THE JOURNAL OF COMPARATIVE NEUROLOGY 397:118–138 (1998)

Axonal Connections of the High Vocal Center and Surrounding Cortical Regions in Juvenile and Adult Male Zebra Finches

ELIZABETH F. FOSTER[1]* AND SARAH W. BOTTJER[2]
[1]Department of Psychology, Florida State University, Tallahassee, Florida 32306-1051
[2]Department of Biology, University of Southern California, Los Angeles, California 90089-2520

ABSTRACT

Neuronal connections of the High Vocal Center (HVC), a cortical nucleus of songbirds necessary for learned vocal behavior, and the region adjacent to HVC called paraHVC (pHVC), were studied in adult and juvenile male zebra finches. Extremely small injections of fluorescent dextran amines or biocytin were made within subregions of HVC and pHVC to define the precise nature and development of these pathways. In adults, all HVC injections produced an even, nontopographic distribution of retrograde label throughout the medial magnocellular nucleus of the anterior neostriatum (mMAN), the interfacial nucleus (NIf), and the uvaeform nucleus of the thalamus (Uva) and an even distribution of anterograde label within area X of the striatum and the robust nucleus of the archistriatum (RA). These same patterns of projections were present in juvenile birds 20–23 days of age, including the projection from HVC to RA, which has previously been reported to develop only after 25–30 days of age. Results also establish a novel efferent projection from HVC to pHVC in both juvenile and adult birds. Injections into pHVC indicate that this region receives afferent input from song control areas HVC, mMAN, medial regions of the parvicellular shell of lateral MAN, NIf, and Uva and projects to Area X, caudomedial regions of striatum, and regions of the caudomedial neostriatum (NCM). Thus, neuronal connections of pHVC are highly integrated with circuitry important for vocal behavior and are distinct from those of HVC. Such differences establish HVC and pHVC as separate brain areas and suggest that each may serve a different function in vocal behavior. Control injections in both juveniles and adults produced specific patterns of projections from areas outside of HVC to areas outside of RA, illustrating an overall spatial organization of projections from HVC and neighboring cortical areas. Further, although neuronal connections of HVC are not topographic, projections of HVC, pHVC, and surrounding areas demonstrate a broad spatial organization of efferents to striatum and regions surrounding RA, thus defining a level of organization beyond that of individual song control nuclei. J. Comp. Neurol. 397:118–138, 1998.

Indexing terms: songbirds; cortex; striatum; development; vocal learning

Birdsong is a learned behavior performed by male birds for courtship, breeding, and territorial defense. In zebra finches, young males learn to sing during a period of juvenile development from approximately 20 days of age until about 80–90 days of age. After this time, the bird produces a fixed, unmodified song pattern throughout adult life. The neural substrate controlling song learning and vocal production consists of an interconnected set of brain nuclei referred to as the song control system (Nottebohm et al., 1976; Bottjer et al., 1989). The High Vocal Center (HVC), a cortical song control nucleus, is critical for production of learned vocalizations in adults and is thought to play a role in song learning (Nottebohm et al., 1976; Simpson and Vicario, 1990). HVC receives its major afferent input from two cortical nuclei, the medial magnocellular nucleus of the anterior neostriatum (mMAN) and the interfacial nucleus (NIf), and from the uvaeform nucleus of

Grant sponsor: NIH; Grant number: DC00190.

*Correspondence to: Elizabeth F. Foster, Department of Psychology, Florida State University, Tallahassee, FL 32306-1051.

Received 10 November 1997; Revised 26 March 1998; Accepted 27 March 1998

the thalamus (Uva; Nottebohm et al., 1982; Bottjer et al., 1989). Although some evidence suggests that HVC also receives direct input from auditory cortex (Field L; Fortune and Margoliash, 1995), anterograde tracer injections into Field L fail to produce distinct terminal label within the Nissl-defined borders of HVC (Kelley and Nottebohm, 1979; Vates et al., 1996).

The HVC sends an efferent projection to the motor-cortical robust nucleus RA (robust nucleus of the archistriatum; Nottebohm et al., 1976, 1982; Bottjer et al., 1989), and this pathway is necessary for song production because lesions of either HVC or RA in adults induce severely disrupted vocal behavior (Nottebohm et al., 1976; Simpson and Vicario, 1990). The HVC-to-RA pathway may also play a role during vocal learning, but this hypothesis has not been tested directly (for review, see Bottjer and Johnson, 1997). HVC also projects to a specific region of rostral striatum called Area X (Nottebohm et al., 1976, 1982; Bottjer et al., 1989). Area X neurons project to the dorsolateral nucleus of the medial anterior thalamus (DLM), which projects in turn to the lateral magnocellular nucleus of the anterior neostriatum (lMAN), which makes efferent projections to RA and Area X (Nottebohm et al., 1976; Okuhata and Saito, 1987; Bottjer et al., 1989; Vates and Nottebohm, 1995). This latter forebrain pathway emanating from HVC is important for normal song learning in juvenile birds but not in the maintenance of adult song production (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991).

Abbreviations

A	archistriatum
Ad	dorsal archistriatum
Area X	area X of avian striatum
Cb	cerebellum
CoA	anterior commissure
Cb	cerebellum
CP	posterior commissure
DLM	dorsolateral nucleus of the medial anterior thalamus
DMP	dorsomedial nucleus of the posterior thalamus
FDA	fluorescein dextran amine
FPL	lateral forebrain bundle
GP	globus pallidus
Hpc	hippocampus
HVC	high vocal center
ICo	intercollicular nucleus
IPC/IM	parvicellular portion of nucleus isthmi
LAD	dorsal lamina of the archistriatum
LFM	supreme frontal lamina
LFS	superior frontal lamina
LH	hyperstriatal lamina
lMAN	lateral magnocellular nucleus of the anterior neostriatum
LMD	medial dorsal lamina
LPO	parolfactory lobe/medial striatum
mMAN	medial magnocellular nucleus of the anterior neostriatum
N	neostriatum
NCM	caudomedial portion of neostriatum
NIf	interfacial nucleus
OM	occipitomesencephalic tract
Ov	ovoid nucleus of thalamus
pHVC	paraHVC
RA	robust nucleus of the archistriatum
RDA	rhodamine dextran amine
Rt	rotund nucleus
S	septum
Str	striatum
TeO	optic tectum
Tn	taeniae nucleus
TrSM	septomesencephalic tract
Uva	uvaeform nucleus
V	lateral ventricle

In addition to receiving a projection from HVC, Area X receives input from a thin strip of cells medial to HVC along the margin of the lateral ventricle, called paraHVC (pHVC; Gahr et al., 1987; Nordeen et al., 1987; Johnson and Bottjer, 1995). A recent report has demonstrated that mMAN not only projects to HVC but also projects directly to pHVC (Foster, et al., 1997). These neuroanatomical similarities with HVC raise the question of whether pHVC is actually a subdivision of HVC or is itself a distinct and separate brain region. Furthermore, pHVC appears to lie within dorsal and caudal regions of caudomedial neostriatum (NCM), a cortical area in which immediate early gene expression and complex changes in neuronal firing rate occur in response to hearing song (Mello et al., 1992, 1995; Mello and Clayton, 1994; Nastiuk et al., 1994; Chew et al., 1995, 1996; Jarvis and Nottebohm, 1997; Stripling et al., 1997). However, the precise neuroanatomical and functional relationships between pHVC, HVC, and NCM have not been established. Aside from its efferent projection to Area X and afferent input from mMAN, the neuronal connections of pHVC are unknown.

Tract tracing studies have shown that neuronal connections of the Area X–DLM–lMAN pathway for vocal learning are highly topographic as projections from specific subregions of these brain nuclei terminate within specific domains of their efferent targets (Johnson et al., 1995; Vates and Nottebohm, 1995). Conversely, neuronal connections of HVC in zebra finches do not appear topographic as partial injections of HVC have been reported to produce retrograde label throughout mMAN, NIf, and Uva and an even distribution of anterograde label within RA and Area X (cf. Nottebohm et al., 1982; Vates and Nottebohm, 1995; Vates et al., 1996; Fortune and Margoliash, 1995).

Developmental studies have reported that the projection from HVC to Area X is present in male zebra finches as young as 20 days of age, at the onset of vocal learning (Mooney and Rao, 1994). However, the HVC-to-RA projection is thought to undergo delayed development as HVC efferents are not visible within RA until after approximately 30 days of age (Konishi and Akutagawa, 1985; Mooney and Rao, 1994). Prior to this age, HVC axons appear to terminate in regions immediately surrounding RA and are later reorganized to ramify within the borders of RA by 30–35 days of age (Konishi and Akutagawa, 1985; Mooney and Rao, 1994).

In the present study, extremely small injections of fluorescent dextran amines or biocytin were made within specific regions of HVC and pHVC in adult and juvenile male zebra finches to (1) examine the precise pattern of neuronal connections of HVC and reexamine these pathways in young birds and (2) determine the afferent and efferent connections of pHVC and establish whether these connections are present at the onset of vocal learning.

MATERIALS AND METHODS

Extremely small injections of fluorescent dextran amines (tetramethylrhodamine [RDA] and fluorescein [FDA] dextran amines; 3,000 molecular weight; Molecular Probes, Eugene, OR]), or biocytin (Molecular Probes) were made into HVC and pHVC of adult (>90 days of age) and juvenile (20–23 days of age) male zebra finches. Injections into HVC were categorized as being located in medial, central, or lateral subregions of HVC based on their

TABLE 1. Injection Sites in Adult and Juvenile Male Birds

	Dextran Amines		Biocytin	
Injection site	Adults	Juveniles	Adults	Juveniles
Confined HVC[1]	14	0	2	0
HVC with spread[2]	14	6	3	2
Confined paraHVC[3]	2	0	2	1
ParaHVC with spread[4]	5	5	1	2
ParaHVC margin[5]	8	1	0	2
Control[6]	7	3	5	4
Total	50	15	13	11

[1]Injections confined within the Nissl-defined borders of the High Vocal Center (HVC).
[2]Injections with tracer spread outside the Nissl-defined borders of HVC.
[3]Injections located medial to HVC within 200 µm ventral or lateral to the margin of the lateral ventricle were considered to be within paraHVC.
[4]Injections into paraHVC with tracer spread more than 200 µm from the margin of the lateral ventricle.
[5]Injections at the margin of HVC and paraHVC.
[6]Injections within regions immediately surrounding HVC or paraHVC.

relative position within the Nissl-defined borders of the nucleus (cf. Fortune and Margoliash, 1995). Partial HVC injections that were completely confined to a specific subregion of HVC and that did not extend outside of Nissl-defined HVC allowed for examination of the precise pattern of its neuronal connections. Previously determined stereotaxic coordinates for HVC were modified to target injections within different medial-to-lateral subregions of HVC, and injections into pHVC were targeted 300 µm or 600 µm medial to HVC. Stereotaxic coordinates used to inject HVC and pHVC were similar for adults and juveniles.

Injections of fluorescent dextran amines

A total of 64 injections (32 of RDA and 32 of FDA) were made in 16 adult male zebra finches and 20 injections (14 of RDA and six of FDA) were made in seven juvenile male zebra finches. Adult birds received bilateral injections of both RDA and FDA targeted within immediately adjacent subregions (medial, central, or lateral) of HVC (n = 7 birds) or within subregions of HVC and pHVC (n = 8 birds). One adult bird received injections into HVC and pHVC on the left side of the brain and injections into adjacent subregions of HVC on the right side of the brain. Three juvenile birds received bilateral injections of RDA and FDA within HVC and pHVC and two juvenile birds received bilateral RDA injections into either HVC or pHVC. In addition, two juveniles received unilateral injections of RDA into HVC. Dextran amine injections were counterbalanced so that like tracers were not targeted to the same brain region bilaterally. For example, if an RDA injection was targeted to medial HVC on the left side of the brain, then an FDA injection was targeted to medial HVC on the right side of the brain.

Only those dextran amine injections that produced both anterograde and retrograde label were considered for analysis. Injections of RDA typically produced more reliable anterograde and retrograde label than did injections of FDA. Consequently, 10 FDA injections into adult birds and three FDA injections into juvenile birds were excluded from analysis. In addition, two FDA and two RDA injections into adult birds and two RDA injections into juvenile birds were excluded from analysis because tissue damage at the injection site interfered with determining the exact location of injections. Thus, 50 of a total of 64 dextran amine injections made into adult birds and 15 of a total of 20 injections made into juveniles were used for analysis. Table 1 lists the location of all tracer injections analyzed.

Prior to surgery, birds were anesthetized with Equithesin, a barbiturate anesthetic, and placed in a stereotaxic apparatus. Extremely small volumes of RDA or FDA (2–5 nl; 10% solution in 0.02 M phosphate buffered saline; PBS) were pressure injected by using a Picospritzer (pipette tip diameter = 24–30 µm; 35 psi, 1–2 pulses, 10-ms pulse duration). Following surgery, adult birds were placed individually in cages, and juveniles were returned to their home aviary to be cared for by their parents. Birds were overdosed with Equithesin 48 hours after surgery and perfused transcardially with saline followed by 10% buffered formalin. Brains were removed, postfixed in 10% buffered formalin for 24–48 hours, and then submerged in 25% buffered sucrose overnight. Each brain was frozen sectioned in the coronal plane (40 µm thickness) from the level of the rostral parolfactory lobe to the hypoglossal nucleus. Two series of sections were collected onto gelatin-coated slides; one series was coverslipped with buffered glycerol mountant and examined for fluorescent label, and the other was Nissl counterstained for identification of brain nuclei.

