Characterization and Localization of D1 Dopamine Receptors in the Sexually Dimorphic Vocal Control Nucleus, Area X, and the Basal Ganglia of European Starlings

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SUMMARY

D1 dopamine receptors were pharmacologically characterized and localized by quantitative autoradiography in the basal ganglia of male and female European starlings (*Sturnus vulgaris*). The D1 selective antagonist SCH 23390 was used to label this receptor subtype. Starlings are songbirds and possess a neural circuit implicated in the learning and production of song. This circuit includes a sexually dimorphic nucleus, area X, that is a subregion of the parolfactory lobe of the basal ganglia and is known from work on zebra finches to receive dopaminergic input from the area ventralis of Tsai. We focused our investigation on the D1-like receptor subtype because they are abundant in the basal ganglia. Competition studies indicate that a variety of dopaminergic ligands compete with [^{3}H] SCH 23390 for the binding site in an order of potency characteristic of a D1-like receptor. Autoradiographic studies of the basal ganglia revealed high D1 receptor densities in the avian homologues of the caudate-putamen and relatively low-receptor densities were observed in the avian homologue of the globus pallidus. In male starlings, area X could be reliably discerned on the autoradiograms by the higher density of D1 receptors compared to the surrounding parolfactory lobe (LPO). This was also true for females, though not as reliably as in males. When we compared the mean D1 receptor density in area X for males and females we did not find a significant sex difference. However, we also analyzed the data by comparing sex differences in the degree to which area X has a higher receptor density in comparison with the surrounding LPO. When we normalized D1 receptor density in area X relative to the LPO, we did find a significant sex difference. This sex difference in relative receptor density represents another neural sex difference in the song circuit that may mediate sex differences in the learning and production of song in starlings and other songbirds.

Keywords: **avian, songbird, vocalization, caudate-putamen, sex difference.**

INTRODUCTION

A system of interconnected brain nuclei has been implicated in the learning and production of birdsong (Nottebohm et al., 1976; Bottjer et al., 1989). This song control circuit includes certain morphological features that are unique to songbirds (i.e., members of the suborder passeres or oscines). It thus appears to constitute a specialization of the avian central nervous system associated with the evolution of song in this taxon (Ulinski and Margoliash, 1990; Brenowitz, 1991). This circuit includes motor nuclei that are involved in song production and nuclei that also exhibit auditory characteristics that are presumably involved in the auditory feedback necessary for vocal learning (for reviews see Konishi, 1989; Nottebohm et al., 1990). The motor pathway, that is necessary for the production of song in adult birds (Nottebohm et al., 1976) consists of a series of nuclei in the

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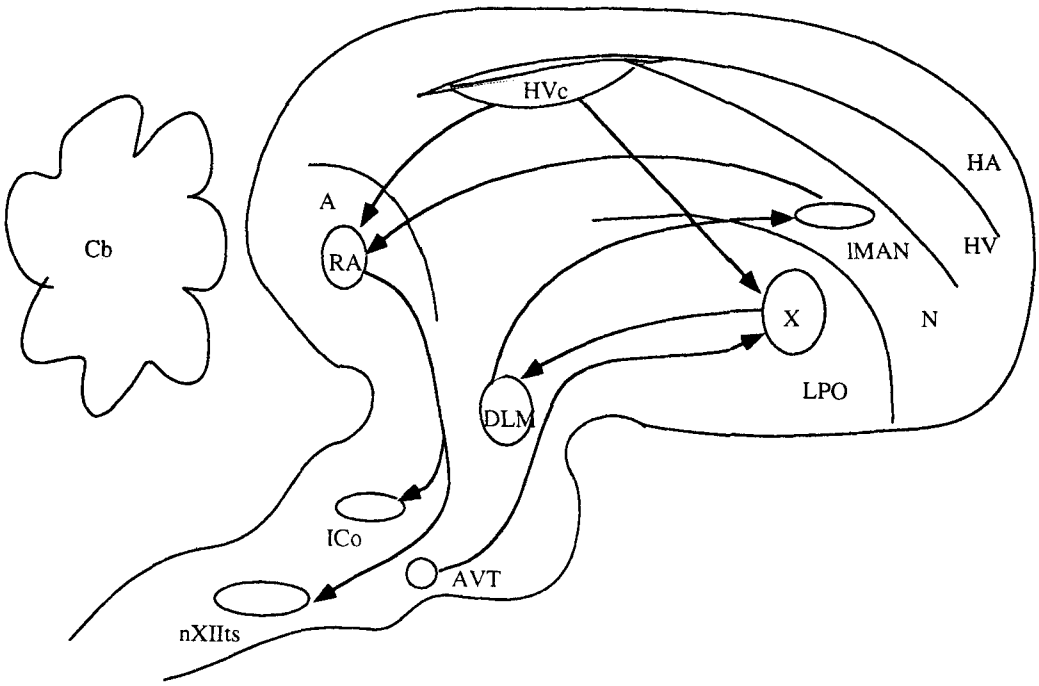

Figure 1 A schematic representation of a "generic" song bird brain illustrating the network of nuclei that controls the acquisition and production of birdsong. The efferent motor pathway, which is involved in song production, consists of the HVc to RA to nXIIts projection. The HVc to X to DLM to MAN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

telencephalon, mesencephalon, and the brain stem that control the neural output to the vocal production organ, the syrinx. This motor pathway is illustrated in Figure 1. It includes a projection from the nucleus hyperstriatum ventrale, pars caudale (HVc or High Vocal Center) to the nucleus robustus archistriatalis (RA) that, in turn, projects to both the nucleus intercollicularis (ICo) and the tracheosyringeal division of the XIIth cranial nerve (nXIIts). Efferent projections from this brain stem nucleus innervate the syrinx.

As illustrated in Figure 1, neural impulses from HVc also reach RA through a more circuitous route than the motor pathway described above (Okuhata and Saito, 1987; Bottjer et al., 1989). This indirect forebrain pathway consists of a projection from HVc to area X of the lobus parolfactorius (LPO) that, in turn, projects to the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM). DLM projects to the lateral portion of the nucleus magnocellularis of the anterior neostriatum (lMAN) that projects to RA. In adult zebra finches, all of the song control nuclei in this rostral forebrain pathway contain cells that display auditory properties in that they respond to the presentation of species-specific song (Doupe, 1993; Doupe and Konishi, 1991). This rostral pathway has been implicated in the process of song learning. For example, lesions of nuclei in the forebrain pathway of zebra finches (especially area X and lMAN) before stereotyped species song is achieved result in song abnormalities, such as lack of note stereotypy and abnormal song length (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Similar lesions, administered after the development of stereotyped song, have no effect on song production in adult male zebra finches. Such studies suggest that the rostral forebrain circuit is not necessary for the transmission of motor signals underlying song production in adult zebra finches; however, this pathway could mediate some motor functions in juveniles.

