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Identification, Distribution, and Developmental Changes of a Melatonin Binding Site in the Song Control System of the Zebra Finch

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ABSTRACT

In many avian species, singing is a circadian or seasonal behavior that appears to be widely dependent on gonadal steroid hormones. To explore the possibility of a further hormone-dependent vocal control mechanism driven by the action of melatonin, we examined the binding of iodinated melatonin (IMEL) in the vocal control network of adult and juvenile (22- and 40-day-old) zebra finches. IMEL binding areas of the zebra finch brain were localized and characterized by using quantitative in vitro autoradiography. In the vocal control system, dense IMEL binding sites were restricted to the nucleus hyperstriatalis ventrale, pars caudalis (HVC). The binding of IMEL to the HVC and to visual areas, e.g., the ectostriatum and the optic tectum, was saturable and showed a single class of high-affinity binding sites with binding affinities (K_ds) in the range of 5–20 pM. Competition experiments with various indols and IMEL showed that the IMEL binding site in the zebra finch brain has properties similar to the high-affinity melatonin receptor described in the chicken, in the house sparrow, and in the mammalian brain and retina. Similar to the zebra finch HVC, the HVC of other songbirds, e.g., male canaries and male house sparrows, has the most intense IMEL binding of all areas of the vocal control network. The IMEL binding in the forebrain vocal control areas of the zebra finch, but not that in the visual processing areas, was sexually dimorphic in correlation with the sexually dimorphic neuroanatomy of the forebrain vocal control areas. In the HVC, there is a developmental increase in the maximal number of binding sites for IMEL and in the protein content, so that the adult phenotype of dense IMEL binding develops between day 40 and day 80. The distribution and developmental pattern of IMEL binding in the song system suggests that melatonin has a role in the motor control of singing. Melatonin binding sites in HVC could link HVC-based song control to circadian and circannual changes in the photoperiod independent of gonadal steroids.

Indexing terms: songbird, HVC, area X, sexual dimorphism

A neural network of discrete brain nuclei controls the song motor pattern and its development in songbirds such as the zebra finch and the canary (Fig. 1; Konishi, 1989; Nottebohm, 1991). The vocal control network is one of the best known models to relate cellular morphology and activity to complex behavior in vertebrates. The chemistry and anatomy of neural vocal control areas, of the sound-modulating organ (syrinx), and of the song pattern undergo developmental and adult alterations (Konishi and Akutagawa, 1985; Nottebohm et al., 1986; Sakaguchi and Saito, 1989). Steroid hormones are thought to control most of this ontogenetic and adult plasticity of both the song pattern and the vocal control network (Gurney and Konishi, 1980; Konishi and Akutagawa, 1985, 1988; DeVoogd, 1986; Nottebohm et al., 1986, 1987; Nordeen et al., 1986; Barclay and Harding, 1988; Güttinger et al., 1993; Gahr, 1994). For example, estrogen treatment during the posthatching period induces song development and the male-like differentiation of the forebrain vocal control areas in female zebra

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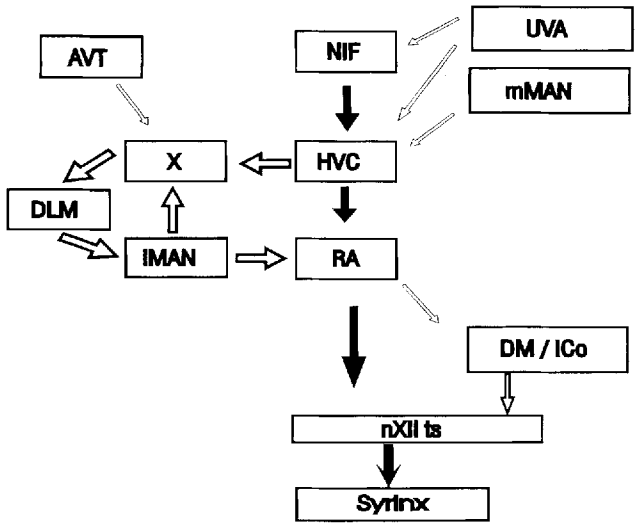

Fig. 1. Nuclei and connections of a vocal control network of the zebra finch. Solid arrows indicate anterograde connections in the descending motor pathway, and open arrows indicate anterograde connections in an auditory loop of the forebrain. The syrinx is a sound-modulating organ of songbirds. AVT, area ventralis of Tsai; DLM, nucleus dorsolateralis thalamis, pars medialis; DM, nucleus dorsomedialis of ICo; HVC, nucleus hyperstriatalis ventrale, pars caudale; ICo, nucleus intercollicularis; MAN, nucleus magnocellularis of the anterior neostriatum (l, lateral part; m, medial part); NIF, nucleus interfacialis; nXIIts, nucleus hypoglossus pars tracheosyringealis; RA, nucleus robustus archistriatalis; UVA, nucleus uvaeformis; X, area X (modified after Konishi, 1989; Perera et al., 1995).

finches, whereas untreated females do not sing (Gurney and Konishi, 1980; Konishi and Akutagawa, 1985, 1988). In adult male zebra finches, song activity (Pröve, 1974) and the type of song pattern are influenced by testosterone and estrogens (Pröve, 1974; Walters et al., 1991). In the adult canary, singing undergoes seasonal changes that are paralleled by seasonal changes in the production of testosterone (Nottebohm et al., 1987). Testosterone treatment of female or of castrated male canaries induces the song that is typical of reproductively active males (Shoemaker, 1939; Heid et al., 1985). Correlated with this behavior are the neuronal phenotypes of vocal control areas, which are sensitive to testosterone treatment (Nottebohm et al., 1986, 1987; Gahr, 1990). Testosterone treatment, however, fails to induce the typical male singing during the moult (DeVoogd, 1986; Gahr, unpublished data). Furthermore, certain song patterns persist after castration in the adult canary (Heid et al., 1985) and in the zebra finch (Pröve, 1974; Arnold, 1975) and appear to be controlled by neural mechanisms that do not require steroids.

In certain species, singing is a circadian behavior (for review, see Welty and Baptista, 1988; Pohl, 1994), and, in many oscine species, it is a seasonal behavior (Saunders, 1947, 1948). Circadian rhythms are frequently regulated by the hormone melatonin (Gwinner et al., 1981; Gwinner, 1991). Melatonin is produced by the pineal gland and, in some species, by the retina during the night and is degraded during the light period, resulting in a pronounced circadian rhythm with peak values of 330 pg/ml plasma during the night and of 19 pg/ml plasma during the midday in the case of the zebra finch (Van't Hoff and Gwinner, personal communication). Beside its general function for circadian rhythms, melatonin is involved in the neural control of periodically occurring reproductive behaviors in some vertebrate species (Crews et al., 1988; Hastings et al., 1988; Lincoln and Maeda, 1992), in neural control of sexual maturation (Carlson et al., 1989), and in the activity of gonadal steroid receptors (Danforth et al., 1983). Therefore, it is appealing to study the role of melatonin for the function of the vocal control network of songbirds. The local action of melatonin in the vocal control system would present a new hormone control mechanism of singing in addition to the steroid dependent mechanisms.