The borders of HVC are accurately defined in Nissl-stained tissue (Johnson and Bottjer, 1993; Bernard et al., 1993; Johnson and Bottjer, 1995; Bernard and Ball, 1995; Ball et al., 1995; Smith et al., 1997; cf. Gahr, 1990, 1997). The location of injection sites with respect to HVC boundaries was determined by making camera lucida drawings of HVC in Nissl-stained sections, and the fluorescent injection site in non-Nissl sections was traced onto these same drawings. Dextran amine injections confined within HVC (adults: n = 14) were analyzed separately from injections that included areas outside the Nissl borders of HVC (adults: n = 14; juveniles: n = 6) or control injections (adults: n = 7; juveniles: n = 3). Tracer injections into HVC of juvenile birds were concentrated within its Nissl-defined borders; however, all injections also contained a small amount of tracer ventral to HVC (Table 1).

Injections located medial to the Nissl-defined border of HVC that did not extend more than approximately 200 µm ventral or 200 µm lateral to the dorsomedial margin of the lateral ventricle at rostral and central levels HVC were considered as confined within pHVC (adults: n = 2). Analysis of the pattern of label from such injections was categorized separately from those targeted to pHVC, which spread ventrally into underlying cortex (adults: n = 5; juveniles: n = 5). Several injections were located in pHVC at the medial border of Nissl-defined HVC (adults: n = 8; juveniles: n = 1). It was difficult to determine the extent to which these injections may have included caudomedial portions of HVC, therefore, the pattern of label is summarized separately (Table 1).

Biocytin injections

A total of 13 biocytin injections (three unilateral, five bilateral) were made in eight adult male zebra finches, and 11 biocytin injections (one unilateral, five bilateral) were made in six juvenile male zebra finches. Biocytin injection sites were categorized similarly to dextran amine injections and are listed in Table 1.

For these injections, birds were anesthetized with Equithesin prior to surgery, placed in a stereotaxic apparatus, and injected iontophoretically with biocytin for 15–20 minutes (5% solution in 0.05 M Tris-HCl, pH 7.2–7.4; 5–6-µA current, pulsed 6 seconds on/off). Twenty-two to thirty hours following surgery, birds received an overdose

of Equithesin and were perfused transcardially with bird saline and chilled in 4% paraformaldehyde/0.4% glutaraldehyde (pH 7.4). Brains were removed, postfixed for 24 hours in 4% paraformaldehyde, and then immersed in 25% buffered sucrose overnight. Each brain was frozen sectioned at 30 μm from rostral parolfactory lobe to the hypoglossal nucleus caudally, and two series of sections were collected into free-floating wells. One series was mounted onto gelatin-coated slides and counterstained for Nissl substance, and the other series was processed by using an immunohistochemical reaction. For the reaction series, sections were rinsed with 0.02 M PBS and endogenous peroxidase activity was quenched by using a 1.0% H_2O_2 PBS solution. Sections were again rinsed in PBS, treated with 5% normal rabbit serum in 0.02 M PBS containing 0.3% Triton X-100 for 1 hour, and then left in goat anti-biotin (1:20,000; Vector Laboratories, Burlingame, CA) overnight at room temperature. The following day, sections were incubated in biotinylated anti-goat IgG (1:200 in 0.3% Triton X-100 in PBS) for 1 hour followed by a 1-hour incubation in avidin-biotin reagent (Vector Elite Standard Kit). For the peroxidase reaction, the concentration of H_2O_2 in the chromagen solution was increased incrementally to enhance the resolution of labeled fibers (cf. Bernard et al., 1993). Sections were first incubated in 0.05% 3,3′diaminobenzidine tetrahydrochloride (DAB) in PBS for 15 minutes. Sections were then treated in a solution of 0.003% H_2O_2 in 0.05% DAB-PBS for 5 minutes, followed by 0.015% H_2O_2 in 0.05% DAB-PBS until the reaction was complete (approximately 5–10 minutes). Sections were then given a series of final rinses, mounted onto gelatin-coated slides, and allowed to dry overnight. Slides were briefly submerged in a series of dehydrating alcohols, defatted in xylenes, and coverslipped with Permount. Alternate sections were mounted onto gelatin-coated slides, air dried for 24–48 hours, and counterstained with buffered thionin.

Birds were treated according to guidelines established by the Animal Care and Use Committee of the University of Southern California.

RESULTS

Adults

Confined HVC injections. Sixteen injections were localized within subregions of HVC and did not extend outside its Nissl-defined boundaries in adults (14 of dextran amine and two of biocytin). Of these injections, eight were localized to lateral HVC (seven of dextran amine and one of biocytin), four were within central HVC (three of dextran amine and one of biocytin), and four were located in medial HVC (all dextran amine injections).

Afferent input to HVC. All dextran amine injections confined within specific subregions of HVC produced an even distribution of retrogradely labeled cells in two cortical nuclei, mMAN and NIf, and the thalamic nucleus UVA (Fig. 1). The distribution of cells in mMAN extended from the lateral ventricle to the medial portion of the song control area $1MAN_{shell}$, a parvicellular region surrounding the magnocellular core of 1MAN (cf. Johnson et al., 1995; Fig. 1F). In addition, most dextran amine injections within lateral HVC (six of seven) and one of the four medial HVC injections produced retrogradely labeled cells in the medial portion of $1MAN_{shell}$. None of the injections confined to central HVC produced retrogradely labeled cells in any region of 1MAN. Retrogradely labeled cells with extensive dendritic labeling were observed throughout NIf (Fig. 1E). All injections confined within HVC produced an even distribution of retrogradely labeled cells throughout the ventromedial portion of Uva. Labeled cells were not visible within other regions of Uva (cf. Wild, 1994; Williams and Vicario, 1993; Fig. 1D). The even distribution of label within mMAN, NIf, and Uva after injections into different subregions of HVC, regardless of location within HVC, confirms reports that afferent input to HVC is not topographically organized (Fortune and Margoliash, 1995; Vates and Nottebohm 1995).

Efferent targets of HVC. The majority of dextran amine injections confined within HVC (13 of 14) produced anterograde label throughout Area X, although the intensity of label varied across brains. Only two of these 13 injections (both located in lateral HVC) produced intense fluorescent label in Area X, whereas the remaining 11 injections produced relatively dim label. Although frequently dim, highly branched labeled fibers were evenly distributed throughout Area X. Biocytin injections into HVC produced sparsely distributed fibers throughout Area X.

All HVC injections produced evenly distributed anterograde label throughout the Nissl-defined borders of RA (Fig. 1C). In addition, six of the seven injections into lateral HVC and all injections into HVC with spread ventral to HVC (n = 14) produced anterograde label in areas immediately rostral, rostroventral, and dorsal to RA (Fig. 2A,B). This distribution of label outside RA appears to correspond to the region previously defined as the "RA cup," an area that receives afferent input from regions immediately ventral to HVC and from auditory cortex (Kelley and Nottebohm, 1979; Vates et al., 1996). Descending HVC-to-RA fibers exited HVC from its caudal aspect, and the vast majority of fibers were fasciculated in fiber bundles (Fig. 3A; Nottebohm et al., 1982). Individual axons could be seen exiting fiber bundles as they descended into RA and branched extensively only when innervating the nucleus.

The even distribution of anterograde label within Area X and RA after injections localized to subregions of HVC is consistent with reports that HVC efferent projections are nontopographic (Fortune and Margoliash, 1995; Vates and Nottebohm, 1995). Although there was no evidence of a topographic projection from HVC to RA, labeled HVC-to-RA fibers descended into RA with a distinct mediolateral topography. Specifically, medial HVC injections labeled fibers that entered RA from its dorsomedial border and lateral HVC injections labeled fibers that entered RA from its dorsolateral border (Figs. 1C, 2C).

All dextran amine and biocytin injections confined within HVC also produced well-labeled fibers throughout HVC itself, indicating that subregions of HVC are highly interconnected (cf. Nottebohm et al., 1982; Figs. 1B, 4). Individually labeled processes could be traced within HVC extending away from the injection site and branching extensively throughout the Nissl-defined borders of the nucleus. Interestingly, injections confined to lateral HVC did not produce label within a small region at the dorsal border of HVC, immediately ventral to the lateral ventricle (Figs. 1B, 4C). This unlabeled area may define a region separate from HVC or a distinct subregion of HVC in close proximity to the lateral ventricle.

All injections of dextran amine and biocytin confined within medial and lateral HVC produced anterogradely

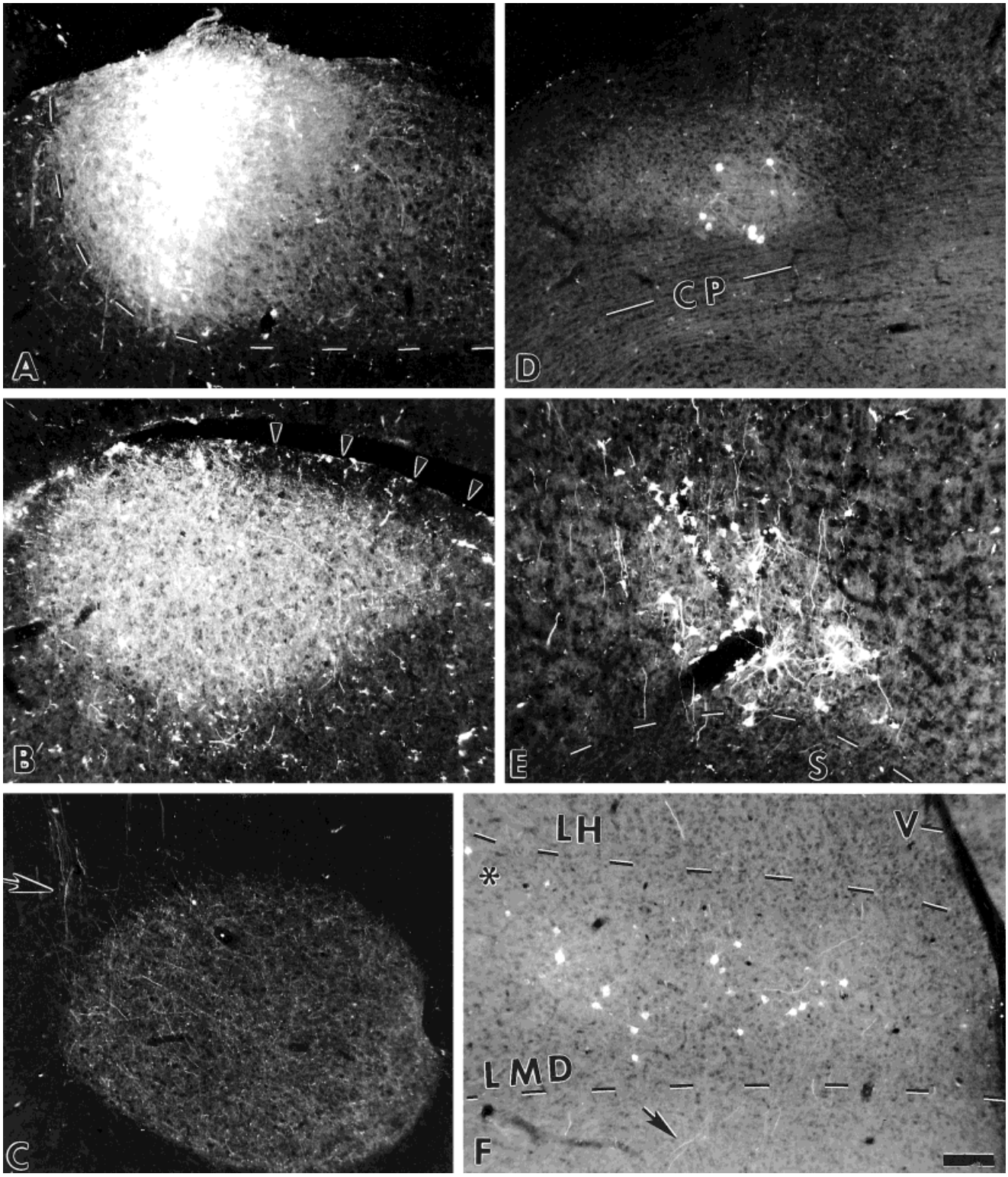

Fig. 1. Photomicrographs of an injection of rhodamine dextran amine in an adult male zebra finch confined in lateral High Vocal Center (HVC; **A**) and the resulting pattern of label (**B–F**). Dashed lines in A delineate the ventral border of HVC. B: Anterograde label in HVC rostral to the injection site. Note the absence of label within HVC just beneath the margin of the lateral ventricle (arrowheads). C: Anterograde label in the robust nucleus of the archistriatum (RA). The HVC-to-RA fibers are visible entering RA exclusively from its dorsolateral border (arrow). D: Retrograde label in the uvaetorm nucleus of the thalamus (Uva), located immediately dorsal to the posterior commissure (CP). E: Intense retrograde label throughout the interfacial nucleus (NIf), located immediately dorsal to the medial dorsal lamina (LMD) fiber tract (dashed lines). F: Retrograde label throughout the medial magnocellular nucleus of the anterior neostriatum (mMAN), located between hyperstriatal lamina (LH) and LMD fiber tracts and adjacent to the lateral ventricle (V). Injections into medial, central, or lateral subregions of HVC produced the same overall pattern of label, indicating that neuronal connections of HVC are not topographically organized. Medial is right; coronal plane. For abbreviations, see list. Scale bar = 115 µm.