Although there have been a number of neuroanatomical and cytoarchitectural studies of the song system (e.g., Nottebohm et al., 1982; Bottjer et al., 1989), comparatively less attention has been paid to understanding the chemical neuroanatomy of the song system (see Ball, 1990 for a review). Investigations of this sort are an important next step in gaining a complete understanding of the neural basis of song learning and production.

Several of the song control nuclei are known to contain catecholamines and the enzymes necessary for their production (Ball, 1990; Barclay and Harding, 1988, 1990; Bottjer, 1993; Lewis et al., 1981; Sakaguchi and Saito, 1989). However, the source of catecholaminergic innervation has only been identified for area X. By injecting horseradish peroxidase (HRP) into area X, Lewis and colleagues (1981) demonstrated that this nucleus receives a brain stem projection from the area ventralis of Tsai (AVT, the homologue of the mammalian ventral tegmental area). They also argued that the projection from AVT to area X was most likely dopaminergic based on observations that catecholamine histoflourescence was reduced by treatment with tetrabenazine, a monoamine depletor, and α-methyl-para-tyrosine, an inhibitor of catecholamine synthesis, but was not reduced by treatment with disulfiram, an inhibitor of norepinephrine synthesis. Barclay and Harding (1988), using high-pressure liquid chromatographic (HPLC) techniques, measured very high levels and rates of turnover of dopamine in area X of male zebra finches, thus, providing independent confirmation of a dopaminergic input to area X. Ball, Allen, and Luine (unpublished data) also measured dopamine in area X of European starlings. At present, the AVT to area X projection is the only identified catecholaminergic projection in the song control system. The function of this projection is unknown. However, because area X is necessary for normal song learning in zebra finches (Scharff and Nottebohm, 1991; Sohrabji, et al., 1990), the projection may be involved in the regulation of song learning.

European starlings are a well-suited species in which to investigate the dopaminergic innervation of area X for two reasons. First, male and female starlings are age-independent learners that continue to learn song as adults (Adret-Hausberger, 1989; Eens et al., 1991, 1992; Böhner et al., 1990). Because they can continue to learn song as adults, there is a large temporal window within which mechanisms underlying the regulation of song learning can be studied. Second, starlings exhibit a moderate sex difference in singing behavior (Hausberger and Black, 1991). Male starlings sing more often and have larger repertoires than females. Related to this behavioral sex difference is the fact that the volume of three of the starling song control nuclei, HVc, RA and area X, have been shown to be approximately two times larger in males than in females (Bernard et al., 1993). Such behavioral dimorphisms may result from differences in the capacity for, or regulation of, song learning, and dopamine may be involved in these processes.

Dopamine, like all monoaminergic neurotransmitters, is a chemical signal that must bind to receptors in order to play any functional role. As many as six dopamine receptors subtypes have now been identified based on molecular cloning studies (Gingrich and Caron, 1993). These six receptors have been divided by Gingrich and Caron (1993) into two broad categories: the "D1-like" receptors, that include two subtypes (D1A and D5/D1B) and the "D2-like" that include four receptor subtypes (D2s, D2L, D3, and D4). Both D1- and D2-like subtypes are known to be enriched in the basal ganglia (Gingrich and Caron, 1993). The two types are thought to act both synergistically and independently (Clark and White, 1987). Studies with mammals also suggest that these two subtypes mediate at least certain types of memory in the basal ganglia (Packard and White, 1991).

In this study, we describe the use of quantitative *in vitro* receptor autoradiography to characterize and map the binding sites of a D1 dopamine receptor antagonist, SCH 23390, in the basal ganglia of male and female European starlings (*Sturnus vulgaris*). Although dopamine receptors in the avian brain have been mapped using autoradiographic procedures in pigeons (Dietl and Palacios, 1988; Richfield et al., 1987), characterization of dopamine receptor binding in the avian brain using *in vitro* receptor binding studies such as saturation and competition analyses have not yet been completed. The focus of the present study was twofold. First, the binding characteristics of [^{3}H] SCH 23390 in the starling forebrain were assessed. Second, localization of [^{3}H] SCH 23390 binding in the basal ganglia was determined. The basal ganglia of birds consists of a pallidal component, the paleostriatum primitivum (PP) and striatal components, the LPO, and the paleostriatum augmentatum (PA). Special emphasis was placed on the identification of sex differences in the distribution of receptors in area X, in order to elucidate the

neurochemical substrate mediating sexually dimorphic song behavior.

MATERIALS AND METHODS

Subjects and Tissue Preparation

European starlings (n = 8 males and n = 8 females) were trapped in Baltimore, Maryland, between February 25 and March 16, 1992 and were housed in our colony at Johns Hopkins University until sacrificed. The natural photoperiod in Baltimore at the time the birds were trapped ranged from 11.2 L:12.8 D to 11.9 L:12.1 D (photoperiods are based on sunrise and sunset and do not include civil twilight). All birds were group housed in similar photoperiodic environments (11 L:13 D). Birds were maintained on this light regime until they were sacrificed (approximately 10 weeks). At the time of sacrifice, birds were judged to be photostimulated to a similar degree based on measurement of gonadal volume after the birds were killed. The mean left testis volume of males was 627.4 mm^3 (S.E.M. = 95.02). The mean diameter of the largest ovarian follicle in females was 2.05 mm (S.E.M. = 0.18). In both males and females these measures were similar to reported field measurements of adult (beyond first year) male and female starlings, taken in March just 1 month prior to the onset of breeding (Bullough, 1942).

It is difficult to determine precisely the age of wild-trapped starlings. However, methods exist to determine if a starling is a first-year bird or an adult (beyond first year). Bullough (1942) reported an unambiguous dichotomy in springtime testis volume between first-year and adult male starlings, such that first-year males have significantly smaller testis volumes than do adults. The testis measurements in our birds were substantially larger than mean measurements reported from first-year male starlings and roughly equivalent to those of adult male starlings in the field between the months of March and June (Bullough, 1942). This suggests that our male birds were at least in their second year. The ovarian follicle measurements from our females were substantially larger than reports of those taken in the field from first-year females in March, however, they were smaller than April and May follicle measurements from first-year females (Bullough, 1942), and thus could not be used to unambiguously determine female ages. Instead, the length of iridescence on the hackles of the foreneck was used to distinguish first year from adult females (Kessel, 1951). The iridescence on the hackles of first-year females rarely extends more than 5.5 mm; however, adult females routinely exhibit more than 6 mm of iridescence. Based on these criteria, all females were adults.