Melatonin affects cells by binding to a membrane-bound receptor that is a member of the G protein-coupled receptor family (Rivkees et al., 1989a; Ebisawa et al., 1994; Reppert et al., 1994). A requirement for a direct role of melatonin for singing and song development that is not mediated through the control of gonadal steroid production is the presence of melatonin binding sites in the vocal control network. Melatonin binding sites can be localized in the brain in in vitro autoradiographic techniques using the isotope 2-^{125}I-iodomelatonin (IMEL; Dubocovich and Takahashi, 1987). Here, we report the identification, distribution, and developmental changes of an IMEL binding site in the vocal control nucleus hyperstriatalis ventrale, pars caudalis (HVC) of the zebra finch. To indicate whether the IMEL binding in the HVC is a general pattern of songbirds, we report further on the HVC of the canary and the house sparrow.

MATERIALS AND METHODS

Animals

Zebra finches, canaries, and house sparrows were housed in a mixed colony under a light-dark cycle of 16 hours light and 8 hours dark. Nine adult male and four adult female zebra finches, five 40-day-old male and two 40-day-old female zebra finches, three 22-day-old male and three 22-day-old female zebra finches, three adult male canaries, and three adult male and one adult female house sparrows were killed by decapitation. Of the adult male zebra finches, three birds were 80 days old, and six were 100 days old. Because there were no differences between these birds, they were analyzed as one age group. Results from the chicken show that the affinity of the IMEL binding site in the brain is independent of the light-dark cycle, whereas the binding capacity undergoes daily alterations (Brooks and Cassone, 1992). Although zebra finches are not reported to be photosensitive, all birds were kept under similar light conditions and were killed at the same time in the morning to avoid circadian alterations of the IMEL mapping. The results reported here are similar to the results obtained from male zebra finches maintained in a light cycle of 12 hours light and 12 hours dark (Gahr and Kosar, unpublished).

For unambiguous localization of the vocal control area HVC in juvenile male zebra finches, the HVCs of the 40-day-old male juveniles were retrogradely labeled with the fluorescent dye rhodamine-coated latex microspheres (LumaFluor, NY). The microspheres (50 nl) were pressure injected into the vocal control nucleus area X of Isoflurane-anesthetized birds 3 days before killing the birds. Area X is a major efferent area of HVC (Fig. 1; Nottebohm, 1991).

Preparation of tissue for autoradiography

After decapitation, brains and syrinx musculatures were removed, quickly frozen, and stored at −80°C until section-

ing. The right halves of the brains and the entire syrinx were cut on a cryostat into 20 μm parasagittal sections, mounted onto gelatin- or lysin-coated slides, and stored at −80°C until the in vitro binding assays were performed. Adjacent sections were mounted onto different slides, and we obtained five series of sections that could be stained separately. Three series were used for total binding of IMEL, one series was used for unspecific IMEL binding, and one series was used for histologic identification of the vocal control areas in Nissl-stained sections or by means of retrograde labeling (see above). To control for left-right differences of the IMEL binding, left and right brain halves were analyzed in coronal sections of two brains. There were no hemispheric asymmetries in the IMEL binding in the analyzed areas of the vocal control network.

In vitro autoradiography

Slide-mounted tissue sections were warmed to room temperature and incubated with 20 pM IMEL (Amersham IM215; specific activity, 2,200 Ci/mmol) in 50 mM Tris-HCl buffer, pH 7.4, with 4 mM $CaCl_2$ in either the presence (nonspecific binding) or the absence (total binding) of 1 μM melatonin (Sigma; M 5250) for 1 hour at room temperature. No IMEL binding was found in any brain area in the presence of 1 μM melatonin. Slides were rinsed in ice-cold Tris-HCl buffer (once for 2 minutes and twice rapidly) and were dried on a hot plate. Autoradiographs were generated by apposition of sections to Hyperfilm-^{3}H (Amersham) for 10 days. Longer exposure times did not change the distribution of IMEL binding areas. During exposure, the cassettes were stored at −80°C. The hyperfilms were developed with Kodak D19 (2 minutes at 20°C) and were fixed with Kodak Unifix. After autoradiography, the sections were fixed in 4% paraformaldehyde, stained with the Nissl stain thionin, and examined by light microscopy to verify the location of IMEL binding brain structures. The sections of the 40-day-old zebra finches were photographed on a fluorescence microscope for identification of the retrogradely labeled HVC before Nissl staining.

Saturation experiments and studies of specificity

To use serial parasagittal sections for saturation and pharmacological studies, we verified whether there was a mediolateral gradient in the IMEL binding (Fig. 2). No mediolateral differences in the IMEL binding densities were found throughout the optic tectum, ectostriatum, nucleus isthmi pars magnocellularis, lobus parolfactorius, or HVC.

For saturation studies and for pharmacological analysis of the IMEL binding sites, serial sections of various brain areas were cut. For each concentration of melatonin (saturation study; 0.1–200 pM) or for each concentration of competitors (study of specificity), three parallel sections of a brain area were mounted on a slide. Saturation experiments were carried out on five adult and five 40-day-old zebra finches. Dissociation constant [binding affinity (K_d)] and maximal number of binding sites (B_{max}) were calculated from Scatchard plots analyzed by a computer using a linear model-fitting program (Munson and Rodbard, 1980). For the study of binding specificity, from 10^{-12} to 10^{-6} molar concentrations of 2-iodomelatonin (Research Biomedical Inc.), melatonin, N-acetylserotonin, 5-metoxytryptamin, and serotonin (Sigma) were used in competition with 20 pM IMEL.

Data analysis

For densitometry of the IMEL binding, optical densities of selected areas were calculated with a computer-based image analysis system (Imatec, Munich, Germany). For standardization of the densitometry, the image of a nonspecific binding section was first subtracted from the adjacent total binding section. Optical density values were converted into pmol bound melatonin/0.1 mm^3 tissue or into nCi/mg polymer with standard curves.

For radioactive standards, we used 20-μm-thick ^{125}I-microscales (Amersham) and a home-made standard on each film. The home-made standard consisted of 20-μm-thick brain sections mounted on slides. On each section, 1 μl of a certain concentration of IMEL was added and dried. This procedure resulted in a defined circle of radiolabeling on each brain section of the standard. From the optical density of these radiolabeled areas (average of five measurements), the size of the radiolabeled areas, and the section thickness (20 μm) we calculated a standard curve of optical density as a function of fmol IMEL/0.1 mm^3 tissue. These values were converted into fmol IMEL/mg protein by measuring the protein content of various brain parts and age groups (Table 1). Various brain areas were dissected out of 300 μm brain slices. Protein concentrations were measured by the method of Lowry. The home-made ^{125}I-standard and the microscales (Amersham) were exposed to the hyperfilm and developed along with the sections. Both standards produced similar results. Because the quantifications using the home-made standard are based on zebra finch brain sections and are more biological than the microscale values (nCi/mg polymer), we report here only the values calculated from the home-made standard curve. Photomicrographs of the original autoradiograms are shown in Figures 2–6. Differences in the protein content, in the IMEL binding density, and in the B_{max} between juvenile and adult male zebra finches were tested with a two-tailed Student's t test.

RESULTS

The main findings of this study were 1) that the vocal control nucleus HVC contains a high level of high-affinity melatonin binding sites and 2) that the amount of this binding site in HVC increases during the period of song development and maturation of the vocal control system.