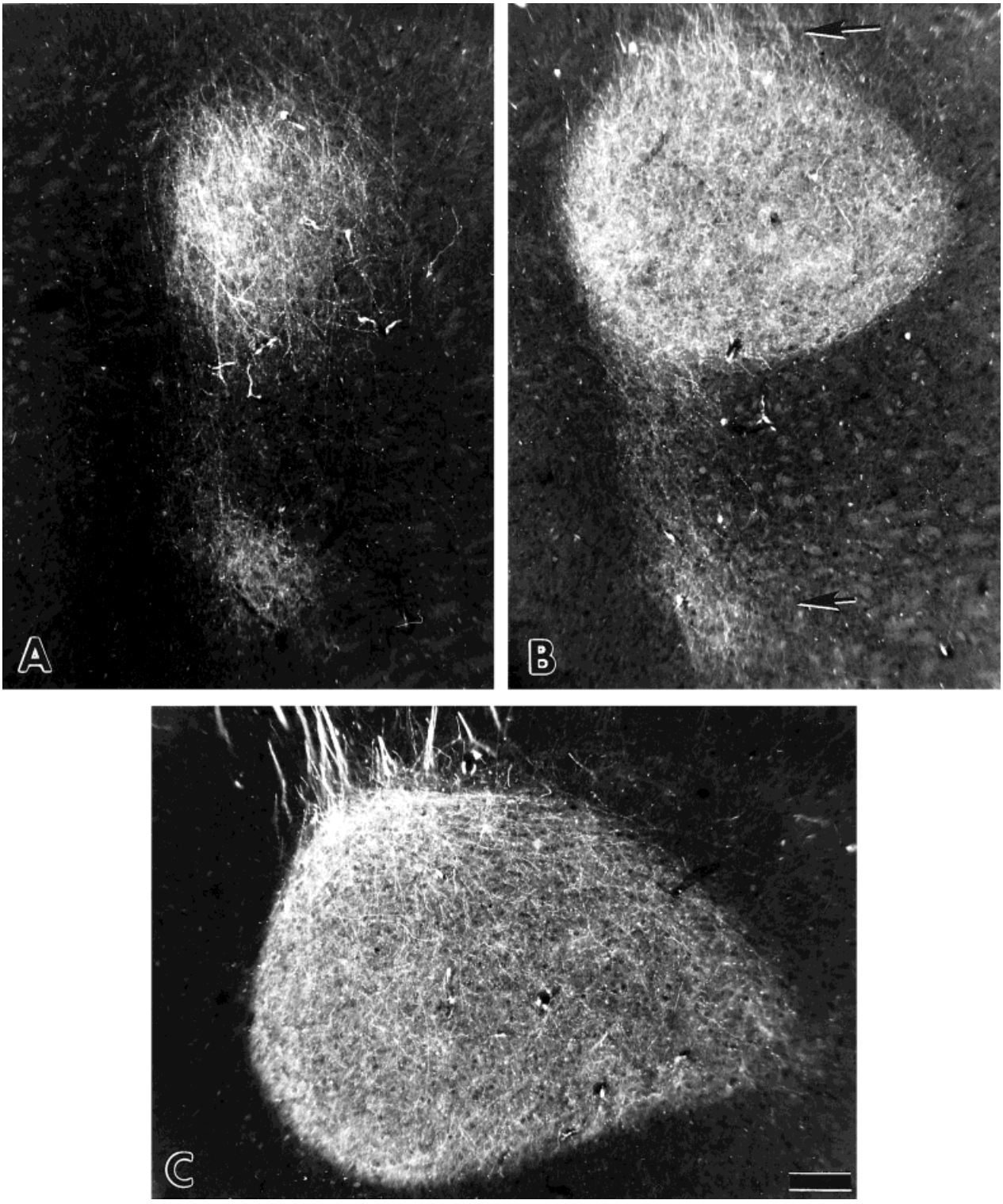

Fig. 2. Photomicrographs of anterograde label (**A**) rostral and ventral to the Nissl-defined borders of the robust nucleus of the archistriatum (RA), (**B**) within RA and dorsal and ventral to RA (arrows), and (**C**) throughout central RA after an injection into the medial High Vocal Center (HVC), which also contained a small amount of tracer spread ventromedial to HVC. Note that labeled fascicles of HVC axons descend into RA from its medial border (C). Medial is left; coronal plane. Scale bar = 115 μm.

labeled fibers in pHVC (Fig. 5). Label consisted of a thin band of fibers medial to HVC that coursed along the margin of the ventricle at the same rostrocaudal extent as HVC. Labeled fibers could be traced entering pHVC as they exited HVC medially except at a distinct region medial to rostral HVC, where there were no labeled fibers

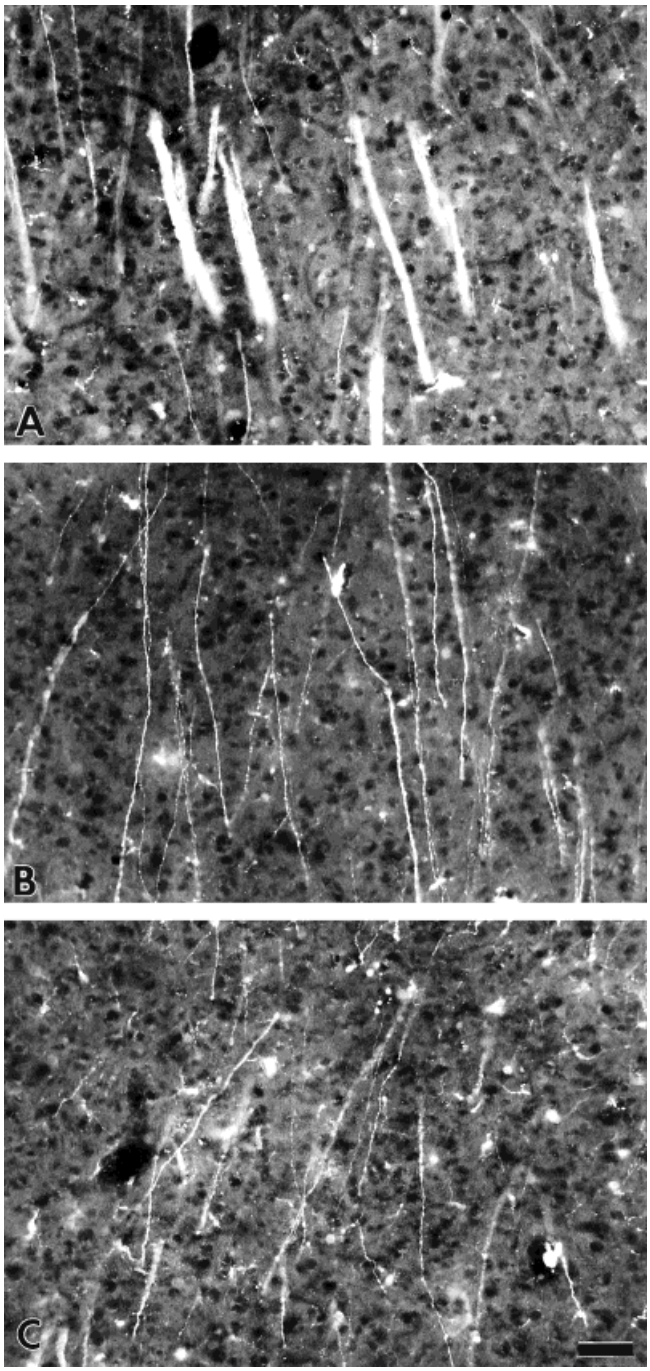

Fig. 3. Photomicrographs of labeled fibers from the High Vocal Center (HVC) to the robust nucleus of the archistriatum (RA) in adult (**A**) and juvenile (**B,C**) male zebra finches. The majority of labeled HVC-to-RA fibers are arranged in fasciculated bundles in adult birds but not in juveniles. Coronal plane. Scale bar = 100 μm.

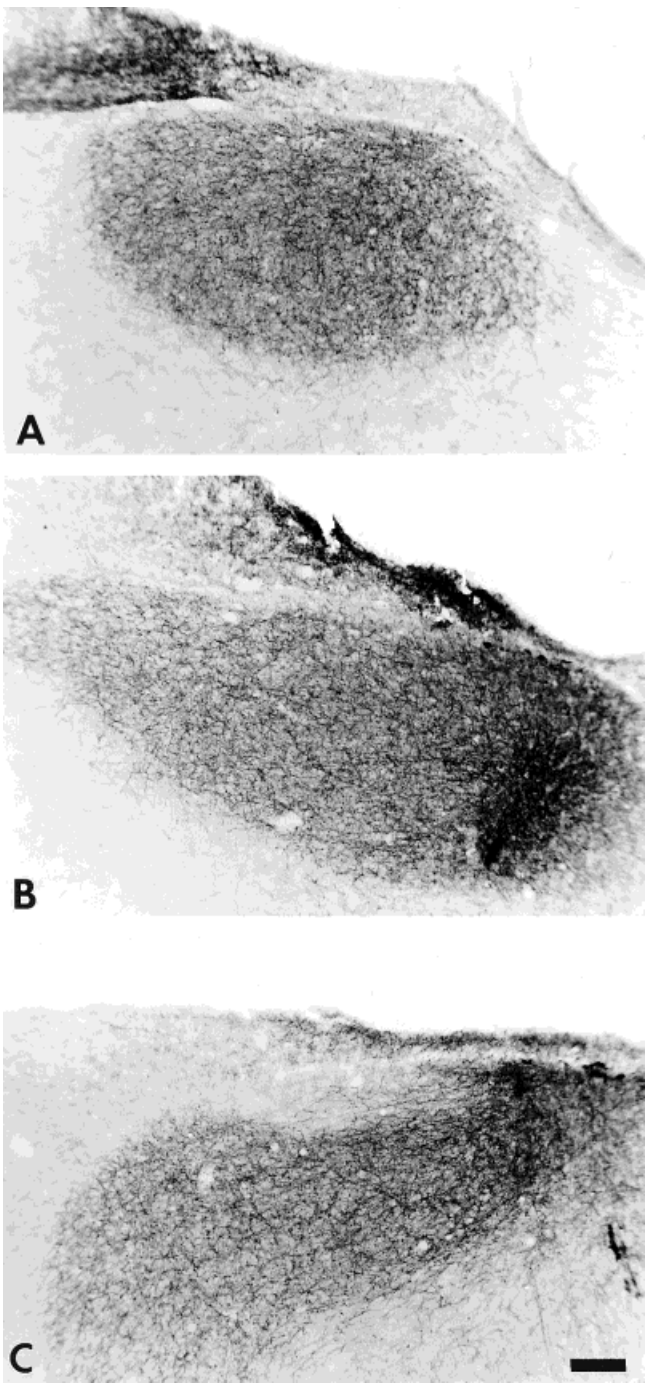

Fig. 4. Photomicrographs of an injection of biocytin into the lateral High Vocal Center (HVC; B,C) and the distribution of label throughout rostral (**A**), central (**B**), and caudal (**C**) regions of HVC, indicating that regions throughout HVC are highly interconnected in zebra finches. Note the relative absence of label in regions of dorsal HVC (C). This region was consistently unlabeled after injections localized to lateral HVC. Medial is left; coronal plane. Scale bar = 115 μm.

(Fig. 5A). This gap of HVC efferents to pHVC at rostral HVC corresponds with a gap of intense Nissl staining at the rostromedial margin of HVC (Fig. 6). This gap region appeared to be continuous with the cytoarchitectonically distinct region ventral to HVC that receives direct input from auditory cortex (the HVC shelf; Kelley and Nottebohm, 1979; Fortune and Margoliash, 1995; Vates et al., 1996), suggesting that the unlabeled gap corresponds to the rostromedial extent of the HVC shelf.

pHVC injections. Injections located medial to the Nissl-defined border of HVC that did not extend more than 200

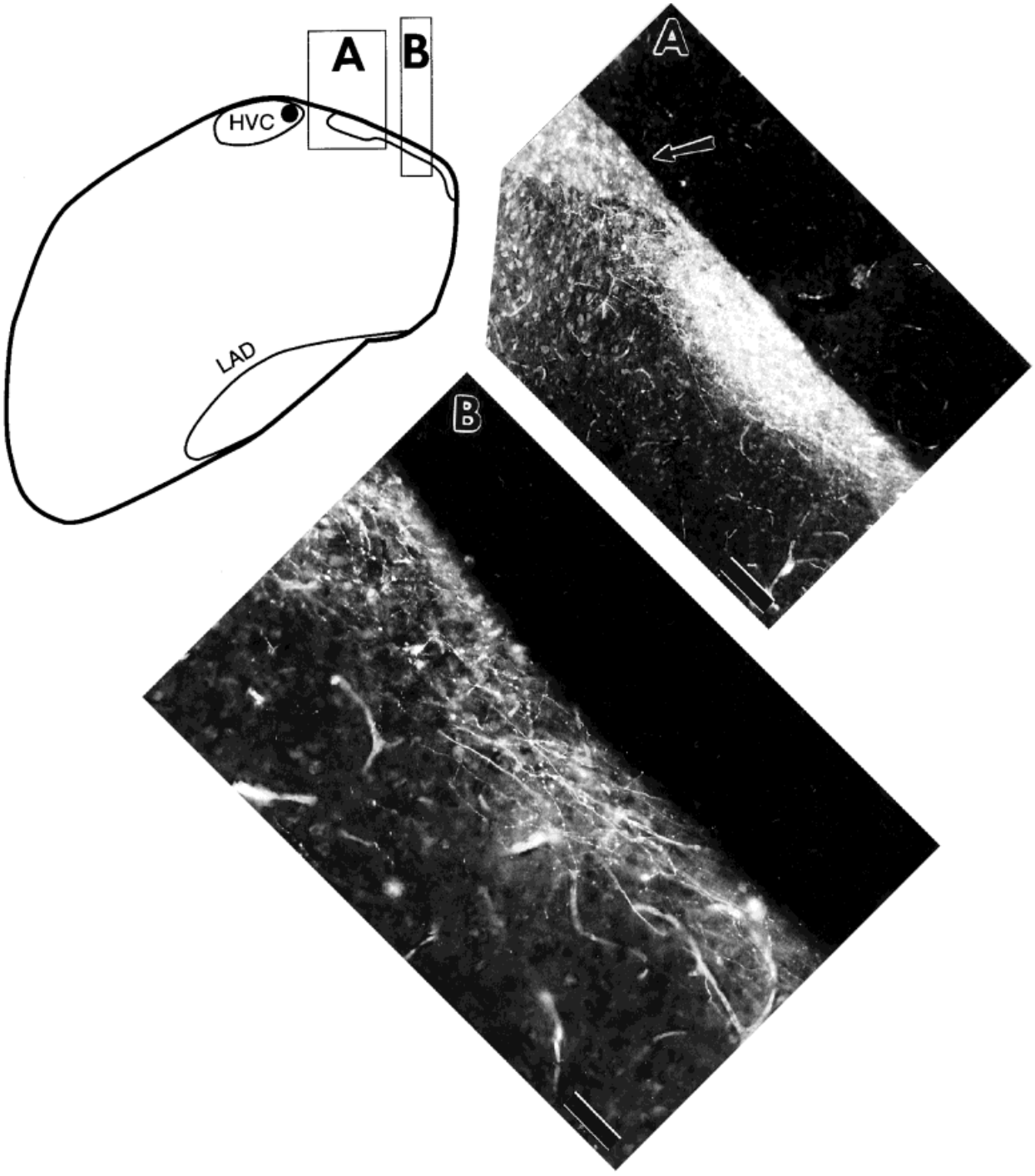

Fig. 5. Schematic illustration of an injection of dextran amines into the medial High Vocal Center (HVC) and location of anterograde label in pHVC. Insets **A** and **B** show the location of label shown in corresponding photomicrographs. A: Note the relative absence of label immediately medial to HVC (arrow). Medial is right; coronal plane. Scale bar = 115 μm in A, 100 μm in B.

