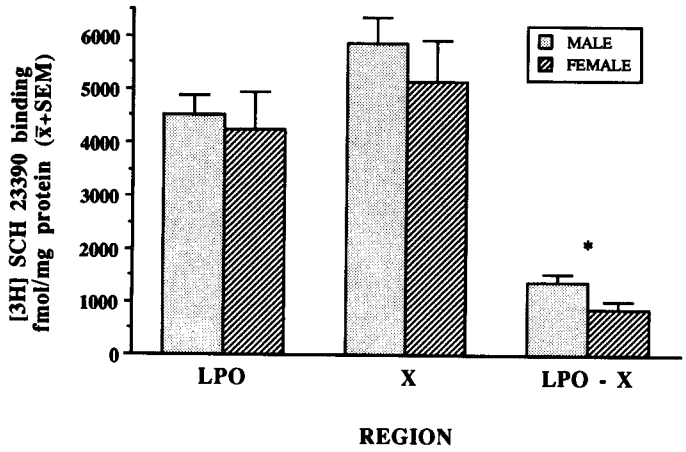

FIG. 5: The density of D_1 dopamine receptors in LPO and area X of male and female European starlings. In both sexes area X has a higher density than the surrounding LPO. The receptor density in area X is not different in males in females in LPO or area X but the difference score is significantly different. $*p < .05$. See text for more details.

binding in comparison to the surrounding LPO which also exhibits substantial [^{3}H] SCH 23390 binding. Unlike [^{3}H] PAC binding and [^{3}H] NMS binding, [^{3}H] SCH 23390 binding has not been used to reconstruct the volume of area X, due to the fact that there is an inconsistent resolution of the ventromedial borders of the nucleus. However, based on the number of sections in which area X is present and the relative proportion of the LPO that it occupies, a sex difference in [^{3}H] SCH 23390 defined area X volume in starlings is suggested.

Upon visual inspection of autoradiographic images of area X, it appeared that area X relative to the surrounding LPO has a higher receptor density in males than in females. Because the LPO is present in non-songbirds as well as songbirds, and area X is a specialized subregion of the LPO found only in songbirds, differences between area X and LPO are an appropriate focus to gain insight into the functional relevance of receptor density. During avian evolution, different areas of the brain have been co-opted into specialized circuits of song control nuclei that mediate the learning and production of song (Brenowitz, 1991a). These circuits of song control nuclei must be considered separately from the neural substrate from which they became specialized to detect and fully comprehend what could be meaningful differences between males and females. In this regard, receptor density in LPO was subtracted from area X receptor density for each individual, to derive a receptor density difference score between area X and LPO. Males had significantly higher difference scores in D_1 receptor density between area X and LPO than did females (see Fig. 5). The functional significance of such a sex difference in the difference score between area X and the surrounding LPO is at present unclear, however in other songbird species area X appears to play a role in song learning (Scharff & Nottebohm, 1991; Sohrabji et al., 1990). Dopamine might act in area X to regulate the sex differences in the propensity of starlings to learn song.

Preliminary studies in male zebra finches, suggest that [^{3}H] SCH 23390 binding defines area X in much the same ways as in starlings; area X can be discerned from the surrounding LPO by a higher density of [^{3}H] SCH 23390 binding. However, in female zebra finches

[^{3}H] SCH 23390 does not define area X, thus binding is homogeneous throughout LPO (Casto, Balthazart, & Ball, unpublished results). The lack of a discernible area X in females zebra finches has been documented in studies which utilized Nissl stained tissue (Nottebohm & Arnold, 1976). Immunoreactive tyrosine hydroxylase (TH) clearly outlines the borders of area X in male zebra finches but area X can not be distinguished from the surrounding LPO when female zebra finch brains are stained for TH (Bottjer, 1992). Thus, Nissl staining, immunoreactive TH, autoradiography for D_1 dopamine receptors in zebra finches appear to define area X similarly, in male zebra finches. The repeated failure to define a structure in female zebra finches that corresponds to area X of males is intriguing. Area X in female zebra finches is an excellent model system in which to study mechanisms of sexual differentiation. Herrmann & Arnold (1990) have recently shown that lesions to HVc block the masculinization of area X (as defined by a Nissl stain) by estradiol. It would be useful to determine if other neurochemical dimorphisms in area X of zebra finches rely on similar transsynaptic mechanisms of sexual differentiation, or on the initiation of a dimorphic cascade of neurochemical differentiation in area X that is triggered by innervation by HVc projections.

CONCLUSIONS AND FUTURE PROSPECTS

Species diversity among the over 4,000 living species of songbirds in the degree of sex difference in brain and behavior provides a great opportunity for understanding how the brain can mediate sex differences in behavior. A general relationship between the degree that there is a sex difference in vocal behavior and the degree that there is a sex difference in the volume of the song control nuclei has emerged. However, future refinements in methodology of three sorts are required to exploit this potential: 1) improved methods of comparison; 2) identification of critical behavioral variables that differ between the sexes; and 3) improved definition and measurement of sexually dimorphic nuclei to identify more precisely what aspects of neural function are different between the sexes. The first issue will require that future comparisons employ the proper taxonomic level to insure that comparisons are not confounded by inter-specific variability that is not directly related to sex differences in vocal behavior and the song control nuclei (Harvey & Pagel, 1991). The second issue will require increased attention to female behavior and to possible sex differences in song perception to more accurately estimate behavioral sex differences. One approach to the third issue is illustrated by the studies we have described here employing a diversity of markers to define the boundaries of a sexually dimorphic nucleus, area X, in starlings and other songbirds. This type of study represents a first step towards a more comprehensive definition of brain nuclei that can provide insight into the neurochemical consequences of sex differences in nuclear volume.

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