Characterization of a melatonin binding protein in the zebra finch brain

Scatchard analyses of saturation binding in specific brain areas were carried out for the HVC, the surrounding neostriatum, the nucleus robustus archistriatalis (RA), the surrounding archistriatum, area X, the surrounding lobus parolfactorius, the lateral portion of nucleus magnocellularis anterioris (lMAN), the surrounding neostriatal tissue, the nucleus dorsolateralis thalamis, pars medialis (DLM), the ectostriatum, the optic tectum, and the nucleus isthmi pars magnocellularis by using concentrations of IMEL ranging from 0.1 to 200 pM. The K_d and B_{max} values of these brain areas of adult males are listed in Table 2. A representative densitometric analysis of the saturation binding from the HVC of adult males is shown in Figure 3.

Specific binding of IMEL to the optic tectum, ectostriatum, and HVC increased linearly with concentrations of IMEL from 0.1 to 200 pM and reached saturation at 50 pM.

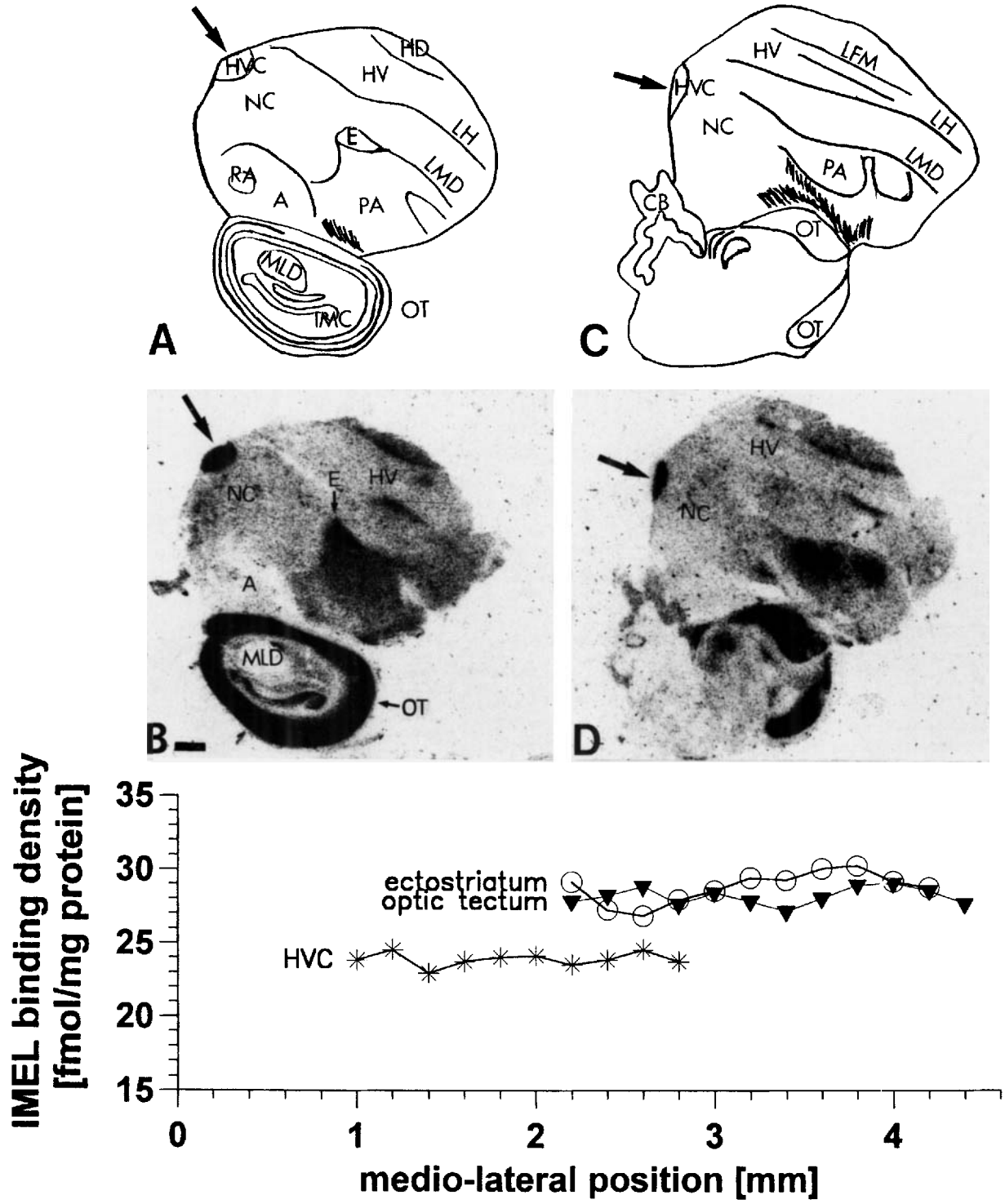

Fig. 2. **A–D:** Binding density of 2-^{125}I-iodomelatonin (IMEL) throughout the mediolateral extent of the vocal control nucleus HVC (*), the optic tectum (OT) (▼), and the ectostriatum (E) (○) of a representative adult male zebra finch. Sequential sagittal 20 μm sections were incubated with 20 pM IMEL as described in Materials and Methods. The abscissa shows the mediolateral position of the sections relative to the midline of the brain (=0 μm). A and C are line drawings of the sections shown in B and D after Nissl staining. There were no mediolateral differences in the IMEL binding density within the HVC, OT, or E. For example, the photomicrographs (B,D) show different mediolateral levels of the HVC. Note the intense labeling of the HVC (large arrows) in both its medial (D) and its lateral (B) portions. A, archistriatum; CB = cerebellum; HD, hyperstriatum dorsale; HV, hyperstriatum ventrale; IMC, nucleus isthmi, pars magnocellularis; LFM, lamina frontalis suprema; LH, lamina hyperstriatica; LMD, lamina medullaris dorsalis; MLD, nucleus mesencephalicus lateralis, pars dorsalis; NC, neostriatum caudale; PA, paleostriatum augmentatum; RA, nucleus robustus archistriatalis. Scale bar = 380 μm.

TABLE 1. Protein Content of Various Areas of the Brain of Juvenile and Adult Male Zebra Finches (mg protein/grams Tissue, Mean ± S.E.M.)[1]

Age	Optic tectum	Ectostriatum	HVC
Forty days	134 ± 2	120 ± 3	106 ± 2
Adults	139 ± 8	121 ± 2	121 ± 4

[1]There is a significant ($P < 0.01$; n = 5) increase in the protein content of the nucleus hyperstriatalis pars caudale (HVC) between day 40 and adulthood. In contrast, protein content of visual areas, such as the optic tectum and the ectostriatum, is already adult like at day 40.