μm ventral or 200 μm lateral to the margin of the lateral ventricle were considered to be confined within pHVC (two dextran amine and two biocytin; Figs. 7, 8). In addition, six injections (five dextran amine and one biocytin) encompassed pHVC and a small area ventral to pHVC and hippocampus dorsal to pHVC. Eight dextran amine injections were located in pHVC at the caudomedial border of Nissl-defined HVC. The pattern of label from the latter injections is summarized separately as injections may have included a small area of medial HVC (see below).

Afferent input to pHVC. All dextran amine injections confined within pHVC and injections with a small amount of spread outside pHVC produced a similar overall pattern

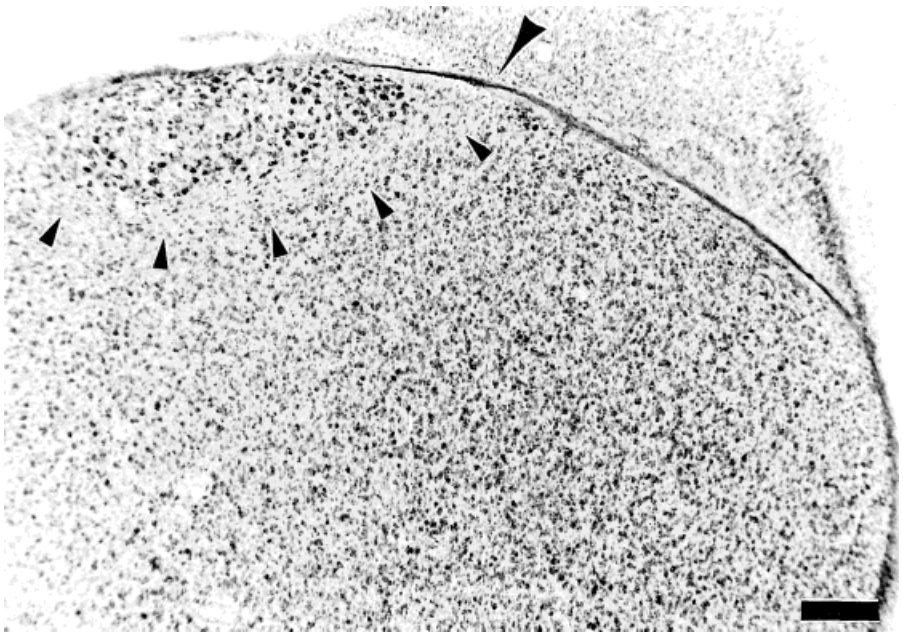

Fig. 6. Photomicrographs of the rostral High Vocal Center (HVC) in Nissl-counterstained tissue of an adult male zebra finch. Note that intense Nissl-staining is discontinuous between the medial border of HVC and the start of paraHVC (pHVC; large arrowhead) and appears similar to the definition of the auditory-recipient region along the ventral border of HVC ("HVC shelf"; small arrowheads). Injections into HVC produced anterograde label throughout pHVC except within this region immediately medial to rostral HVC (cf. Fig. 4A). Medial is right; coronal plane. Scale bar = 225 μm.

of retrograde label, although the amount and intensity of label varied across injections. All pHVC injections produced labeled cells throughout mMAN and in medial regions of $1MAN_{shell}$ (Fig. 7D). Medial injections produced fewer retrogradely labeled cells between mMAN and $1MAN_{shell}$ than did more lateral injections (i.e., those closer to medial HVC). In addition, four injections (two confined and two injections with ventral spread) produced sparsely distributed labeled cells in rostromedial cortex caudal to mMAN between the medial dorsal lamina (LMD) and the hyperstriatal lamina (LH). The distribution of labeled cells from the latter injections extended caudally to the level of NIf.

Although a small number of intensely labeled cells were visible in NIf after injections into pHVC, label was substantially less than that produced by similarly sized injections confined within HVC (Fig. 7B). A few retrogradely labeled cells were also visible within the ventromedial portion of Uva and immediately surrounding regions (Fig. 7C). Because so few labeled cells were visible in NIf and Uva, it was difficult to determine precisely whether these projections to pHVC are topographically organized. A small number of intensely labeled cells were visible throughout HVC after pHVC injections, indicating that HVC sends a direct projection to pHVC (Fig. 7E). In addition, faintly labeled cells were sparsely distributed within dorsomedial regions of NCM immediately ventral to pHVC.

Efferent targets of pHVC. Injections confined to pHVC and the majority of injections that included regions ventral to pHVC produced anterogradely labeled fibers throughout Area X (Fig. 8A). Anterograde label in Area X confirms previous retrograde studies demonstrating a projection from pHVC to Area X (Nordeen et al., 1987; Johnson and Bottjer, 1995). Labeled fibers in Area X were sparsely distributed and frequently faint, likely the result of extremely small injections that only partially filled pHVC. Although label was visible throughout Area X, label was consistently more prominent in the dorsomedial portion of the nucleus.

The pHVC injections produced anterograde label in caudomedial cortex from the ventral margin of HVC to the dorsal archistriatal lamina (LAD) at the same rostrocaudal extent as HVC (Figs. 8, 9). At the level of rostral HVC, labeled fibers were visible leaving the injection site in pHVC and extending to regions ventral to HVC. A very few fine labeled processes were located within the Nissl-defined borders of caudal HVC, but most labeled fibers were distributed ventral and medial to HVC. The presence of only a few, lightly labeled fibers within caudal HVC indicates that pHVC does not send a substantial projection to HVC. Rather, pHVC efferents were located in regions ventral to HVC. This distribution of label clearly overlaps dorsal and caudal aspects of the NCM, a cortical area that demonstrates immediate early gene induction and complex changes in neuronal firing rate in response to song stimuli (Mello et al., 1992, 1995; Mello and Clayton, 1994; Nastiuk et al., 1994; Chew et al., 1995, 1996; Jarvis and Nottebohm, 1997). It is difficult to determine the precise correspondence of pHVC efferent label within NCM because this area is not well delineated in Nissl-stained tissue.

Injections produced well-labeled fibers throughout pHVC, indicating that neurons within pHVC are interconnected. Interestingly, label extended throughout pHVC except at a small region medial to rostral HVC. Fiber label within pHVC ended abruptly near the rostromedial margin of HVC, leaving an unlabeled gap at this level. Anterograde label was absent from this same region corresponding to

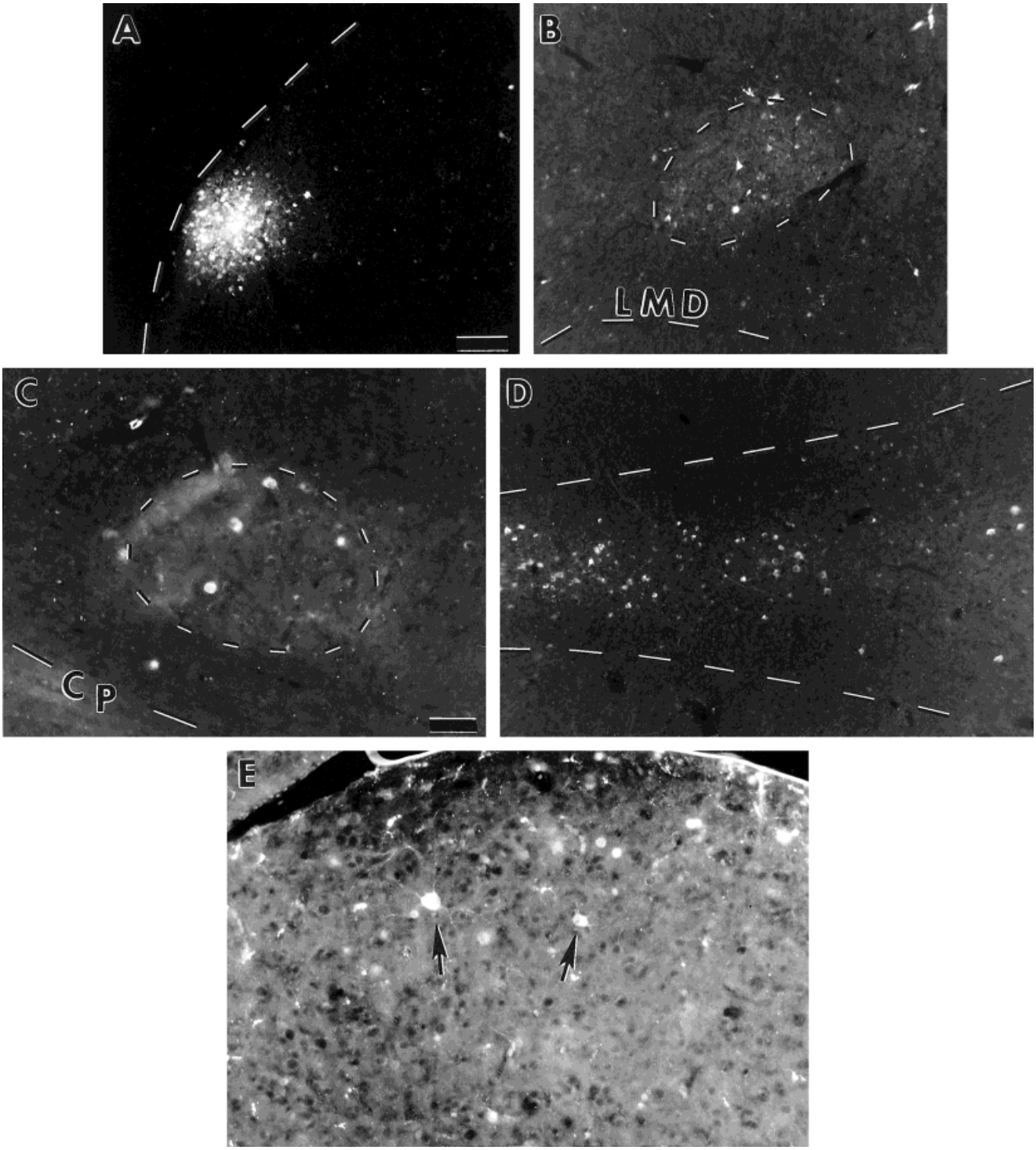

Fig. 7. Photomicrograph of an injection of rhodamine dextran amine confined within the para-high vocal center (pHVC) of an adult male zebra finch (**A**) and the distribution of retrograde label in interfacial nucleus (NIf; **B**), uvaeform nucleus (Uva; **C**), medial magnocellular nucleus of the anterior neostriatum (mMAN; **D**), and HVC (**E**). Dashed lines delineate the dorsomedial margin of the telencephalon in A and the borders of NIf in B and Uva in C. Dashed lines in D delineate the hyperstriatal lamina (dorsal) and the medial dorsal lamina (LMD; ventral) fiber tracts. E: Intensely filled cells were located within HVC (arrows), indicating that cells within HVC project to pHVC. CP, posterior commissure. Medial is left; coronal plane. Scale bars = 100 μm for A,B,D, 115 μm for C,E.

the medial extent of the HVC shelf after HVC injections (see above).