Birds were killed by decapitation, and their brains were quickly removed and frozen on powdered dry ice. Frozen brains were cut on a cryostat and coronal sections (16 μm) were collected throughout the entire basal ganglia. Single sections were thaw mounted on gelatin-coated slides, dried at room temperature, and stored at −70°C until receptor binding assays were performed. Brain sections from all 16 birds were used in the assay. The sampling protocol for brain sections from each bird used in the assay consisted of using two adjacent sections (one for determination of total binding, and one for determination of nonspecific binding; see below), then skipping six sections and repeating this sampling procedure throughout the basal ganglia.

Drugs

[^{3}H] SCH 23390 (S.A. 60.3 ci/mmol, New England Nuclear, Boston, MA), a specific D1 receptor antagonist, was the ligand selected to label D1 dopamine receptors. The following unlabeled ligands were used as cold competitors in the general autoradiography procedure and/or in the validations of binding characteristics: (+) butaclamol HCl (+BUT, a general D1/D2 antagonist) and its inactive enantiomer (−) butaclamol HCl (−BUT), (+) SCH 23390 HCl (+SCH, a D1-specific antagonist) and its inactive enantiomer (−) SCH 23390 HCl (−SCH), (−) sulpiride (−SULP, a D2-specific antagonist) and its inactive enantiomer (+) sulpiride (+SULP), (−) apomorphine HCl (−APO, a general D1/D2 agonist) and its inactive enantiomer (+) apomorphine HCl (+APO), (+) SKF 38393 HCl (+SKF, a D1-specific agonist) and its inactive enantiomer (−) SKF 38393 HCl (−SKF), (−) propylnorapomorphine HCl (−PNA, a D2-specific agonist) and its inactive enantiomer (+) propylnorapomorphine HCl (+PNA), and dopamine HCl (DOP). All unlabeled drugs were obtained from Research Biochemicals Incorporated (RBI, Natick, MA). These drugs are expected to compete with [^{3}H] SCH 23390 for the binding site with an order of potency characteristic of a D1-like receptor (D1-specific compounds: +SCH and +SKF > D1/D2 general compounds: +BUT and −APO > D2-specific compounds: −PNA and −SULP). The inactive enantiomers are expected to compete less well than their respective active enantiomers.

Quantitative Receptor Autoradiography

Assay procedures were adapted from a previously described procedure (Dawson et al., 1986). A 50-mM Tris HCl (pH 7.4) + 120-mM NaCl, 5-mM KCl, 2-mM $CaCl_2$, and 1-mM $MgCl_2$ buffer was used in this assay. During the assay, slides (as described above) were removed from the freezer and fan dried for 30 min. Slides were then preincubated in buffer at room temperature for 30 min in order to wash out bound endogenous dopamine from the receptors. Next, slides were transferred to slide mailers containing 7 ml of the incubation medium which consisted of 2.0 nM [^{3}H] SCH 23390 in buffer. Slides were incubated at room temperature for 60 min. Next, two 5-min washes in ice-cold buffer were used to

wash any unbound ligand from the sections. A final dip in ice-cold-distilled water was used to remove any excess buffer salts from the slides. The slides were then fan dried. Nonspecific binding was estimated by incubating adjacent sections in a medium containing 2.0 n*M* [^{3}H] SCH 23390 and a cold competitor, +BUT (2 μM concentration). Once slides were dried, they were placed in X-ray cassettes and apposed to tritium-sensitive Hyperfilm (Amersham Corps, Arlington Heights, IL) for 13 days, after which the film was developed for 5 min in Kodak D-19 developer, and fixed for 2 min with Kodak rapid fixer.

Because [^{3}H] SCH 23390 has been reported to bind to 5-HT2 receptors (see Dawson et al., 1986), the competitive 5-HT2 receptor antagonist ketanserin (200 n*M* concentration, RBI, Natick, MA) was added to a small subset of the slide mailers containing the incubation medium and a subset containing the incubation medium plus butaclamol. By comparing the autoradiograms of sections incubated with and without ketanserin, we assessed the amount of nonspecific and total binding resulting from the ligand binding to 5-HT2 receptors.

Autoradiograms were analyzed with the aid of a computerized image analysis system, consisting of a Chroma Pro 45 light box (Byers Photographic Equipment Company, Portland, OR) that projected an image of the autoradiogram to a video camera (Sony Model XC-77, Japan) interfaced via a Data Translation Quick Capture digitizing board (Model DT2255, Marlboro, MA) to an Apple Macintosh IIci microcomputer (Cupertino, CA). Digitized images were measured and analyzed with *Image*, an image analysis software package (version 1.41, Wayne Rasband, NIH, Bethesda, MD). All binding data were determined from film density in areas of interest (see below). Standards containing various concentrations of tritium (American Radiolabeled Chemicals Inc., St. Louis, MO) were co-exposed on the film and used to convert optical density to femtomoles of tritiated ligand specifically bound per milligram. Mean protein content has been previously determined in quail and songbirds (Ball et al., 1990) and is approximately 10% of tissue wet weight. Thus, specific activities per equivalent wet weight provided by the tritium standards were transformed into specific activities per mg protein using a ratio of protein versus wet weight of 10%. Specific binding in areas of interest was defined as the density of binding in the absence of +BUT (total binding) minus binding in the presence of +BUT (nonspecific binding) in adjacent sections. Optical density measurements of total binding and nonspecific binding were obtained by digitizing sections and tracing the perimeters of the areas of interest. Once the perimeter of an area of interest was traced, the computer calculated the average optical density for that area and converted that optical density to femtomoles of [^{3}H] SCH 23390/mg protein. In order to determine the receptor density for an entire nucleus, specific binding determined from each pair of adjacent sections in which the area of interest appeared were averaged and reported as the mean receptor density for that nucleus.

Specific binding of [^{3}H] SCH 23390 was assessed in four structures: the paleostriatum augmentatum, the paleostriatum primitivum, the LPO, and area X. We had originally planned to assess the volume of area X based on its autoradiographic definition, and expected that area X volumes would be larger in males than females, as they are when defined by Nissl staining and muscarinic receptor autoradiography (Bernard et al., 1993). However, volumetric reconstruction of area X was not always feasible because clear definition of the ventromedial boundaries of the nucleus was not possible in all sections that contained area X. Despite the inability to reconstruct area X volume, the entire boundary of area X could be clearly defined in at least three sections per bird (male range = 7–16; female range = 3–14). In structures where no systematic heterogeneity in receptor density in the rostral/caudal, ventral/dorsal, or medial/lateral extent of the structure was observed, (i.e., PA, PP, and area X) the receptor densities for that structure were measured in all sections from a single bird in which that structure appeared and were averaged and reported as the mean receptor density for that structure. In LPO, where receptor density appeared heterogeneous, the LPO was subdivided into a dorsolateral part which encompassed area X when it was present and a ventromedial part which did not encompass area X. Because LPO extends further than area X, both rostrally and caudally, many sections that contained LPO did not contain area X. When area X was present in a section, the optical density measurement for the dorsolateral LPO was taken just medially to Area X, and this measurement included no part of area X. The use of only a small part of the dorsolateral LPO to determine this region's receptor density (when area X is present) seemed a reasonable way to proceed because the only heterogeneity in [^{3}H] SCH 23390 binding that is evident in the dorsolateral LPO is the presence of area X. Receptor densities for each of the two subregions of LPO were calculated using the same procedure described above for PA, PP, and area X. Measurement of all structures was performed bilaterally in order to test for hemispheric differences in receptor density; however, repeated measures analyses of variance (ANOVAs) revealed no significant hemispheric differences in [^{3}H] SCH 23390 binding in any of the structures measured. Thus, density measures from the right and left sides were averaged to yield one mean receptor density value per region.