TABLE 2. Specific Binding of 2-^{125}I-Iodomelatonin in Discrete Areas of the Brain of Juvenile and Adult Male Zebra Finches

	Adults		Forty days	
Areas	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)
Vocal control system and surrounding areas				
HVC	27.1 ± 1.9	18.5 ± 2.4	18.8 ± 1.5	26.5 ± 3.4
NC	19.9 ± 3.0	27.5 ± 3.1	18.7 ± 1.7	26.5 ± 3.4
RA	14.0 ± 1.5	23.5 ± 2.9	18.0 ± 2.0	21.8 ± 1.0
A	nd	nd	22.5 ± 0.5	49.3 ± 5.1
Area X	16.6 ± 4.0	64.2 ± 5.1	22.7 ± 1.0	52.5 ± 4.3
LPO	27.5 ± 3.2	24.7 ± 2.3	27.3 ± 2.5	13.3 ± 0.6
IMAN	25.5 ± 0.5	52.8 ± 8.0	22.5 ± 0.5	59.8 ± 5.0
Visual system				
Ectostriatum	28.7 ± 3.6	9.7 ± 2.9	28.0 ± 4.5	9.7 ± 0.5
Optic tectum	38.5 ± 2.9	12.4 ± 2.5	35.7 ± 5.2	9.5 ± 1.2
N. isthmi pars magnocellularis	28.5 ± 2.7	16.1 ± 2.5	nd	nd

[1]The binding affinity (K_d) and the maximal number of 2-^{125}I-iodomelatonin binding sites (B_{max}) are the mean ± S.E.M. of independent determinations, each performed in duplicate, of five juvenile and five adult male zebra finches (three sections per slide). Concentrations of 2-^{125}I-iodomelatonin ranged from 0.1 to 200 pM. The K_ds of all areas are in the same range, indicating one high-affinity melatonin binding site in the zebra finch brain. The B_{max} of the HVC of adults is significantly higher ($P < 0.01$) compared to the 40-day males (nd = values not determined). See Table 4 for abbreviations.

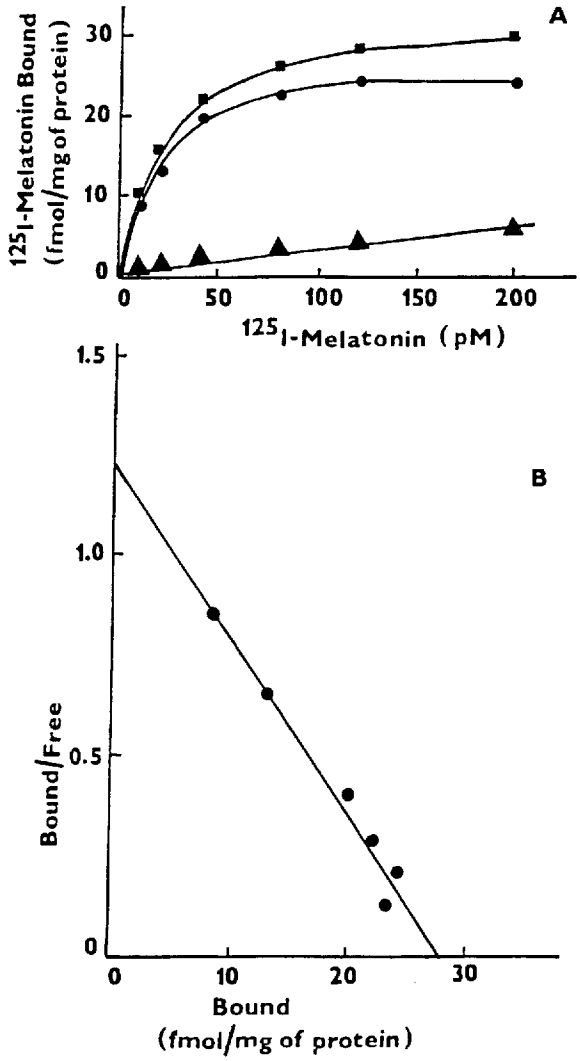

Fig. 3. Characterization of a melatonin binding protein in the zebra finch brain. **A:** Densitometric analysis of saturation binding of IMEL in the HVC of the adult male zebra finch using quantitative autoradiography. Specific binding (circles) was obtained after subtracting nonspecific binding (triangles) from total binding (squares). Specific binding was saturable. **B:** Scatchard plot of the saturation data of the IMEL binding in the HVC. Data points are the means of triplicate determination from one experiment and are representative for five such studies. The HVC of the depicted male has a binding affinity (K_d) of 18.3 pM and a maximal number of binding sites (B_{max}) of 26.2 fmol/mg protein.

Scatchard plots for the various brain areas that bound IMEL were linear, indicating one class of high-affinity binding sites (Fig. 3). The K_ds of all analyzed areas were in the same general range (9–65 pM; Table 2). Among the areas of the vocal control system, the highest affinity for IMEL was found in HVC (K_d = 18.5 ± 2.4 pM). This affinity was similar to the K_ds of the visual processing areas, such as the nucleus isthmi pars magnocellularis, the ectostriatum, and the optic tectum (Table 2).

The specificity of the binding site for melatonin was tested in binding competition experiments with various indols that are chemically related to melatonin. These experiments showed that the IMEL binding site has a high affinity for melatonin and iodomelatonin and a low affinity for other indols (Table 3). Because the pharmacological experiments (see below) showed similar specificity in all brain areas, and because the K_ds of all areas were in the same range of affinity, we suggest that there are no area differences in the type of the melatonin binding site. Because the Scatchard analysis and the competition studies showed a high-affinity IMEL binding site, we analyzed the spatial distribution of this binding site in the zebra finch brain with emphasis on the areas of the vocal control network.

Distribution of IMEL binding sites in the vocal control network of zebra finches

The IMEL binding in nuclei of the vocal control network, in the tissues that surround these nuclei, and in the syrinx that contains the sound-modulating muscles were quantified (Table 4). For comparison to brain areas that commonly bind IMEL, we analyzed the binding of three visual areas: the ectostriatum, the optic tectum, and the nucleus isthmi pars magnocellularis (Table 4; Karten, 1969; Nixdorf and Bischof, 1982). The ectostriatum and optic tectum of adult males showed the highest densities of IMEL binding among all brain areas (Table 4).

Of the vocal control areas of adult zebra finches, IMEL binding was strongest in the HVC, with 23.8 ± 3 fmol IMEL/mg protein (Figs. 2, 4, Table 3). The density of IMEL binding in HVC was similar to areas of the visual systems. Comparison of the Nissl-stained sections to the autoradiograms showed an exact fit between the area of dense melatonin binding in the caudale neostriatum and the Nissl-defined HVC of adult males (Fig. 4A–C).

TABLE 3. Specificity of 2-^{125}I-Iodomelatonin (IMEL) Binding to Brain Areas of the Zebra Finch[1]

Indol	Inhibition Concentration (50%; IC_{50}; pM)
Melatonin	0.75 ± 0.1
2-Iodomelatonin	30.0 ± 1.5
N-acetylserotonin	2×10^5
5-Hydrotryptamine	$> 10^6$
5-Methoxytryptamine	$> 10^6$

[1]Brain sections were incubated with six concentrations (1 pM to 1 μM) of unlabeled indoles and 20 pmol IMEL. IC_{50} values are the concentrations of compounds capable of reducing the IMEL binding by 50%. The values were obtained from IMEL binding in the optic tectum and the HVC. Results are the means of triplicate determinations from two experiments per indol. The specificity of the binding site for melatonin and iodomelatonin, but not for N-acetylserotonin, indicates that the IMEL binding site of the zebra finch brain is similar to the high-affinity melatonin receptor reported in other avian and mammalian species.