All confined pHVC injections and those that included regions ventral to pHVC produced a thin band of faint anterograde label that extended along the dorsomedial border of RA but did not penetrate within the Nissl-defined borders of RA (Fig. 8F). Although confined pHVC injections did not produce anterograde label within RA, four of the

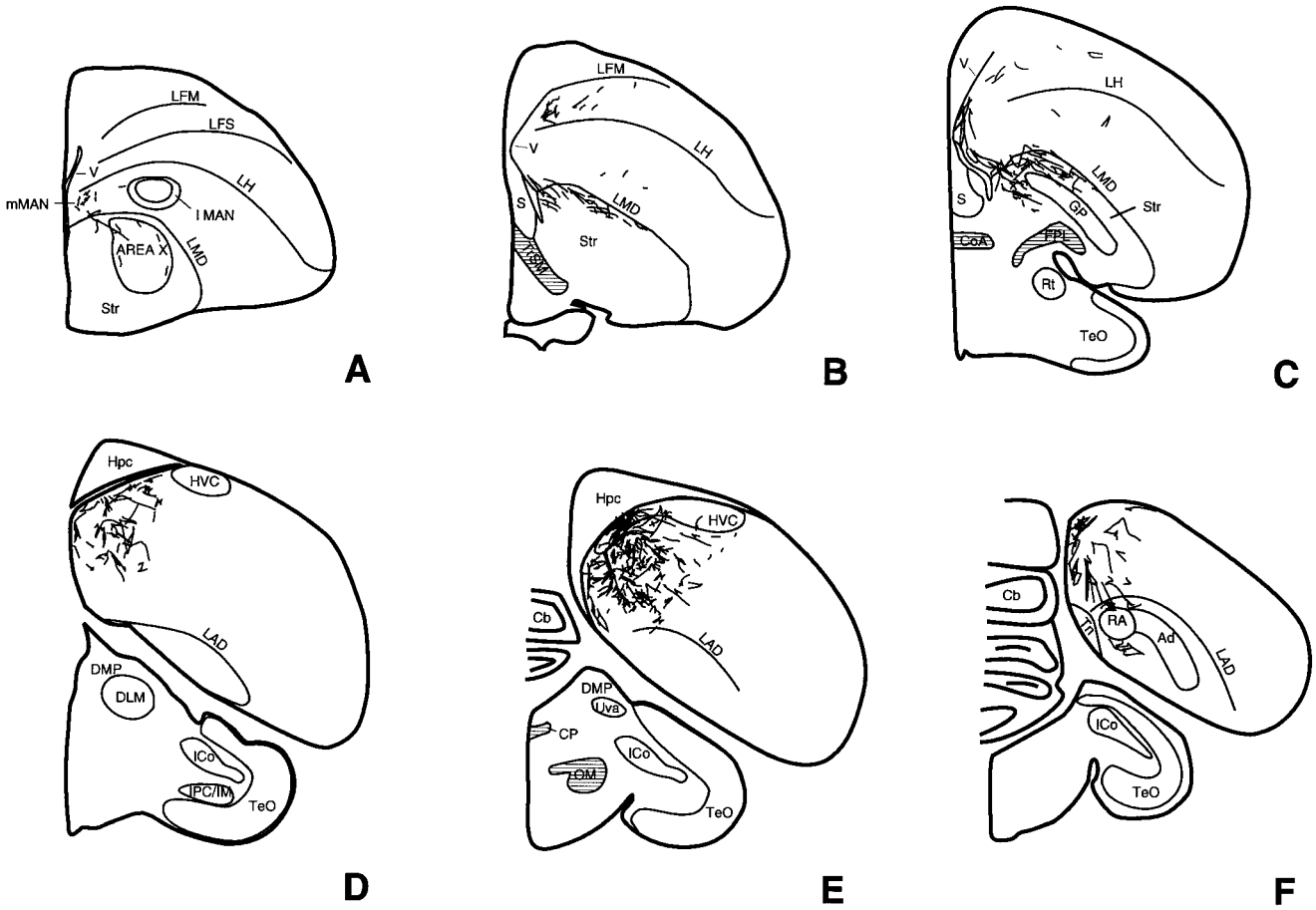

Fig. 8. **A–F:** Schematic illustrations of anterograde label after an injection of biocytin confined within para-High Vocal Center (pHVC) of an adult male zebra finch. The injection site was located along the dorsal margin of the telencephalon and did not extend ventrally more than 200 µm into underlying cortex (E). Anterograde label was visible in area X (A), medial magnocellular nucleus of the anterior neostriatum (mMAN; A), dorsomedial striatum (B,C), and throughout medial regions of the dorsocaudal cortex (D–F). Label in caudal cortex at least partially overlaps with the auditory responsive region called the caudomedial neostriatum (NCM). Anterograde label was not prominent in HVC, indicating that pHVC sends a major projection to regions immediately outside of HVC but not to HVC proper. A to F, rostral to caudal. Medial is left; coronal plane. For abbreviations, see list.

five dextran amine injections that spread caudoventral to pHVC produced faint anterograde label within the Nissl-defined borders of RA, likely due to spread of tracer into HVC-to-RA fibers of passage.

All biocytin injections into pHVC produced a distinct pattern of labeled fibers concentrated within dorsal regions of medial striatum (parolfactory lobe [LPO] in birds) and dorsomedial regions of lateral striatum at the level of the anterior commissure (accessory paleostriatum [PA] in birds). The distribution of anterograde label excluded globus pallidus (Fig. 8B,C). Striatal label extended from the level caudal to Area X to the level of the anterior commissure, coexistent with the rostrocaudal extent of the septum. Labeled processes extended ventrally from the injection site along the margin of the lateral ventricle to the level of the anterior commissure, where they entered the striatum.

All pHVC injections with spread into overlying hippocampus produced bilateral label in fornix and septum. This pattern of label was not seen after any injection confined within pHVC, and the distribution of label was identical to that produced by control injections confined within hippocampus (see below). In addition, two of the five dextran amine injections into pHVC with spread into overlying hippocampus also produced dim anterograde label within nucleus taeniae (Tn), located medial to rostral RA.

Injections into the margin of HVC and pHVC. Eight dextran amine injections were located along the margin of medial HVC and pHVC and also contained a small amount of tracer spread ventral to medial HVC (Fig. 10). All of these injections produced well-labeled somata within mMAN, NIf, and Uva. Six of the eight injections also labeled cells distributed in regions of medial cortex at the same rostrocaudal level as NIf, presumably in medial regions of Field L. Four injections produced faint anterograde label within Area X, and the remaining four injections did not produce any detectable label in Area X. Seven injections produced faint anterograde label within RA.

Fig. 9. Brightfield photomicrographs of an injection of biocytin confined within the para-High Vocal Center (pHVC) of an adult male zebra finch (**A**) and the distribution of label within regions of the caudomedial portion of neostriatum in darkfield illumination (**B**). Dashed lines in A define the ventral and medial borders of HVC. Medial is left; coronal plane. Scale bars = 225 µm for A, 115 µm in B.

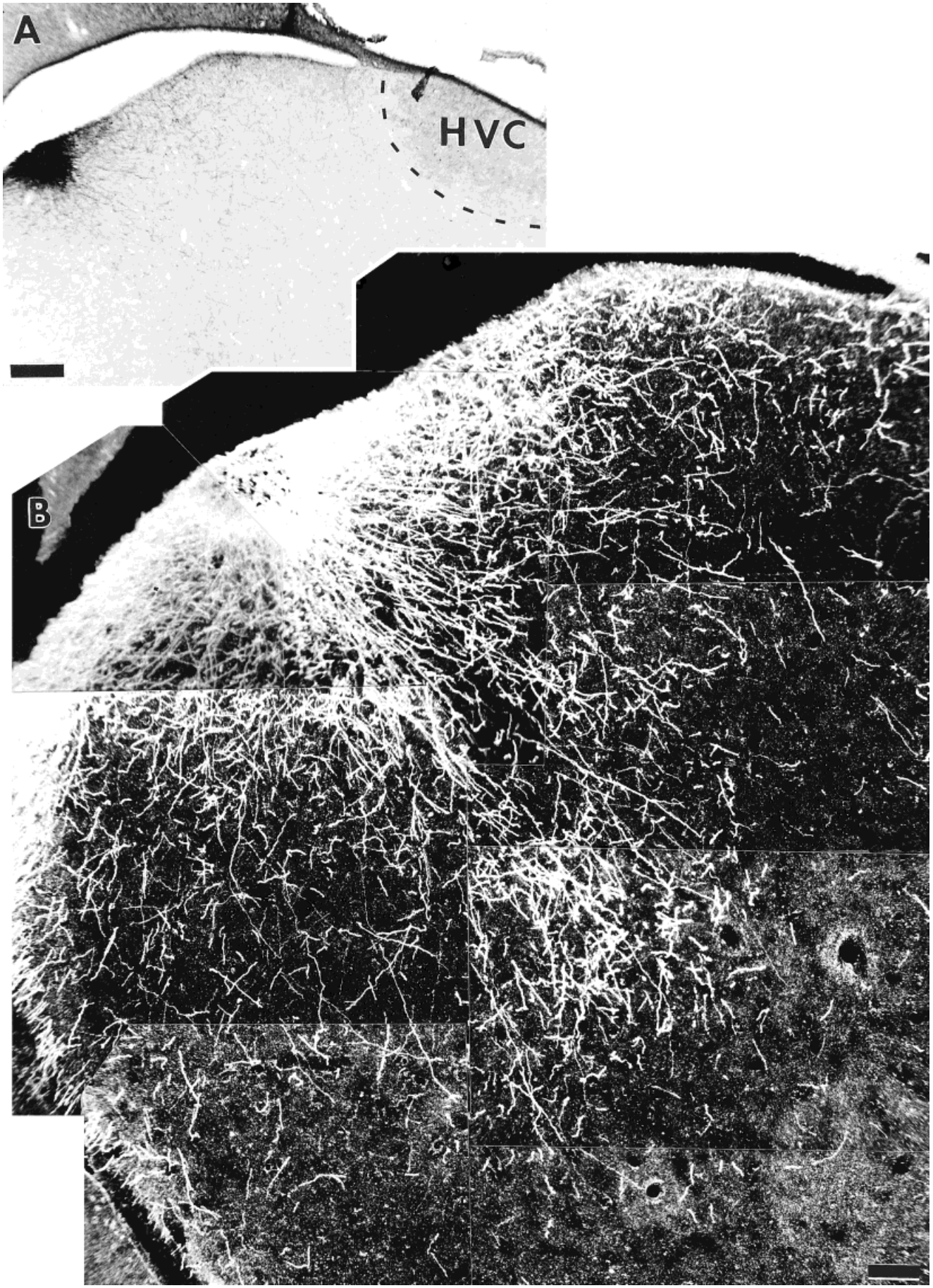

Figure 9

Faintly labeled fibers were also visible within the caudomedial cortex ventral to HVC and pHVC, similar to the pattern of label within regions of NCM following pHVC injections.

Control injections. A total of 12 control injections were made into regions surrounding HVC (seven of dextran amine and five of biocytin). Dextran amine injections were located lateral to HVC (n = 3), ventrolateral to HVC (n = 1), ventromedial to HVC (n = 1), and caudal to HVC (n = 2). Control injections of biocytin were located ventromedial to HVC (n = 1) and within medial hippocampus dorsal to HVC (n = 4).

Control injections of dextran amines located lateral and ventrolateral to HVC (n = 4) produced retrogradely labeled cells in mMAN, NIf, and Uva (Fig. 11). This pattern of label is likely the result of tracer entering fibers of passage from these nuclei as they approach HVC from its ventrolateral border (Wild, 1994; Fortune and Margoliash, 1995; Vates et al., 1996; Foster et al., 1997). Lateral control injections also produced retrogradely labeled cells in the medial portion of $1MAN_{shell}$. Recent tract tracing experiments have indicated that medial regions of $1MAN_{shell}$ project to regions lateral to HVC (Brady and Bottjer, unpublished data), which may account for the presence of labeled cells in $1MAN_{shell}$. Label may also be the result of hitting the $1MAN_{shell}$-to-dorsal archistriatum (Ad) fibers of passage, which travel lateral to HVC before descending ventrally to the archistriatum (Johnson et al., 1995). Lateral and ventrolateral control injections produced retrograde label dorsolateral to NIf, presumably within regions of Field L. The control injection located ventromedial to HVC produced retrogradely labeled cells concentrated dorsomedial to NIf, presumably in medial regions of Field L.

Control dextran amine injections located lateral and ventrolateral to HVC produced anterograde label in areas immediately rostral-lateral, rostroventral, and dorsal-lateral to RA, presumably within lateral areas of RA cup, and within regions dorsal to medial Ad (Fig. 11D), the area lateral to RA that receives input from $1MAN_{shell}$ (Johnson et al., 1995; Fig. 11E). The control injection of biocytin located ventromedial to rostral HVC produced dense anterograde label exclusively dorsomedial to RA, presumably within medial regions of RA cup. Thus, injections placed at different mediolateral coordinates outside of HVC produced anterograde label immediately outside of RA and medial Ad, corresponding to areas defined as RA cup, which was organized with a distinct medial-to-lateral pattern.

Biocytin and dextran amine injections restricted to the hippocampus produced intensely labeled fibers within the fornix that terminated in the lateral septum. Labeled fibers could also be traced from the fornix to the pallial commissure and into contralateral hippocampus.

Juveniles

HVC injections. Six dextran amine injections were concentrated within medial (n = 1), central (n = 4), or lateral (n = 1) subregions of HVC in juvenile male birds 20–23 days of age. Although one injection was localized to lateral HVC, medial and central HVC injections were not restricted solely to these subregions as intense fluorescence was visible throughout the nucleus. All injections also included a small amount of tracer spread ventral to the Nissl-defined borders of HVC. In general, injections into HVC of juvenile birds were not as localized to specific subregions of HVC or restricted within the Nissl-defined borders of HVC compared with similarly sized injections in adults, an unavoidable difference due to the small size of HVC in young birds (Bottjer et al., 1985; Nordeen and Nordeen, 1988a).

Afferent input to HVC. Retrogradely labeled cells were distributed throughout mMAN, NIf, and Uva after all HVC injections in juveniles (Fig. 12). In addition, four of the six injections produced labeled cells within medial regions of $1MAN_{shell}$, and the lateral HVC injection also produced labeled cells in the dorsomedial portion of $1MAN_{core}$. The presence of label in mMAN, NIf, and Uva is consistent with a previous report suggesting that HVC afferent inputs are present in 20-day-old male zebra finches (Mooney and Rao, 1994).