Binding Characteristics

Although [^{3}H] SCH 23390 binding characteristics have been well described in mammals (e.g., Dawson et al., 1986; Richfield et al., 1987), similar binding characteristics have not been verified in birds. Preliminary experiments were performed in order to assure that this ligand binds with similar characteristics in birds. To minimize

variance in receptor density between sections in these experiments, 16-μm brain slices were sectioned from minced brain tissue prepared from the forebrain of a male starling and formed in a mold with a diameter of 11 mm. Assays for these experiments were carried out using the same procedures as that in receptor autoradiography except that at the end of the assay, sections were wiped from the slides with glass fiber filters (Whatman International Ltd., Maidstone, England) and radioactivity bound per slice was determined by scintillation counting. This variation in the assay procedure is often used for preliminary autoradiographic studies as it circumvents time delays associated with the generation of autoradiograms.

To determine the affinity (apparent dissociation constant, K_d) and saturability (maximum number of binding sites, B_{max}) of [^{3}H] SCH 23390 binding, brain-mash sections were incubated with a range (0.1–9.6 nM) of [^{3}H] SCH 23390 concentrations that bracketed the incubation concentration used in the receptor autoradiography (described above). Nonspecific binding was estimated at every other concentration by incubating sections with 2 μM butaclamol. In these saturation experiments, three individually mounted brain-mash sections were incubated with each concentration of [^{3}H] SCH 23390. Sections were wiped off slides with glass fiber filters and placed in individual scintillation vials along with 5 ml of scintillation fluid. Sections were counted twice in a scintillation counter, average disintegrations per minute (dpm) were calculated for each section, and the dpm for the three sections from each concentration of ligand were then averaged, and reported as the average total dpm or nonspecific dpm for each concentration of the ligand. These dpm values were then transformed into femtomoles of [^{3}H] SCH 23390.

Competition experiments were performed in order to evaluate the specificity of [^{3}H] SCH 23390 for D1 dopamine receptors. Three sections of brain mash mounted individually on gelatin-coated slides were incubated with a 2-nM concentration of [^{3}H] SCH 23390 and one of two different concentrations (10^{-6} and 10^{-8} M) of several unlabeled dopamine receptor-specific compounds or their inactive enantiomers (described previously). These compounds were selected because mammalian studies (Andersen and Jansen, 1990; Richfield et al., 1987; Alburges et al., 1992; Dawson et al., 1986) suggest that they will differentially compete with [^{3}H] SCH 23390 for the binding site and by examining the order of potency in which these compounds compete with [^{3}H] SCH 23390, we can assess whether the binding exhibits the characteristics of a D1-like receptor. As in the saturation experiments, sections were wiped off slides with glass fiber filters and placed in individual scintillation vials along with 5 ml of scintillation fluid. Sections were counted twice in a scintillation counter, average dpm were calculated for each section, and the dpm for the three sections from each concentration of ligand were then averaged and reported as the mean dpm for a specific competitor at a specific concentration. Three sections of brain mash, were incubated with a 2-nM concentration of [^{3}H] SCH 23390 in the absence of any cold competitor, and binding was assessed as previously described. The total binding (mean dpm) from these three sections served as the standard of 100% [^{3}H] SCH 23390 binding to which cold competitors could be compared. In addition, four concentrations (10^{-5} to 10^{-8} M) of unlabeled dopamine were also used to compete with [^{3}H] SCH 23390. For each of these four concentrations, five individually mounted brain-mash sections were incubated with a 2-nM concentration of [^{3}H] SCH 23390 and the specified dopamine concentration. Binding was assessed as previously described.

RESULTS

Binding Characteristics

No differences in specific or nonspecific signals were detected in autoradiograms of sections incubated with ketanserin and sections incubated without ketanserin, suggesting that, at a 2-nM concentration, significant amounts of [^{3}H] SCH 23390 do not bind to 5-HT2 receptors in starlings. Thus, ketanserin binding will not be discussed further in this paper.

Results of saturation and competition experiments suggest that [^{3}H] SCH 23390 binding to homogenized brain tissue was saturable, specific, and of a high affinity. Scatchard analysis of the binding data indicated a K_d of 1.24 nM and a B_{max} of 31.54 fmol/slice [see Fig. 2(A)].

These values conform with similar studies performed on striatal tissue preparations in rats (Dawson et al., 1986). However, this saturation study in starlings indicates that [^{3}H] SCH 23390 shows substantial nonspecific binding at high-incubation concentrations. In fact, specific binding exceeded nonspecific binding in only a limited range of concentrations [0.1–6.0 nM, see Fig. 2(B)].

Competition studies using dopamine and a variety of D1- and D2-selective compounds indicate that [^{3}H] SCH 23390 binds selectively to D1-like receptors in starlings (see Fig. 3). The endogenous ligand dopamine competed for binding sites with [^{3}H] SCH 23390 in a linear manner [Fig. 3(A)]. Both D1-selective ligands, +SKF and +SCH, demonstrated the highest affinity for [^{3}H] SCH 23390 binding sites at micromolar concentrations as indicated by their ability to compete with the binding of [^{3}H] SCH 23390 [Fig. 3(B,C)]. Both +BUT and −APO, two general D1/D2 ligands also showed high affinities for [^{3}H] SCH 23390 binding