TABLE 4. Densities of 2-^{125}I-Iodomelatonin (IMEL) Binding in Areas of the Zebra Finch Brain and in the Syrinx[1]

Area	Abbreviations	Binding density (fmol/mg protein) Adult males	Forty-day males
Vocal control system and surrounding areas			
Hyperstriatalis ventrale, pars caudale	HVC	23.8 ± 3.0	14.8 ± 2.0
Neostriatum caudale	NC	9.0 ± 1.2	14.5 ± 1.5
N. robustus archistriatalis	RA	9.1 ± 4.0	12.5 ± 3.8
Archistriatum	A	6.0 ± 4.1	4.2 ± 2.4
Area X	X	3.6 ± 2.8	7.3 ± 2.5
Lobus parolfactorius	LPO	18.2 ± 3.8	24.2 ± 3.2
N. magnocellularis anterioris lateralis	lMAN	3.0 ± 2.5	2.8 ± 2.6
N. dorsomedialis thalami	DLM	3.2 ± 2.2	3.7 ± 2.6
N. hypoglossus pars tracheosyringealis	nXIIt	4.1 ± 2.6	3.5 ± 2.1
Syrinx		3.1 ± 1.4	2.5 ± 1.5
Visual processing areas			
Ectostriatum	E	29.0 ± 4.0	30.8 ± 2.2
Optic tectum	OT	27.8 ± 2.4	32.0 ± 4.1
N. isthmi magnocellularis	IMC	26.9 ± 3.1	27.8 ± 2.9
Auditory processing areas			
Field L		5.1 ± 3.0	nd
N. ovoidalis		2.8 ± 2.0	nd
N. mesencaphalius dorsalis lateralis	MLD	3.2 ± 1.0	nd

[1]The values give the binding densities of IMEL at each site calculated as explained in Materials and Methods. The nonspecific binding in the presence of 1 μM melatonin was less than 1 fmol/mg protein. Means are based on triplicate determination per animal and on a total of seven adults and of five 40-day-old males. The weakest binding of all brain areas was 3 fmol/mg protein. Note that IMEL binding in the HVC is significantly ($P < 0.01$) higher compared to all other areas of the vocal control system. It is statistically similar to the binding of LPO and of visual areas. Furthermore, the binding density of the HVC of adults is significantly higher ($P < 0.01$) compared to the HVC of 40-day-old males, whereas the binding in all other areas does not change during late ontogeny (nd, not determined).

Moderate IMEL binding in RA was detectable only in one of the nine adult male zebra finches. In this bird, the binding density was similar to the binding in the caudale neostriatum outside the HVC but was lower than the binding in the HVC and in the visual areas (Fig. 4, Table 3). Despite the weak IMEL binding in RA, RA was visible in the autoradiograms, because the binding in the surrounding archistriatum of adult zebra finches was even lower. Very low binding densities were found in the nucleus hypoglossus (nXII), DLM, lMAN, area X, and the syrinx. lMAN, area X, and DLM were visible as light spots in the anterior neostriatal tissue, the lobus parolfactorius, and the thalamus, respectively (Fig. 5A, Table 4). Similar to the zebra finch, the HVC of the canary (19.2 ± 1.7 fmol/mg protein) and of the house sparrow (16.5 ± 1.5 fmol/mg protein) showed dense IMEL binding.

In a previous study of the house sparrow, Cassone and Brooks (1991) did not report IMEL binding in the HVC. The difference in the IMEL binding pattern in the vocal control network of the zebra finch and the house sparrow study of Cassone and Brooks (1991) is probably due to sex differences in the house sparrow. The one female house sparrow that was included in our study had no elevated IMEL binding in HVC and bound IMEL in the entire lobus parolfactorius, similar to female zebra finches (see below). In the study of Cassone and Brooks (1991), only female house sparrows were analyzed.

Outside the vocal control network, specific IMEL binding sites were found in numerous brain areas of the adult male zebra finches, including structures that are involved in the processing of visual information at several levels of neural organization and in areas of the limbic system. Prominent IMEL binding areas in these systems are the nucleus suprachiasmaticus of the circadian system; the tectum opticum, nucleus rotundus, nucleus isthmi pars magnocellularis, nucleus isthmi pars parvocellularis, and ectostriatum of the tectofugal system; and the nucleus lateralis anterior, nucleus dorsolateralis anterior thalami, and nucleus geniculatus lateralis pars ventralis of the thalamofugal system. This overall distribution of IMEL binding was similar in all adult birds and was similar to the distribution reported for the house sparrow, another songbird species (Cassone and Brooks, 1991), for the chicken (Rivkees et al., 1989b; Stehle, 1990; Siuciak et al., 1991), and for the quail (Cozzi et al., 1993). These studies of the house sparrow, chicken, and quail describe the general pattern of IMEL binding in the avian brain in detail. Therefore, we restrict our report to the IMEL binding in areas of the vocal control network that are present in songbirds only or that have not been analyzed previously in the house sparrow (see above).

Sexually dimorphic IMEL binding

IMEL binding was sexually dimorphic in the forebrain vocal control areas HVC and area X, but not in the optic tectum, the ectostriatum, or the nucleus isthmi pars magnocellularis. Dense IMEL binding was not found in either the caudale neostriatum (binding density, 9.3 ± 1.8 fmol/mg protein) of adult female zebra finches (where the HVC of males is located) nor was there an area of reduced IMEL binding similar to the male area X in the female lobus parolfactorius (Fig. 5). The IMEL binding density in male (18.2 ± 3.8 fmol/mg protein) and female (17.6 ± 2.9 fmol/mg protein) lobus parolfactorius was similar. Like the female zebra finches, the caudale neostriatum of the one female house sparrow we analyzed did not contain the subarea (HVC) delineated by high IMEL binding that was seen in the male birds.

Developmental changes of the IMEL binding

The forebrain vocal control areas develop postnatally. In histological (Nissl-stained and fiber-stained) sections of the zebra finch forebrain, vocal control areas are first detectable around posthatching day 10 and differentiate the adult male typical phenotype between 40 and 80 days (Herrman and Bischof, 1986). The HVC of adults (i.e, 100 days) was clearly defined by its IMEL binding pattern (Fig. 4), whereas the HVC of 22- and 40-day-old juveniles was not (Fig. 6). Below, we compare the IMEL binding capacity, affinity, and density of juveniles and of adults.

In 40-day-old males, the K_d of the HVC and of the visual areas was in the range of the adult males (Table 2). In contrast, the IMEL binding density ($P < 0.01$) and the maximal binding capacity (B_{max}; $P < 0.01$) of the HVC