Efferent targets of HVC. All dextran amine and biocytin injections in juveniles produced anterograde label within the Nissl-defined borders of RA and immediately dorsal to RA (Fig. 13). Although the intensity of fluorescent label within RA was less than that in adult birds, anterograde label was visible throughout the nucleus. Individual HVC-to-RA fibers were not fasciculated in bundles as in adults (Fig. 3B,C) and remained unbranched until reaching the level of dorsal RA, where they branched extensively when entering the nucleus. All juvenile HVC injections also produced faint anterograde label rostral, rostroventral and dorsal to rostral RA. Individually labeled axons entering areas surrounding RA could be traced passing through medial regions of RA before reaching their final targets. This pattern of label surrounding RA is similar to the distribution of label in RA cup produced by injections that spread outside HVC and the majority of injections restricted to lateral HVC in adults.

Injections into HVC produced an even distribution of highly branched labeled fibers throughout Area X. Similar to the pattern of label produced by HVC injections in adult birds, Area X label in juvenile birds was evenly distributed throughout the nucleus and was frequently dim. Injections concentrated within central HVC produced dim to moderately intense fluorescent label in pHVC, whereas the injection into lateral HVC produced intense fluorescent label in pHVC. The pHVC was not anterogradely labeled after the medial HVC injection. Similar to the pattern of label seen in pHVC of adults, anterograde label in pHVC of juveniles was continuous along the margin of the lateral ventricle at the same rostrocaudal extent as HVC except at a region medial to rostral HVC (see above). This gap of HVC-to-pHVC efferents at the level of rostral HVC also corresponds with a gap in intense Nissl-staining at the level of rostral HVC in juvenile birds.

Control injections. Control injections of dextran amines in juveniles were located lateral to caudal HVC (n = 2) and ventral to rostral HVC (n = 1). These injections produced retrogradely labeled cells in mMAN, Uva, NIf, medial regions of $1MAN_{shell}$, and regions of cortex at the same rostrocaudal level as NIf. Overall, there were fewer labeled cells in each of these areas compared with the amount of label produced by injections concentrated within HVC. As described above, label in mMAN, NIf, and Uva from control injections outside HVC was likely caused by uptake of tracer by fibers of passage from these nuclei, which enter HVC at its ventrolateral border.

Control injections produced anterograde label immediately rostral, rostroventral, and dorsal to the Nissl-defined

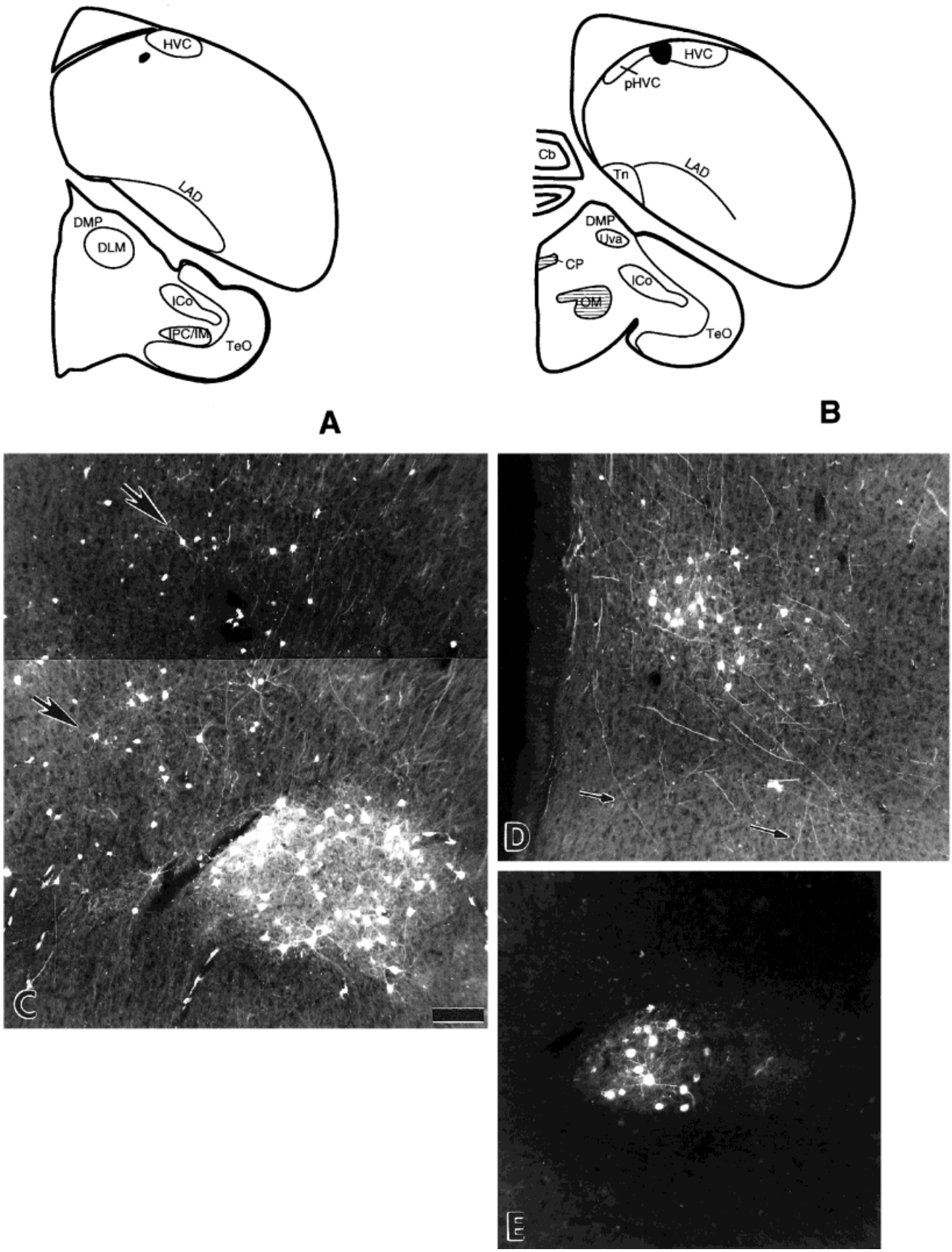

Fig. 10. Schematic illustrations of an injection of rhodamine dextran amine at the margin of the High Vocal Center (HVC) and paraHVC (pHVC) with a small amount of tracer spread ventromedial to HVC in an adult male zebra finch (**A,B**), and the resulting pattern of retrograde label in interfacial nucleus (NIf) and Field L (**C**), medial magnocellular nucleus of the anterior neostriatum (mMAN; **D**) and uvaeform nucleus (Uva; **E**). Label in Field L is concentrated in regions dorsal and medial to NIf (C; arrows). Arrows in D indicate Area X-projecting axons. Medial is left; coronal plane. Scale bar = 115 µm.

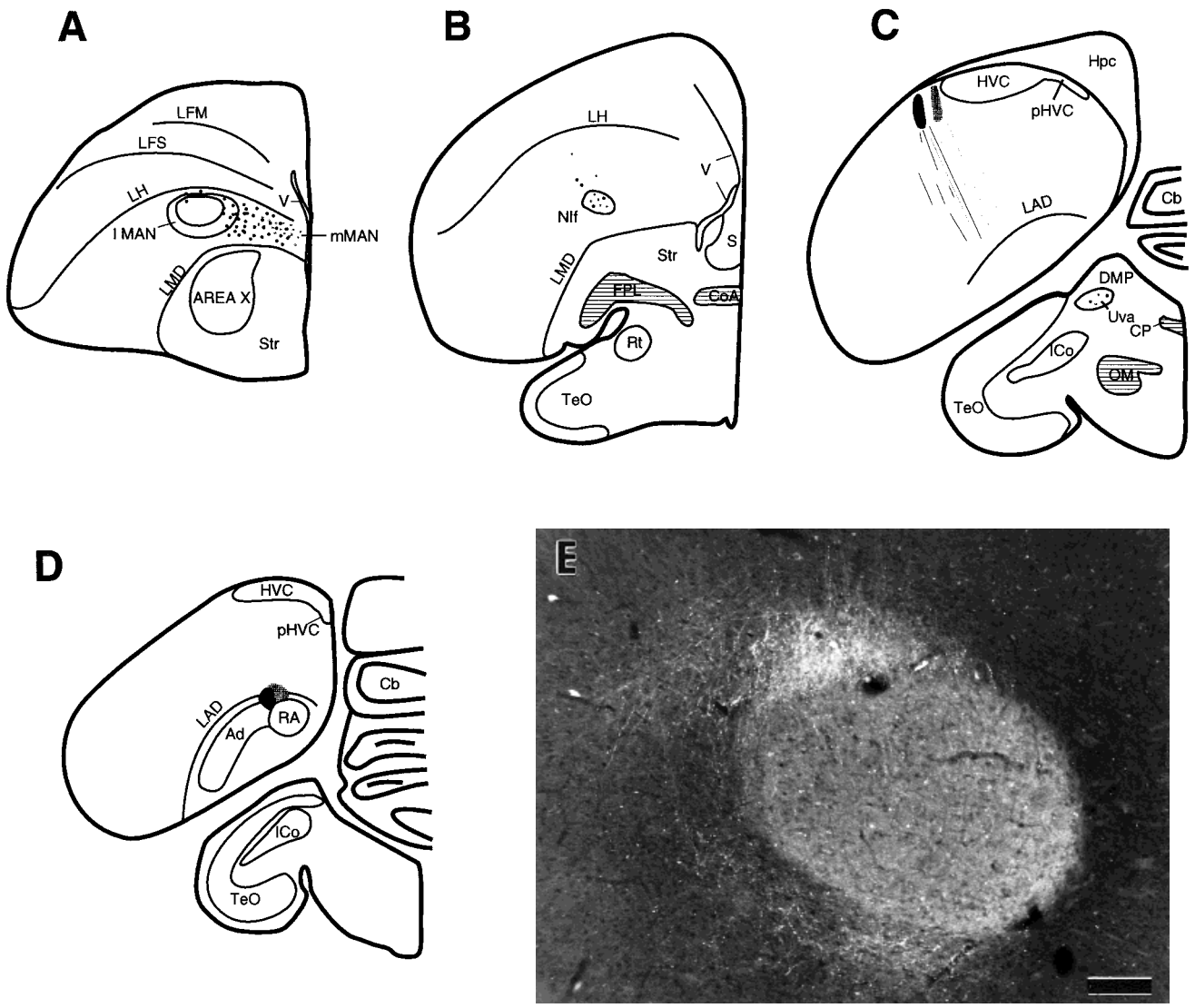

Fig. 11. **A–D:** Schematic illustrations of control injections of rhodamine dextran amine (black) and fluorescein dextran amine (gray) injections lateral to the High Vocal Center (HVC) in an adult male zebra finch and the resulting pattern of label. **E:** Photomicrograph showing anterograde label dorsolateral to robust nucleus of the archistriatom (RA) and immediately ventral to RA. Labeled fibers descended to these regions from the dorsolateral aspect of RA. A–E: Rostral to caudal. Medial is right; coronal plane. For abbreviations, see list. Scale bar = 115 μm.

borders of RA (Fig. 14). Occasionally, individually labeled, unbranched fibers were seen within RA, whose trajectory could be traced to regions ventral to RA. Anterograde label immediately surrounding RA after injections that did not encompass any portion of HVC indicates that in juveniles, as in adults, regions outside of HVC do not project to neurons within the Nissl-defined borders of RA. Label surrounding RA in juveniles corresponds to an area defined as the "waiting compartment" immediately outside of RA, which as been reported to receive input from HVC before rearrangement and growth of HVC axons into RA (Konishi and Akutagawa, 1985; Mooney and Rao, 1994). However, label surrounding RA corresponds to a similar distribution of label after control injections in adult birds, demonstrating that areas surrounding HVC send specific projections to areas surrounding RA in both juveniles and adults.

pHVC injections. A total of five dextran amine and three biocytin injections were made into pHVC of juvenile male zebra finches. Although pHVC injections were extremely small, it was difficult to prevent tracer injections from spreading into regions ventral or dorsal to pHVC. Thus, each of the five dextran amine injections and two of the biocytin injections into pHVC of juvenile birds also contained a small amount of tracer within cortex ventral to pHVC and hippocampus overlying pHVC. One dextran amine injection and two biocytin injections were located at the margin of HVC and pHVC in juveniles.