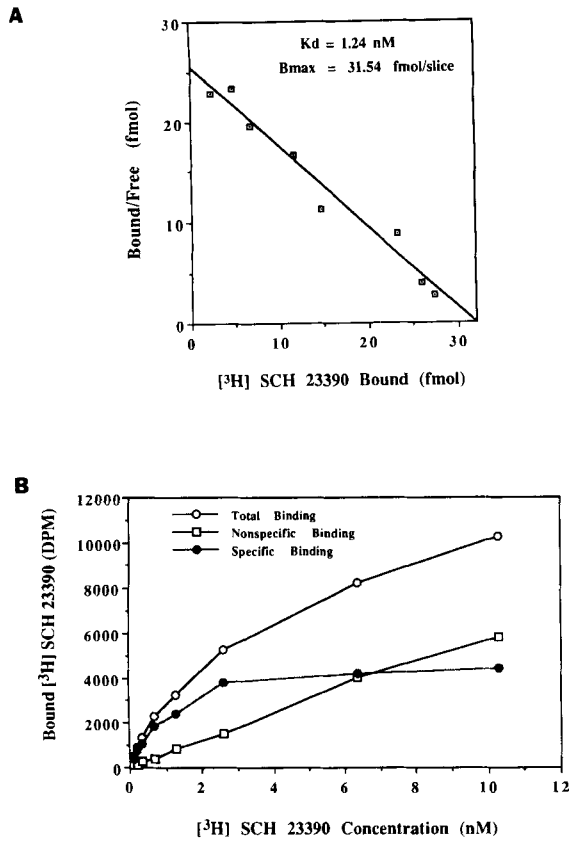

Figure 2 (A) Scatchard plot of saturation binding data for [^{3}H] SCH 23390 in the starling forebrain. (B) Direct plot of the saturation binding for [^{3}H] SCH 23390. [^{3}H] SCH 23390 was measured at equilibrium conditions (60 min at 23°C) with specific binding defined as excess over blanks containing 2 μM (+)-butaclamol.

sites [Fig. 3(B,C)], though lower than that exhibited by the D1-selective ligands. The two D2 receptor-specific compounds, −PNA and −SULP, did not demonstrate a high affinity for [^{3}H] SCH 23390 binding sites at either concentration [Fig. 3(B,C)]. In all cases, the efficacy of the inactive enantiomers of these dopaminergic ligands in competing with [^{3}H] SCH 23390 was lower than the active isomers. These data conform to previous reports of D1 receptor selectivity of [^{3}H] SCH 23390 in mammals (Andersen and Jansen, 1990; Richfield et al., 1987; Alburges et al., 1992; Dawson et al., 1986).

Distribution of [^{3}H] SCH 23390 Binding in the Basal Ganglia of Male and Female European Starlings

Autoradiograms illustrating [^{3}H] SCH 23390 binding for a male and female starling are shown in Figure 4. There was a high-receptor density in the LPO and PA of both males and females and a lower density in the PP (Fig. 4). The area X subregion of the LPO could be discerned by the higher

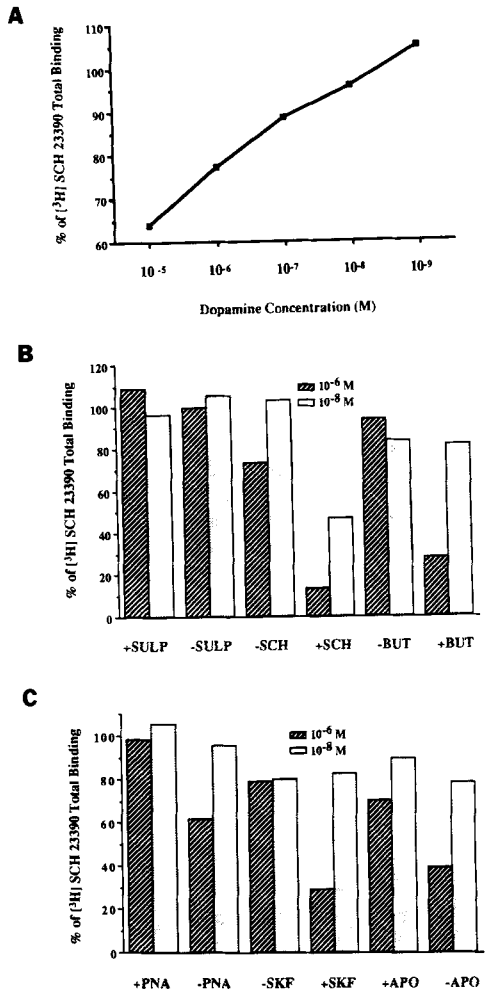

Figure 3 (A) A graph illustrating the ability of varying concentrations of dopamine to displace a 2-nM concentration of [^{3}H] SCH 23390. The competition was run with thin (16-μm) sections derived from the starling forebrain. Sections were wiped off the slides and counted in the beta counter. See methods for more detail. Increasing concentrations of dopamine linearly inhibited the binding of [^{3}H] SCH 23390. (B) Bar graph illustrating the ability of a high and low dose of a selection of dopaminergic antagonists to compete for binding sites with a 2-nM concentration of [^{3}H] SCH 23390. Only D1-specific compounds are able to inhibit [^{3}H] SCH 23390 binding. (C) Bar graph illustrating the ability of a selection of dopaminergic agonists to compete for binding sites with a 2-nM concentration of [^{3}H] SCH 23390. Only D1-specific compounds were able to inhibit [^{3}H] SCH 23390 binding. See methods for a listing of the full names of the compounds.