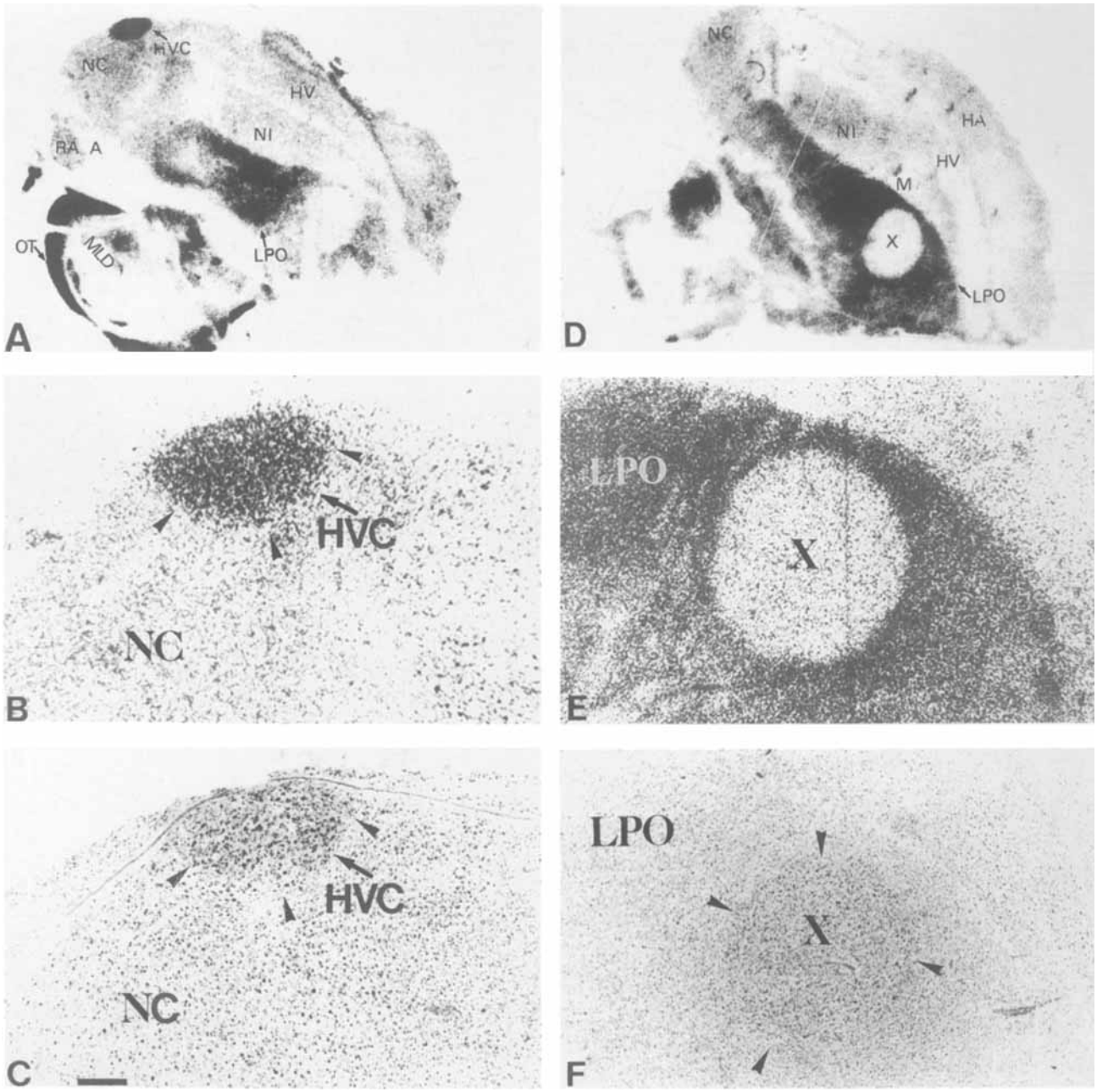

Fig. 4. **A–F:** Intense binding of IMEL in the vocal control area HVC and weak IMEL binding in area X of adult male zebra finches. A, B, D, and E are photomicrographs of the autoradiograms of parasagittal sections. B and E are higher power sections of A and D. C and F are photomicrographs of Nissl-stained sections adjacent to B and E, respectively. In A, strong IMEL binding is shown in the central HVC (a vocal control area), in the OT, and in the lobus parolfactorius (LPO). Weak binding occurs in the NC surrounding the HVC. The RA, another vocal control area located in the archistriatum (A), was weakly labeled in the bird depicted. In B, the HVC from A is shown at higher power. Comparison to the Nissl-stained HVC (C) shows that intense IMEL binding in the caudale neostriatum is restricted to the HVC. Arrowheads in B and C indicate the borders of the HVC. In D, strong IMEL binding is shown in the LPO. The vocal control nuclei area X (X) in LPO and lMAN (M) in the anterior neostriatum bind little IMEL. In E, LPO and area X are shown at higher power. Comparison to the Nissl-stained area X (F) shows that the reduced IMEL binding in the LPO is restricted to area X. Arrowheads in F indicate the border of area X. HA, hyperstriatum accessorium; NI, neostriatum intermedium; MLD nucleus mesencephalicus lateralis dorsalis. Scale bar = 160 μm in B,C,E,F, 0.8 mm in A,D.

increased significantly between 40 and 80 days of postnatal life. At posthatching day 40, the IMEL binding densities in the HVC and in the surrounding neostriatum were similar (Fig. 6, Table 4). This result was derived from the comparison of the Nissl-stained HVC, the retrogradely labeled HVC, and the IMEL autoradiograms of the caudale neostriatum, including HVC of 40-day-old male zebra finches (Fig. 6). Because the juvenile HVC is not always identifiable in Nissl-stained sections (Gahr, 1990), we used retrograde labeling to have an additional marker for the delineation of

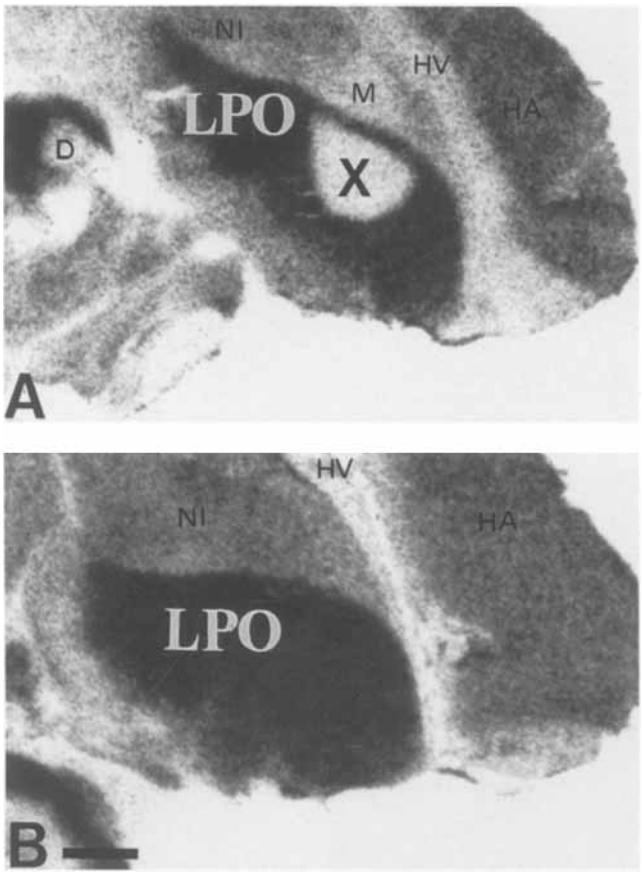

Fig. 5. Sexual dimorphism in the IMEL binding in the LPO. Photomicrographs of the autoradiograms of the anterior forebrain (parasagittal sections) of a juvenile male (**A**) and a juvenile female (**B**) zebra finch (40 days old). Little IMEL binding occurs in area X (X) in the juvenile male, whereas juvenile females bind IMEL throughout LPO similar to adult females. Weak binding was found in lMAN (M) and DLM (D) of juvenile males (A). Scale bar = 40 μm for A, 0.8 mm for B.