The incidence of retrograde label after dextran amine injections into pHVC in juvenile birds was highly variable. Of the five total injections made, two produced labeled cells in mMAN, three produced very few, faintly labeled cells in NIf, two produced well-labeled cells in Uva and one produced faintly labeled cells in Field L. Most injections

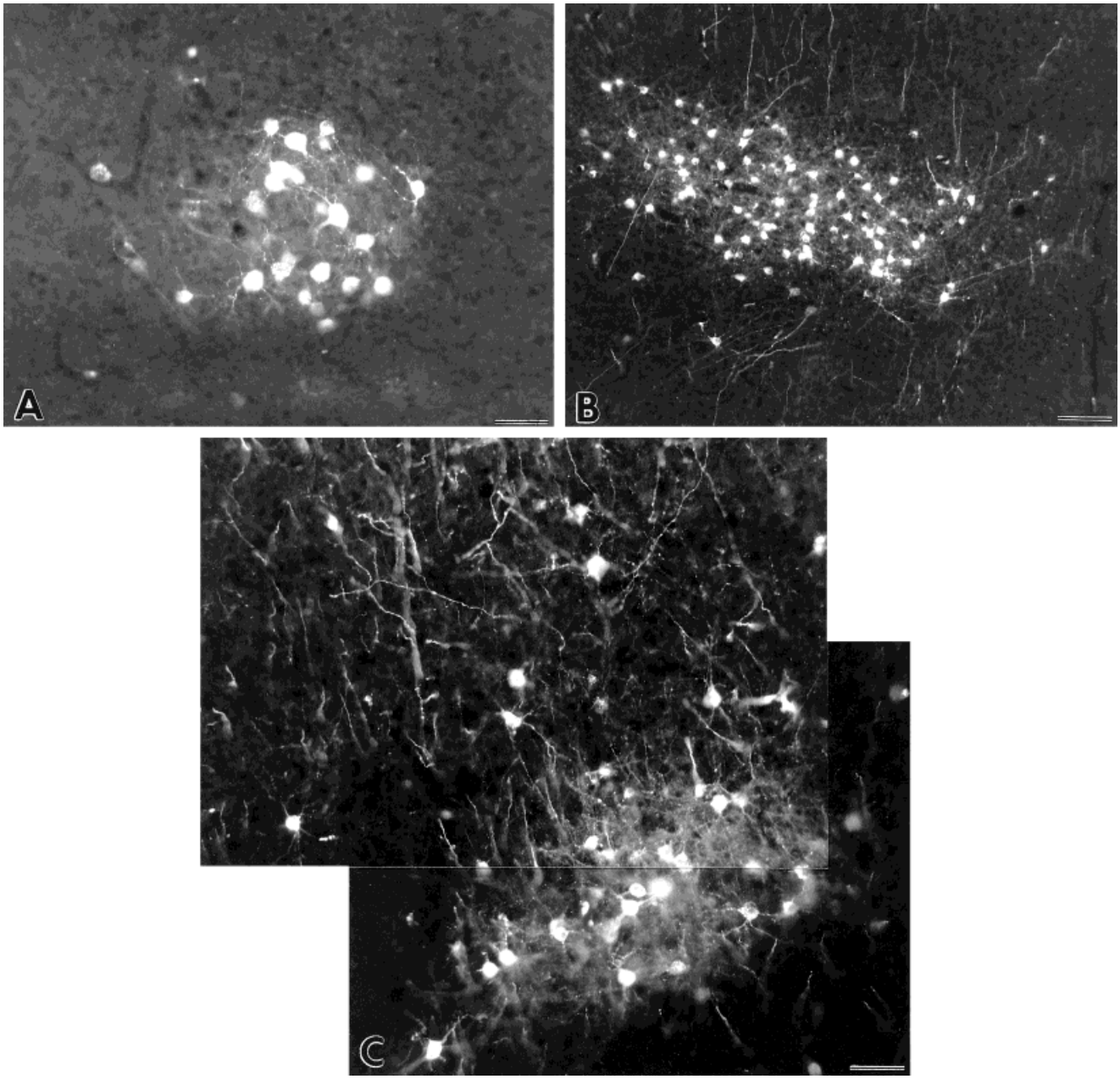

Fig. 12. Photomicrographs of retrogradely labeled cells in the uvaeform nucleus (Uva; **A**), the medial magnocellular nucleus of the anterior neostriatum (mMAN; **B**), interfacial nucleus (NIf) and regions around NIf (**C**) from an injection into the lateral High Vocal Center of a juvenile male zebra finch (23 days of age). Medial is right; coronal plane. Scale bars = 100 μm for A,C, 115 μm in B.

(four of the five) also produced faintly labeled cells within dorsomedial regions of the cortex, within regions ventral to pHVC.

Three of five injections of dextran amines into pHVC produced faint anterograde label in Area X, regions along the dorsomedial border of RA, or within the central region of Tn. However, none of these three injections produced label in all of these areas. Each of the three biocytin injections into pHVC produced the same distribution of label within regions of NCM and the dorsomedial portion of lateral striatum and Area X as seen in adult birds.

DISCUSSION

HVC injections

Injections localized to medial, central, or lateral HVC, confined strictly within the Nissl-defined borders of the nucleus of adult male zebra finches, produced an even distribution of retrograde label in mMAN, NIf, and Uva and an even pattern of anterograde label within the Nissl-defined borders of RA and Area X. These results demonstrate that neither the afferent nor the efferent connections of HVC are topographically organized and in

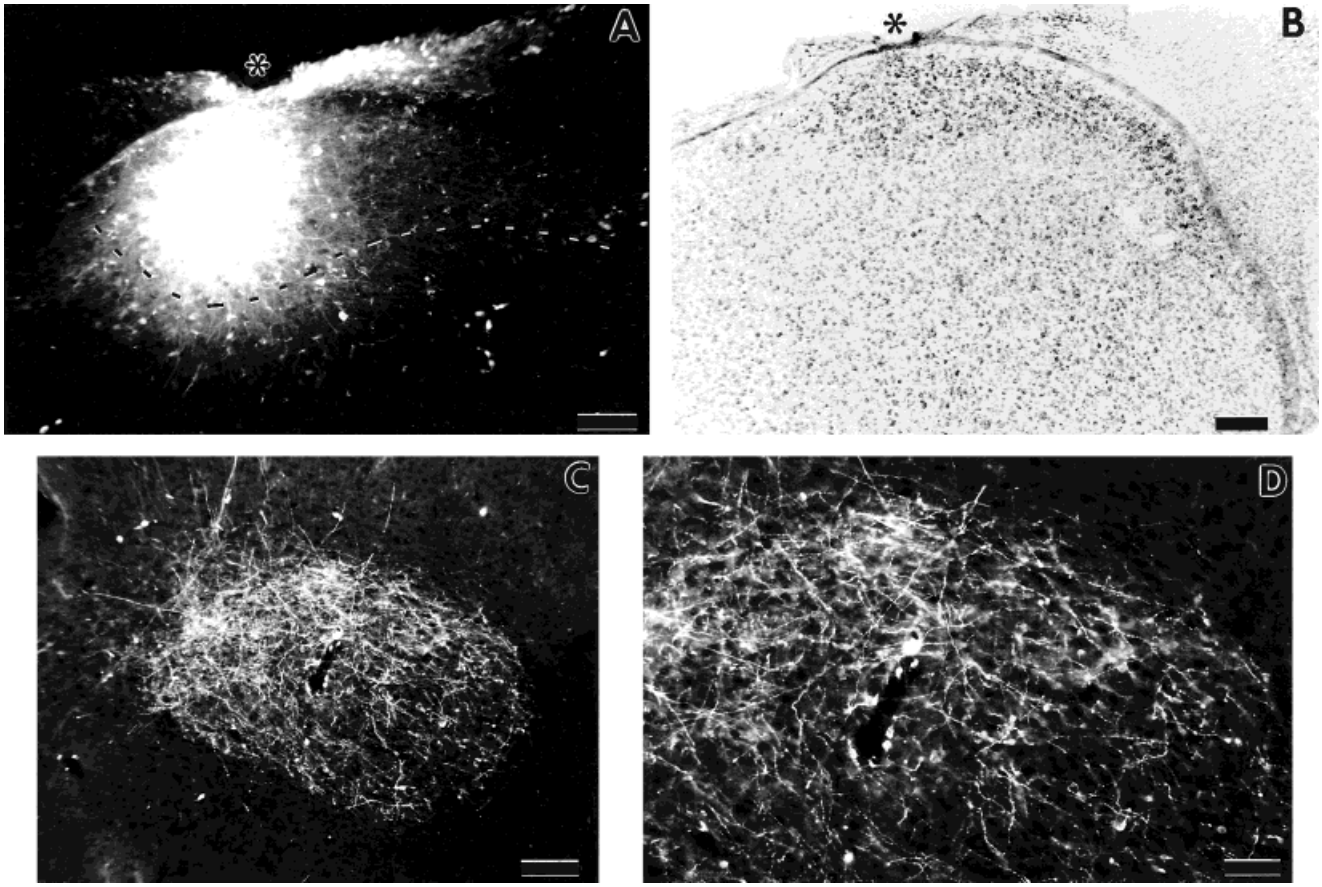

Fig. 13. **A:** Photomicrograph of an injection within lateral High Vocal Center (HVC) of a 23-day-old male zebra finch. **B:** Nissl-counterstained section corresponding to A. Asterisks in A and B indicate the location of the injection relative to the Nissl-defined borders of HVC. **C,D:** Photomicrographs showing the distribution of anterograde label within the robust nucleus of the archistriatum (RA). Medial is right; coronal plane. Scale bars = 115 μm in A,C, 225 μm in B, 100 μm in D.

this respect are consistent with previous reports (Fortune and Margoliash, 1995; Vates and Nottebohm, 1995; Vates et al., 1996). Injections concentrated within subregions of HVC of juvenile males indicate that inputs from mMAN, NIf, and Uva are present by 20 days of age (cf. Mooney and Rao, 1994). Whether these projections are also nontopographic as in adults is uncertain as injections in juveniles tended to encompass all or most of HVC. Injections into HVC of juveniles produced an even distribution of anterograde label throughout Area X, demonstrating that this pathway is present at the onset of vocal learning (cf. Alvarez-Buylla et al., 1988; Nordeen and Nordeen, 1988b).

The finding that injections of dextran amines concentrated within the Nissl-defined borders of HVC in juveniles produced a clear pattern of anterograde label within the Nissl-defined borders of RA prompts a reexamination of the development of the HVC-to-RA pathway. This result contrasts with previous reports indicating that HVC efferents do not ramify within RA until after approximately 25 days of age (Konishi and Akutagawa, 1985; Mooney and Rao, 1994; but cf. Mooney, 1992). The discovery of a connection from HVC to RA in juvenile males may be due to the use of more recently available, highly sensitive anterograde tracers in the present study. The intensity of anterograde label within RA was less in juvenile than in adult males, a finding that would be expected based on the continued ingrowth of axons from newly generated HVC neurons into RA during the course of song learning (Nordeen and Nordeen, 1988b). However, the discovery of a specific projection from HVC to RA in 20-day-old birds at the onset of vocal learning has important implications for the study of vocal learning and production. Namely, HVC neurons may exert some direct role in song behavior (along with 1MAN neurons) even at the onset of vocal learning, as opposed to only during later stages of vocal development (for review, see Bottjer and Arnold, 1997).

Another important finding of the present study is that regions in close proximity to HVC project to regions outside of RA. Control injections immediately surrounding HVC in both juveniles and adults produced anterograde label immediately outside of RA but not within its Nissl-defined borders. This result also argues for a reexamination of the precise pattern and development of neuronal connections of HVC and surrounding regions. Previous reports have indicated that HVC sends a projection to a "waiting compartment" outside of RA prior to the ingrowth of axons into RA (Konishi and Akutagawa, 1985; Mooney and Rao, 1994). In the present study, control injections outside HVC in juvenile birds produced a distinct distribution of label surrounding RA, suggesting that HVC may not project to a "waiting compartment" outside of RA in

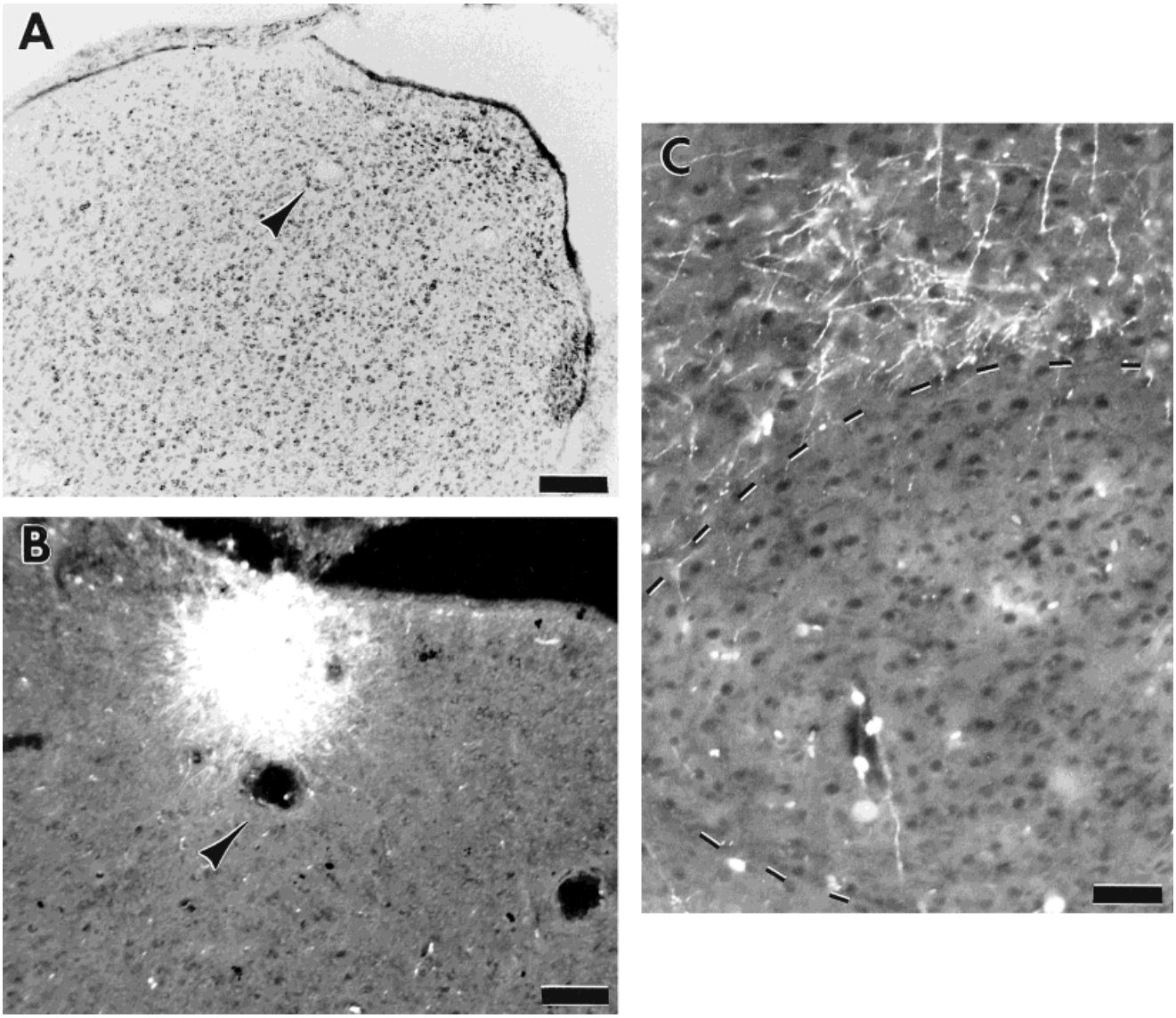

Fig. 14. Photomicrographs of an injection of rhodamine dextran amine caudolateral to the High Vocal Center in a 22-day-old male zebra finch (**A,B**) and the pattern of anterograde label immediately dorsal to the Nissl-defined borders of the robust nucleus of the archistriatum (RA) but not within RA (dashed lines; **C**). Arrowheads identify the same blood vessel in the section containing the injection site and in the Nissl alternate section (A,B). Medial is right; coronal plane. Scale bars = 225 μm in A, 115 μm in B,C.

juveniles. Rather, regions surrounding HVC form distinct projections to regions surrounding RA, and these connections form early in development. Curiously, regions immediately surrounding RA have been referred to as "RA cup" in adults (Kelley and Nottebohm, 1979; Vates et al., 1996), whereas regions surrounding RA have been named the "waiting compartment" in young birds. Because the pattern of label surrounding RA after control injections in adults and juveniles is strikingly similar, areas defined as RA cup in adults may be directly comparable to regions defined as the waiting compartment of juveniles.