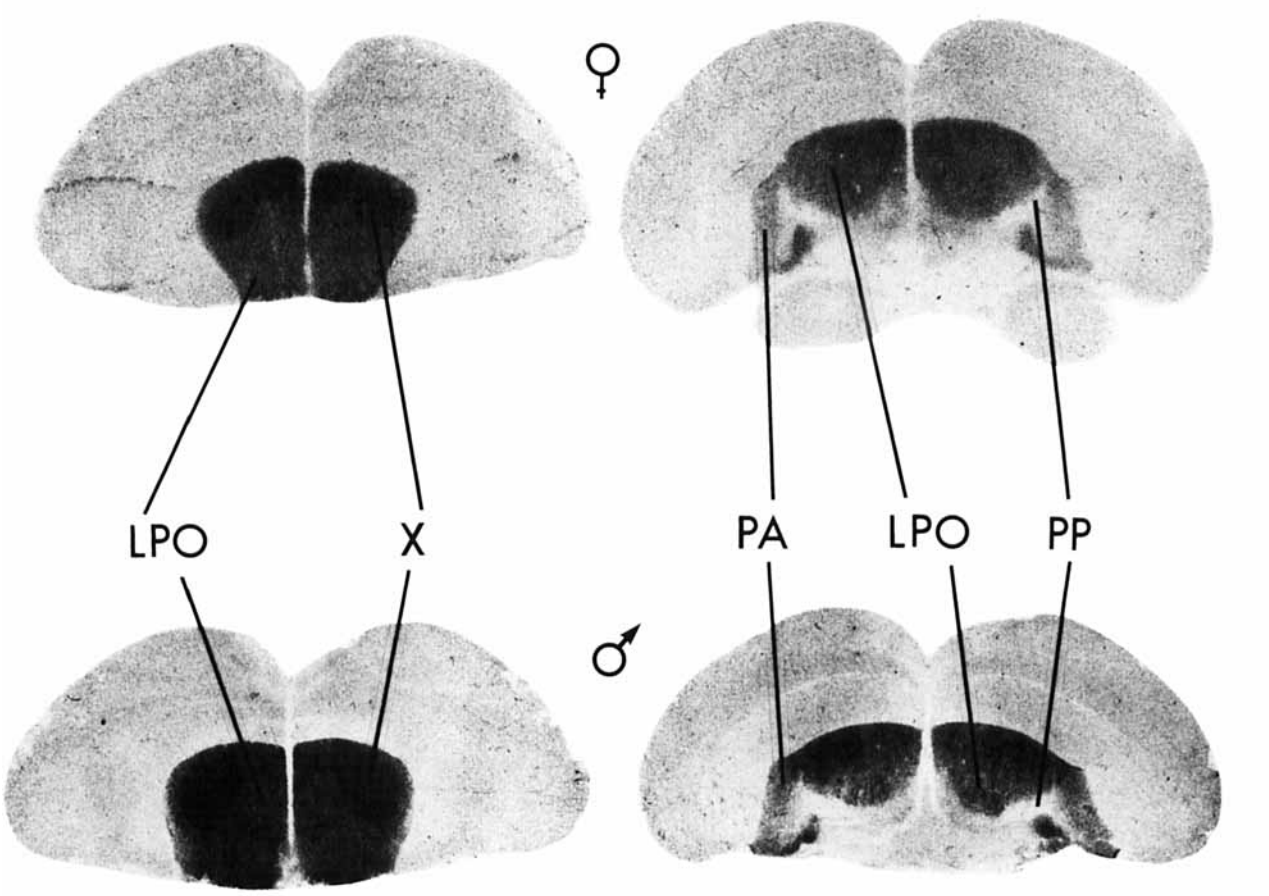

Figure 4 Four autoradiograms in the transverse plane illustrating [^{3}H] SCH 23390 binding at different levels of the basal ganglia in a male and a female starling. Area X can be bilaterally differentiated from the surrounding LPO by its high density of D1 binding sites. High [^{3}H] SCH 23390 binding can be detected in the LPO and PA and low binding is detected in the PP.

receptor density in this structure in comparison to the surrounding LPO (Fig. 4). Although it was present in all birds, area X could be discerned on the autoradiograms more easily and frequently in males than in females. Histograms representing D1 receptor density in the various regions of the basal ganglia of males and females are shown in Figure 5. The highest levels of [^{3}H] SCH 23390 binding were in area X. *T* tests on these data revealed no sex differences in D1 receptor density in any of the measured regions of the starling, including area X.

Although a significant sex difference in receptor density in area X was not detected with the analysis just described, a visual analysis of the autoradiograms suggested that area X relative to the surrounding LPO is more distinct in males than in females. As evidenced by the error bars in Figure 5, there is high interindividual variability in [^{3}H] SCH 23390 binding in the various brain regions that were measured. In addition, individual differences were such that if an individual showed above average binding in one area, that individual showed high binding in other areas as well. For instance, the density of D1 receptors in LPO and area X were positively correlated in the 16 starlings

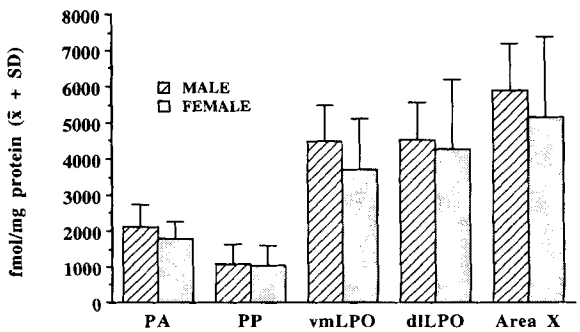

Figure 5 Bar graph illustrating variation in [^{3}H] SCH 23390 binding in the basal ganglia of male and female starlings based upon an analysis of the autoradiograms with an image analysis system. No significant sex differences were detected in receptor density in any structure.

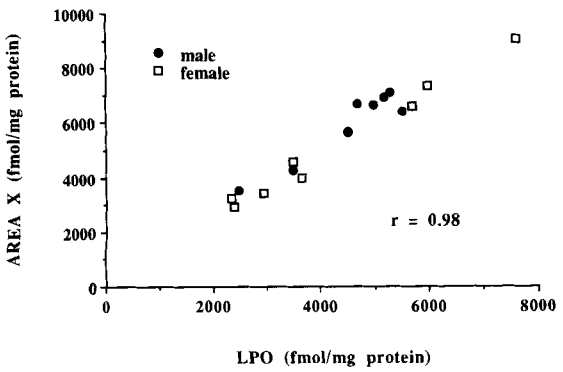

Figure 6 Correlation between [^{3}H] SCH 23390 binding in the dorsolateral LPO and area X in male and female starlings. [^{3}H] SCH 23390 binding was positively correlated in these two structures in both males and females (r = 0.98).

(r = 0.98; Fig. 6). Such interindividual variation in the receptor density of the LPO complex could mask potentially significant sex differences in the density of area X relative to the LPO. Because of this high interindividual variation, we compared D1 receptor density of area X, that had been normalized relative to its surrounding LPO, in male and female starlings. The rationale for using such a normalization procedure is as follows. With the exception of area X, no sex differences in D1 receptor density have been reported in the LPO of an avian species. Because sex differences exist in area X in starlings (Bernard et al., 1993), and area X is a specialized subregion of the dorsolateral LPO found only in songbirds it is appropriate to determine how area X is differentiated from the surrounding LPO in males versus females. In other words, is the neurochemical specialization that is present in area X that distinguishes it from the surrounding LPO, the same in males and in females?

In order to normalize D1 receptor density in area X with respect to LPO, receptor density in the dorsolateral LPO was subtracted from area X receptor density for each individual on the autoradiograms where area X could be discerned from the surrounding LPO by its high receptor density. Similar procedures for normalizing data generated by optical densitometry have been used by other authors (see Coquelin et al., 1991). These difference scores were then subjected to a two-tailed t test which revealed a significant sex difference $t(14) = 2.24$, $p < 0.05$). Males had significantly higher D1 receptor density in area X relative to dorsolateral LPO than did females (see Fig. 7).

DISCUSSION

In this study we have pharmacologically characterized [^{3}H] SCH 23390 binding in adult starlings and found that the characteristics are similar to those that have been described in mammalian species. The results suggest that this compound specifically labels a D1-like dopamine receptor in starlings as it does in mammalian species. We have also demonstrated that area X in starlings can be distinguished from the surrounding LPO by a substantially higher density of D1 dopamine receptors. A sex difference in D1 receptor density in area X relative to the dorsolateral LPO was detected. We will discuss each of these main findings in turn.