HVC. In contrast to the juveniles, the binding density in the HVC of adults was 2.5 times higher than the binding in its surrounding neostriatal tissue, so that the HVC of adult males was characterized by its IMEL binding density in the caudale neostriatum (Fig. 4, Table 4). The increase in the binding in the HVC correlates with a 15% increase of the protein content (Table 1) and a 40% increase in the maximal binding capacity per milligram of HVC protein (Table 2). In summary, the identification of the HVC by means of IMEL binding is due to a drop in the binding in the tissue surrounding HVC and an increase in the binding of HVC after day 40. None of the other brain areas showed a significant increase in the IMEL binding after posthatching day 40. For example, the development of the IMEL binding of visual areas is already completed at day 40. The binding densities, the K_d, and the B_{max} of the ectostriatum and of the optic tectum of 40-day-old and adult zebra finches were similar (Tables 2, 4).

Unlike the HVC, and similar to visual areas, area X of 40-day-old zebra finches already showed the adult pattern of IMEL binding (Figs. 4, 5); 40-day-old males showed low binding in area X but high binding in the surrounding lobus parolfactorius. In contrast to the 40-day-old males, reduced IMEL binding was not found in any subarea of lobus parolfactorius in the 40-day-old females. The binding density of the entire female lobus parolfactorius (20.5 ± 4 fmol/mg protein) was similar to the binding density of the male lobus parolfactorius outside area X (24.2 ± 3.2 fmol/mg protein). In contrast, the lobus parolfactorius of 22-day-old males and females contained a large central subarea of lower IMEL binding compared to the rest of the lobus. This area was similar in location to area X of males. In this area, the binding density was 5.2 ± 2.6 fmol/mg protein in the 22-day-old males and 5.6 ± 3.1 fmol/mg protein in the 22-day-old females. The lobus parolfactorius outside area X of 22-day-old males and females bound 11.5 ± 4.1 and 10.8 ± 2.3 fmol IMEL/mg protein, respectively.

DISCUSSION

Scatchard analysis of IMEL binding of the vocal control nucleus HVC and of visual processing areas showed a single class of IMEL binding sites in these areas, with K_d in the range of 5–30 pM. Pharmacological characterization of the IMEL binding in HVC and in visual areas showed a high affinity of the binding site for melatonin and 2-iodomelatonin (the nonradioactive analog of IMEL) and showed low affinities for related indols, including N-acetylserotonin. Therefore, the IMEL binding site of the zebra finch brain is similar to the high-affinity melatonin receptor that was previously described in defined areas of the chicken, house sparrow, and mammalian brain and of the chicken and rabbit retina (Dubocovich, 1985, 1988; Dubocovich and Takahashi, 1987; Vanecek et al., 1987; Rivkees et al., 1989a; Fang et al., 1990; Chong and Sugden, 1992). The high-affinity melatonin receptor is thought to mediate functional responses regulated by melatonin in the chicken brain (Siuciak et al., 1991).

The presence of dense melatonin receptors in HVC indicates that melatonin may affect neural mechanisms of vocal control by direct action in the HVC. Because lMAN, area X, DLM, RA, nXII, and the syrinx bind little or no IMEL, the importance of melatonin for the function of these structures appear to be limited. Although we cannot exclude the fact that there are a few cells with dense melatonin receptors in the above vocal control areas, the HVC appears to be the main target for melatonin-dependent effects on the song system.

The HVC is a major part of the vocal control network that is involved both in song learning and in motor control of singing (Konishi, 1989; Nottebohm, 1991). Furthermore, in the zebra finch, the differentiation of the HVC appears to play a key role for the differentiation of the entire vocal control network (Konishi and Akutagawa, 1985; Gahr and Konishi, 1988; Herrmann and Arnold, 1991). Female zebra finches develop a song system only if they are treated with estrogens during the first 30 days after hatching. This action could be mediated by estrogen receptor-containing neurons in the HVC area (Gahr and Konishi, 1988). In agreement, the destruction of HVC abolishes the capacity of estrogen to differentiate the vocal control system in the female zebra finch (Herrmann and Arnold, 1991), and estrogen microimplants placed near HVC induce partial masculinization of HVC neurons (Grisham et al., 1994). Melatonin, however, does not seem to play a major role for the early sex-specific differentiation of the vocal control system in the zebra finch, because high densities of melatonin

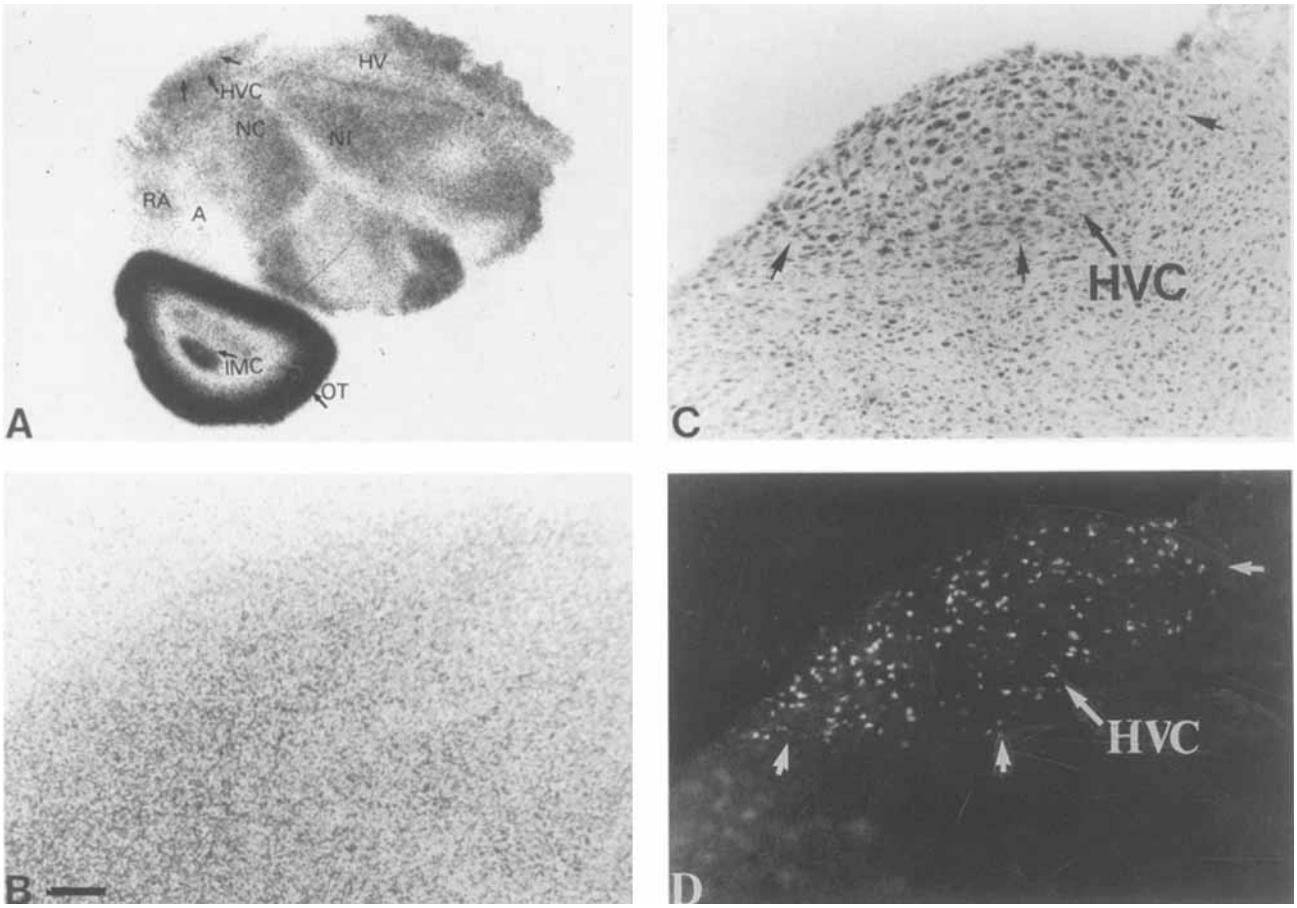

Fig. 6. Weak IMEL binding in the vocal control area HVC of juvenile male zebra finches. **A:** the IMEL binding of the forebrain of a 40-day-old male is shown in a low-power photomicrograph of an autoradiogram. Weak IMEL binding is found in the NC, including the HVC. The position of the HVC is indicated by the arrows. **B:** The HVC area in A shown at a higher power. Note that the HVC does not differ from the surrounding NC in terms of IMEL binding density. **C,D:** The HVC of the section adjacent to A is shown in Nissl-staining (C) and as a field of retrogradely labeled neurons (D; photomicrograph of fluorescent image) that project to area X. With both techniques (C,D), the HVC of this 40-day-old male is clearly delineated, whereas the HVC is not distinguished by its IMEL binding (A,B) in contrast to adult males (see Fig. 4). In contrast to the HVC, the OT and the LPO of 40-day-old males bind high levels of IMEL, similar to adult males. Scale bar = 1 mm in A, 80 μm in B–D.

receptors in the HVC are found only after the end of the period of estrogen-inducible differentiation of the HVC, i.e., after day 40. Similar to the 40-day-old birds included in this study, analysis of earlier stages (15, 22, and 30 days; Gahr and Kosar, unpublished results) did not show intense IMEL binding in the HVC.

Rather than a cause, the development of the sexually dimorphic melatonin binding pattern in the HVC and area X appears to be a consequence of the sexually dimorphic anatomical differentiation of the HVC and area X. Adult female zebra finches do not sing, and they have neither a differentiated HVC and area X (Nottebohm and Arnold, 1976), nor an area of elevated IMEL binding in the caudale neostriatum, nor an area of low binding in the lobus parolfactorius. Outside the vocal control system, the IMEL binding pattern is not sexually dimorphic. The lack of binding in the caudale forebrain of female zebra finches correlates with the degeneration of the HVC in females that is probably due to cell death (Kirn and DeVoogd, 1989). The mechanisms of the sex-specific changes in the IMEL binding of area X of females require further discussion.

The central part of the lobus parolfactorius of females (the area that is homologous to the male area X) first shows a male-like phenotype with a low binding of melatonin in this area at day 22. In the male, the typical adult IMEL binding pattern of area X (low binding) is already seen in the lobus parolfactorius of 22-day-old birds. In females, the area of low IMEL binding in the lobus parolfactorius disappears by posthatching day 40. Studies of the birth and death of cells in the female lobus parolfactorius indicate that cells in the lobus parolfactorius undergo only limited cell death if area X does not differentiate in a male direction (Kirn and DeVoogd, 1989). This suggests that the developmental increase in the IMEL binding in the central part of the lobus parolfactorius (the female area that is homologous to the male area X) is due to the expression of melatonin receptors of former area X cells after day 40 or to an inhibition of the IMEL binding in area X before day 40. Either possibility implies that cells of the central part of the lobus parolfactorius redifferentiate if the vocal control network does not develop in a male direction. Because the IMEL binding in the lobus parolfactorius outside area X of

22-day-old males and females bound little IMEL compared to the adults, more work is needed to manifest a male trait in the presumptive "area X" of juvenile females.

Instead of a role in the control of the sex-specific differentiation of the vocal control network of males, the developmental changes of the IMEL binding in the HVC suggests a role of melatonin for song development and for the motor control of singing in adult males. In the HVC, the typical IMEL binding pattern of adult males develops between day 40 and adulthood (i.e., day 80). Zebra finches learn and memorize their song during the first 30–40 days of posthatching life (Böhner, 1983). Between day 30 and day 80, the young males develop their adult song by trying to match their vocal output to the previously stored memory (Konishi, 1965; Böhner, 1983). Thus, the late ontogenetic increase in melatonin receptors in the HVC correlates with the sensory-motor phase of song learning of the zebra finch. Alternatively, the melatonin receptors may play a role for the control of the stabilized song in adult birds.

To determine the mechanisms of the late developmental increase in melatonin binding of the HVC, the increase in receptors per milligram of protein (40%) and in protein content (15%) of the HVC needs to be explained. Similar to the chicken brain development (Chong and Sugden, 1992), in the zebra finch HVC, the IMEL K_d did not change during the development, whereas the B_{max} increased between 40 and 80 days of postnatal life. The increase in melatonin receptors and protein content of the HVC coincides with the myelinization (Herrman and Bischof, 1986) and, thus, with the addition of oligodendrocytes to the HVC and coincides with the maturation of the motor pathway of the vocal control network and, thus, with the addition of HVC neurons that project to the vocal control nucleus RA (Nordeen and Nordeen, 1988; Kirn and DeVoogd, 1989; it is unknown whether the melatonin receptor is a neuronal protein, a nonneuronal protein, or both). Factors that control the expression of melatonin receptors in the HVC are currently unknown.

In agreement with the hypothesized role of melatonin in the song motor control, lMAN, area X, and DLM, which are all part of a recursive, primarily auditory loop in the vocal control network (Doupe and Konishi, 1991), show little IMEL binding similar to auditory areas such as field L, nucleus ovoidalis, and nucleus mesencephalicus lateralis dorsalis in the zebra finch brain (Table 4). lMAN, area X, and DLM are not required for the control of the crystallized motor pattern of adult zebra finches (Nottebohm et al., 1976; Bottjer et al., 1984; Halsema and Bottjer, 1992). In contrast, the HVC contains auditory neurons, but it is necessary for the production of the species' typical song pattern (Nottebohm et al., 1976; McCasland and Konishi, 1981).

Apart from a motor control function of melatonin, melatonin binding in the HVC could indicate other functions of the HVC in addition to auditory processing and song control, such as visual information processing. An electrophysiological investigation showed visual neurons in the HVC (Bischof and Engelage, 1985). Visual information could reach the HVC via the thalamic nucleus uvaeformis, which receives visual input from the optic tectum (Wild, 1994).

Ongoing experiments should give insights into the aspects of singing that are affected by the local action of melatonin in the vocal control nucleus HVC. This is particularly interesting for birds such as the canary, with seasonal changes in singing and in the neural phenotypes of the song control system (Gahr, 1990; Nottebohm, 1991). Because IMEL was bound in the HVC of both the photoperiod-independent zebra finch and the photoperiod-dependent canary, we need to examine canaries under changing day length to determine whether a circannual regulation of melatonin binding sites could be a basis for seasonal changes in song control. Both changing and constant expression of melatonin binding sites in the HVC could link HVC functions directly to changes in the photoperiod that are independent of gonadal steroids.

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