Binding Characteristics

Using the D1 dopamine receptor antagonist, SCH 23390, we were able to map the distribution of D1 receptors in discrete areas of the European starling basal ganglia. In preliminary studies, [^{3}H] SCH 23390 was found to bind to sites in the starling brain with characteristics similar to those reported in mammals. To our knowledge, this is the first study to characterize pharmacologically the binding of [^{3}H] SCH 23390 to D1 receptors in an adult

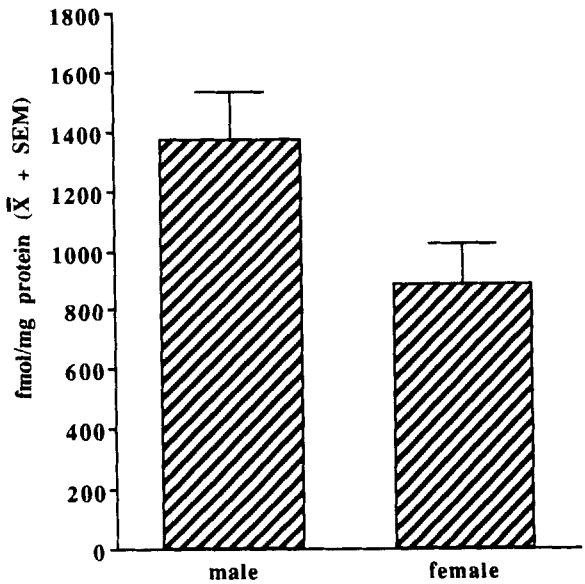

Figure 7 Bar graph illustrating the sex difference in [^{3}H] SCH 23390 binding in area X after subtracting out [^{3}H] SCH 23390 binding of the dorsolateral LPO. A significant sex difference was detected in D1 receptor density in area X relative to D1 receptor density in the dorsolateral LPO.

avian species. Scatchard analysis of binding data from brain-mash slices analyzed by beta counting indicated that observed binding was saturable in the nanomolar range, with an apparent dissociation constant (K_d) of 1.24 n*M*. This value is in good agreement with reports of the dissociation constant for [^{3}H] SCH 23390 in rats, which have ranged from 0.57 to 1.84 n*M* (Richfield et al., 1987; Dawson et al., 1986). The maximum number of binding sites (B_{max} = 31.54 fmol/slice) obtained by scintillation counting is also consistent with reported B_{max} in rats (Richfield et al., 1987; Dawson et al., 1986). In addition to producing dissociation constants and estimates of maximum binding that are comparable to those reported in rats, saturation analysis also confirmed that nonspecific binding increases dramatically as [^{3}H] SCH 23390 concentrations are increased above 2.5 n*M*. In fact, specific binding exceeded nonspecific binding only at concentrations below 6.0 n*M*. Richfield et al. (1987) have reported similar increases in nonspecific binding when [^{3}H] SCH 23390 concentrations are increased above 2.5 n*M* in rats. Thus, the use of low concentrations of [^{3}H] SCH 23390 (<2.5 n*M*) are necessary in order to map successfully the distribution of D1 sites in the avian basal ganglia.

Competition studies revealed that [^{3}H] SCH 23390 does bind preferentially to a D1-like dopamine receptor in starlings. The D1-like receptor subtype in the starling showed a high affinity for benzazepines such as SCH 23390 and SKF 38393; this is one of the defining properties of this class of dopamine receptors in mammals (Gingrich and Caron, 1993). A comparison of the two D1 receptor-specific cold competitors reveals that +SCH is a more effective competitor than +SKF. This may be due to a greater ability of +SCH to compete with [^{3}H] SCH 23390 for serotonin binding sites. Although ketanserin was used to block the binding of [^{3}H] SCH 23390 to 5-HT2 sites in a subset of the brain sections used in the general autoradiography procedure, and did not appear to have an effect on either specific or nonspecific binding, recent reports suggest that [^{3}H] SCH 23390 also binds to 5-HT1c receptors (Alburges et al., 1992; Lidow et al., 1991). Because ketanserin only blocks 5-HT2 receptors, it is possible that the differential efficacy of +SCH and +SKF in competing for [^{3}H] SCH 23390 binding sites was due to differential affinity for 5-HT1c binding sites. The starling D1-like receptor subtype also exhibited a low affinity for substituted benzamides such as sulpiride. These ligand properties are again characteristic of a receptor in the D1-like category based on mammalian studies (Gingrich and Caron, 1993).

Distribution of D1 Sites in the Starling Paleostriatal Complex

The general distribution of [^{3}H] SCH 23390 binding in the starling paleostriatal complex is qualitatively similar to the pattern described in other receptor autoradiography studies on the avian brain that have employed [^{3}H] SCH 23390 as the ligand. Specifically, studies using [^{3}H] SCH 23390 to map D1 receptor distribution in pigeons (*Columba livia*) have shown that the LPO-PA complex has a high density of D1 receptors ranging from 200 to 500 fmol/mg protein, and the PP has a low density of D1 receptors in the range of 10 fmol/mg protein (Dietl and Palacios, 1988; Richfield et al., 1987). In contrast, our data suggest that [^{3}H] SCH 23390 binding is approximately an order of magnitude higher in starlings than has been reported in pigeons. These differences may reflect the phylogenetic diversity of these species. Unpublished preliminary data from our laboratory suggest that zebra finches have D1 receptor densities in the paleostriatal complex that are about two times higher than those in starlings, and Japanese quail (*Coturnix japonica*) have paleostriatal D1 receptor densities that are about one-half as high as that in starlings (Casto, Balthazart, and Ball, unpublished data). European starlings and zebra finches both belong to the order *Passeriformes,* pigeons to the order *Columbiformes,* and Japanese quail to the order *Galliformes.* It appears that passerines, which are the most recently evolved avian order, have a substantially higher density of D1 sites in the paleostriatal complex than birds from any other order studied to date; however, until more species from these and other orders are studied, any conclusions about phylogeny and D1 receptor density in birds will be tentative.

In addition to appearing similar to patterns of D1 receptor density reported in other avian species, [^{3}H] SCH 23390 binding patterns in the starling paleostriatal complex appear similar to those found in mammals. Historically, homologies between the avian and mammalian basal ganglia have been misunderstood (see Karten, and Dubbledam, 1973; Nauta, and Karten, 1970; Parent, 1986; Reiner et al., 1984). It is now generally agreed that the avian homologue of the pallidal component of the mammalian basal ganglia (i.e., the globus pallidus) is the PP; however, striatal homologies are still not fully agreed upon. It has been

suggested that the homologue of the major striatal component of the mammalian basal ganglia (i.e., the caudate-putamen complex) is the LPO-PA complex; however, further structural homologies are uncertain (Karten and Dubbledam, 1973; Reiner et al., 1984; Reiner and Anderson, 1990). Parent (1986) has suggested that based on connectivity, the LPO is similar to the mammalian caudate, and the PA is similar to the putamen. Regardless of this debate, evidence exists in support of phylogenetic conservation of D1 receptor distribution and general neurochemical profile in the basal ganglia of birds and mammals (for a review see Reiner and Anderson, 1990). Specifically, the caudate-putamen complex of a variety of mammals (i.e., rats, cats, and monkeys) has been reported to contain high concentrations of D1 binding sites as determined by [^{3}H] SCH 23390 receptor autoradiography, and the globus pallidae of these species contain comparatively low concentrations of D1 binding sites (Dawson et al., 1986; Richfield et al., 1987). *In situ* hybridization studies in mammals have found that the mRNA for the D1A subtype are enriched in the basal ganglia in contrast to the other D1-like subtypes (i.e., D5/D1B) identified from cloning studies. This suggests that we have identified the receptor protein expressed by the avian homologue of the D1A clone (Gingrich and Caron, 1993).

Superimposed on the evolutionarily conservative pattern of D1 receptor binding in all avian species is a specialized pattern of D1 binding sites in starlings. Area X is discernible from the surrounding LPO by its substantially higher density of D1 binding sites. Unpublished results from our lab suggest that a similar pattern of [^{3}H] SCH 23390 binding is evident in male zebra finches but not in female zebra finches (Casto and Ball, unpublished observations). These findings suggest that high D1 receptor density will most likely be found in all song birds in which area X is present. This phylogenetically distinct distribution suggests that a specialization has evolved in the dopaminergic innervation of the LPO of song birds. Because lesions of area X during early sensitive phases for song learning disrupt the development of normal singing in male zebra finches (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), one might speculate that dopaminergic regulation of neural activity in LPO plays a role in song learning. In so far as male and female starlings exhibit different capacities for song learning and production, it is not completely unexpected that sex differences exist in the density of D1 sites in area X.

When [^{3}H] SCH 23390 binding in area X was considered independently of binding in surrounding structures, no sex differences in binding were detected. However, there is high between-subjects variability in D1 receptor density within the dorsolateral LPO. Because there is high between-subjects variability in overall [^{3}H] SCH 23390 binding, and because binding in area X is positively correlated with binding in the dorsolateral LPO (see Fig. 6), we used a measure of area X receptor density relative to the surrounding LPO. This method allowed us to assess whether D1 dopamine receptor density within area X, relative to the surrounding LPO, is the same in males as compared to females. A significant sex difference in this relative receptor density was detected. This suggests that there is a sex difference in the dopaminergic specialization of the LPO of European starlings.

Possible Causes and Significance of Sex Differences in D1 Receptor Density in Area X

Sex differences in neurotransmitter receptor density are not consistently found in area X of starlings. When area X in male and female starlings is defined by muscarinic receptor autoradiography, sex differences are found in volume of area X, but not in the density of muscarinic receptors in area X (Ball et al., 1990; Bernard et al., 1993). It should be noted that receptor density in area X relative to the surrounding LPO was not assessed in the muscarinic receptor autoradiography; however, area X appeared to be defined in male and female starlings with equal clarity, unlike the present study. Other examples of sex differences in density of neurotransmitter receptors have been demonstrated in birds. In Japanese quail, males have a higher density of α_2-adrenergic receptors as defined by [^{3}H]*p*-aminoclonidine (PAC) binding than do females in the medial mammilary nucleus and the ICo (Ball et al., 1989). These nuclei are involved in the control and regulation of gonadotropin secretion and vocal behavior, respectively. It has subsequently been demonstrated that α_2-adrenergic receptor density and muscarinic receptor density in the ICo of male Japanese quail are modulated by androgens in adulthood (Ball and Balthazart, 1990). Specifically, castration of males leads to a decrease in the levels of α_2-adrenergic and muscarinic binding sites in various subregions of the ICo. The decreases in α_2-adrenergic and muscarinic binding sites associated with castration were blocked by testosterone replacement therapy. These data suggest

that testosterone has activational effects on α_2-adrenergic and muscarinic receptor levels in the ICo of male quail. The possibility exists that similar activational effects of testosterone or its metabolites may be responsible for the sex difference in D1 binding sites in area X relative to LPO in the present study. Because both male and female starlings were maintained in a photostimulated condition, presumably males had higher levels of circulating testosterone than females. However, it is also possible that the sex difference in D1 receptor density resulted from either more permanent effects of gonadal steroids acting early in development, or a combination of both transient and permanent effects of steroids.

Whether activational or organizational effects of sex steroids mediate the sex difference in relative D1 receptor density in area X in adult starlings, the mechanism by which they might exert their effect is not obvious. Numerous attempts have been made at localizing steroid receptors in area X and to date neither estrogen nor androgen receptors have been detected in area X of any song bird studied (cf. Walters et al., 1988). However, HVc, which sends an efferent projection to area X, has been shown to possess both estrogen and androgen receptors in a number of song bird species (Arnold et al., 1976; Arnold and Saltiel, 1979; Brenowitz and Arnold, 1989; Gahr et al., 1987; Gahr et al., 1993; Zigmond et al., 1980). Herrmann and Arnold (1991) have recently shown that juvenile female zebra finches depend on transynaptic input from HVc in order for estrogen to masculinize area X as defined by Nissl stain. It would be useful to determine if other neurochemical dimorphisms such as the sex difference in relative D1 receptor density in area X of starlings reported here rely on similar transynaptic 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. Studies using castrated males and castrated males that receive T replacement, or intact females and females that receive exogenous T would also be useful in teasing apart the exact types of steroidal hormone effects that may underlie this sex difference.

In addition to possible steroid effects on expression of D1 receptors in area X, sex steroids also influence dopamine (DA) levels and DA turnover in area X in male zebra finches. Specifically, castration of adult male zebra finches results in a significant increase in DA levels and DA turnover in area X, and treatment of castrates with the aromatizable androgen, androstenedione, counteracts the effects of castration on DA levels in area X (Barclay and Harding, 1988). The effectiveness of androstenedione in reducing DA levels in castrates appears to be due to its androgenic as opposed to estrogenic metabolites, as administration of dihydrotestosterone to castrates decreases DA levels, whereas administration of estradiol does not (Barclay and Harding, 1990). Although no data regarding the effects of androgens on DA levels in area X of starlings exists, it seems quite possible that such effects would be similar to those reported in zebra finches. If DA turnover in the area X of starlings is decreased by circulating androgens, then it is possible that the sex difference in D1 receptor density in area X relative to the LPO may simply be a result of receptor up-regulation in males to compensate for the DA decrease.

The relevance of a sex difference in D1 receptor density to sexually dimorphic song behavior in starlings is, at present, unknown. In zebra finches and canaries, many aspects of the song control circuit, such as nuclear volume, cell soma size, and cell number have been found to be different between the sexes (Arnold et al., 1986), but the precise function of each of these sex differences remains to be clarified. Because the sex difference occurs in area X, a part of the forebrain pathway which in zebra finches is necessary for song learning (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), one might speculate that dopamine serves a regulatory role in song learning. Such a hypothesis is even more intriguing when one considers that starlings continue to update their song repertoires as adults (Eens et al., 1992).

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