Most lateral HVC injections in adults and the injection confined to lateral HVC in the juvenile bird produced label within RA and in regions dorsolateral to RA, presumably within dorsolateral RA cup. Although the overall pattern of afferent and efferent connections of medial, central, and lateral HVC are similar, lateral HVC is the only region within the Nissl-defined borders of HVC that projects to a region of RA cup. In this regard, lateral HVC is neuroanatomically distinct from other regions of HVC, and this distinction may already be established in young birds.

pHVC injections

The present results establish the complete pattern of neuronal connections of pHVC in adults and juvenile male zebra finches. The pHVC receives its primary sources of afferent input from rostral regions of medial cortex including song control regions of mMAN, medial regions of $1MAN_{shell}$, and regions immediately caudal to these nuclei. Anterograde label in Area X after pHVC injections establishes that, like HVC, pHVC projects directly to Area X

(Nordeen et al., 1987; Johnson and Bottjer, 1995) and that this projection is present in young birds. The pHVC also projects to regions caudomedial to Area X within a specific region of the dorsomedial striatum that appears to include both medial striatum (LPO) and the medial aspect of lateral striatum (PA). In addition, pHVC sends a major projection to areas at the same rostrocaudal level as HVC and pHVC, within a region that overlaps partially with the auditory responsive region NCM. Although the function of NCM in song behavior has not been determined, reports have implicated NCM in higher-order processing of auditory and song-related information. Specifically, immediate early gene induction occurs throughout NCM in response to song stimuli (Mello et al., 1992, 1995; Mello and Clayton, 1994; Nastiuk et al., 1994) and NCM neurons demonstrate complex response patterns to song stimuli (Chew et al., 1995, 1996; Jarvis and Nottebohm, 1997; Stripling et al., 1997). Areas within NCM receive direct projections from regions of the auditory thalamic nucleus ovoidalis and also receive indirect input from regions of auditory cortex (Vates et al., 1996). Interestingly, NCM as defined by intense immediate early gene induction in coronal sectioned tissue appears to encompass pHVC (Mello and Clayton, 1994). If pHVC is considered part of NCM, the HVC-to-pHVC projection would serve as a specific neuroanatomical link between distinct song control nuclei with auditory information processed within NCM. The present results delineate a robust projection from pHVC to regions of NCM, suggesting that pHVC may further influence cortical auditory processing related to song behavior. In addition, the pHVC-to-NCM pathway in juveniles may play a direct role in vocal learning by influencing auditory processing during development, when auditory information is critical for normal song learning (Price, 1979; Nordeen and Nordeen, 1992).

Efferent projections of pHVC to Area X indicate that this nucleus and its associated pathways form a complete circuit of neuronal connections and suggest the possibility that pHVC may direct information to brain areas important for vocal learning (Fig. 15A). Specifically, pHVC receives afferent input from both mMAN and medial regions of $1MAN_{shell}$ and pHVC projects directly to Area X. In turn, Area X sends a projection to the dorsal thalamic nucleus DLM (dorsolateral nucleus of the anterior thalamus; Bottjer et al., 1989; Okuhata and Saito, 1987), and the ventromedial portion of DLM projects specifically to medial regions of $1MAN_{shell}$ (Johnson et al., 1995). A functional corollary of this circuitry is that pHVC may play a direct role in processing information important for vocal learning because Area X and its associated pathways are critical for normal song development (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Further, information processed in the pHVC–Area X–DLM–$1MAN_{shell}$ pathway may be fed back into pHVC and its associated pathways including NCM.

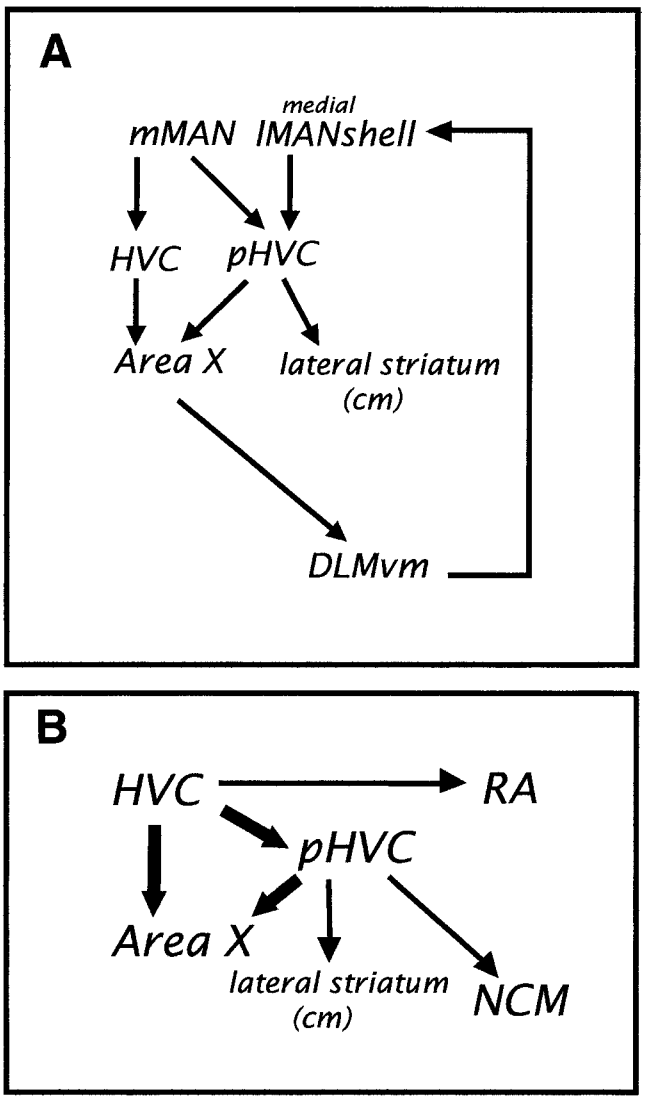

Fig. 15. Schematic illustrations of the feedback pathways of the para-High Vocal Center (pHVC) and medial shell of the lateral magnocellular nucleus of the anterior neostriatum (**A**) (lMAN) (text), and the efferent projections of HVC and pHVC (**B**). The projection from HVC to pHVC provides an indirect pathway by which HVC projects to Area X, as pHVC also projects to Area X (bold lines). ParaHVC also projects to the caudomedial portion (cm) of lateral striatum and to regions within the auditory-responsive region, caudomedial neostriatum (NCM). The differential pattern of efferent projections of HVC and pHVC may have functional implications for the roles of each of these nuclei in vocal behavior. For abbreviations, see list.

Distinctions of HVC, pHVC, and surrounding regions

The present results delineate a novel efferent projection from HVC to pHVC because HVC injections produced a distinct pattern of anterograde label throughout pHVC and pHVC injections produced retrogradely labeled cells in HVC. Because both HVC and pHVC project directly to Area X, the HVC-to-pHVC projection provides an indirect route by which HVC projects to Area X (Fig. 15B, open arrows). Thus, information processed within HVC is presumably directed to Area X and to the pathway important for vocal learning (Area X–DLM–1MAN) via two pathways. The direct source of afferent input to pHVC from HVC further implicates pHVC in processing and distributing song-related information.

Although injections into HVC appeared to label all of pHVC, a conspicuous absence of label was apparent within a distinct region medial to rostral HVC, just below the lateral ventricle in both juveniles and adults. This gap of HVC-to-pHVC efferents corresponds to a gap in intense Nissl staining medial to rostral HVC in both age groups. In

adults, this gap appears to define the rostromedial extent of HVC shelf, the region ventral to HVC that receives afferent input from auditory cortex (Kelley and Nottebohm, 1979; Vates et al., 1996). The absence of label medial to rostral HVC and the distinct cytoarchitectonic features of this same region indicate that this area is likely the HVC shelf that inserts itself between HVC and pHVC at the level of rostral HVC.

HVC injections produced anterograde label throughout HVC, indicating that subregions of HVC are highly interconnected (cf. Nottebohm et al., 1982). Curiously, injections into lateral HVC defined a distinct unlabeled region in the dorsal aspect of HVC, just below the lateral ventricle. This region of dorsal HVC may define a specific subdivision of HVC, the dorsal boundary of the nucleus, or delineate a region separate from HVC. Interestingly, a recent report by Vates et al. (1996) has described the pattern of label following an injection confined within the HVC shelf immediately ventral to HVC. The authors reported that this injection produced anterograde label throughout regions ventral to HVC (i.e., throughout HVC shelf) and within a distinct region of dorsal HVC. The ventral shelf axons did not project within any other region of HVC nor did they travel within the Nissl-defined borders of HVC. Taken together, the present report of the pattern of label delineating a distinct, unlabeled region within dorsal HVC and the report that areas of dorsal HVC are continuous with regions of the shelf ventral to HVC may indicate that the auditory shelf of HVC includes not only a cytoarchitectonically distinct region ventral to HVC but also encompasses a neuroanatomically distinct region of dorsal HVC. Thus, it is possible that areas that receive direct projections from auditory cortex (the HVC shelf) may not only surround HVC ventrally but may also extend along a dorsal arc of HVC. The possibility that auditory regions are present along both the ventral and dorsal borders of HVC may provide a basis for how auditory information is neuroanatomically integrated with circuits controlling vocal behavior.

Interestingly, major auditory regions of birds are organized in a nested configuration similar to that of HVC and the underlying shelf. For example, the auditory thalamic nucleus ovoidalis (Ov) is organized into distinct subdivisions—a central core surrounded by a shell area—and both regions project directly to auditory cortex (Durand et al., 1992; Wild et al., 1993; Vates et al., 1996). In cortex of songbirds, NIf is completely nested within auditory cortex (Field L); RA is surrounded rostrally, dorsally, and ventrally by RA cup, which receives direct projections from auditory cortex (Kelley and Nottebohm, 1979; Vates et al., 1996); and HVC is surrounded by the auditory shelf and is located in close association with the auditory region NCM. One simple mechanism by which auditory information is integrated with song control circuitry may be by means of a cytoarchitectural nested configuration.

Topographic organization of HVC and adjacent cortical regions

Topographically organized patterns of neuronal connections exist among specific nuclei within the pathway important for vocal learning (Area X–DLM–1MAN) and include projections from dorsolateral and ventromedial regions of DLM to $1MAN_{core}$ and $1MAN_{shell}$, respectively (Johnson et al., 1995), projections from $1MAN_{core}$ and $1MAN_{shell}$ to RA and the adjacent region Ad, respectively (Johnson et al., 1995; Vates and Nottebohm, 1995), and projections from $1MAN_{core}$ to specific domains within Area X (Vates and Nottebohm, 1995). Conversely, afferent and efferent projections of HVC are not organized topographically. Nevertheless, the present results demonstrate an overall spatial organization of efferent projections from HVC and adjacent regions to (1) distinct areas of striatum and (2) regions immediately surrounding RA. First, regions within the Nissl-defined borders of HVC project to Area X, which is located in the rostral portion of medial striatum (LPO). The pHVC, located medial to HVC, projects to Area X and to a more caudal region of medial striatum that encompasses the dorsal aspect of LPO and PA. Thus, adjacent cortical regions HVC and pHVC send spatially organized projections to distinct areas of striatum. Second, medial, central, and lateral subregions of HVC project directly within the Nissl-defined borders of RA, whereas regions of lateral HVC and areas immediately outside of HVC project to regions surrounding RA. Although projections from HVC to RA are not topographic with respect to terminations within RA, lateral HVC and regions of dorsal cortex adjacent to HVC terminate in a distinct medial-to-lateral arrangement within regions ventral to the dorsal archistriatal lamina (LAD; i.e. within archistriatum; cf. Veenman et al., 1995). These patterns demonstrate a larger-scale spatial organization of neuronal connections of HVC and surrounding areas that extends beyond the level of individual song control nuclei.

LITERATURE CITED

Alvarez-Buylla, A., M. Theelen, and F. Nottebohm (1988) Birth of projection neurons in the higher vocal center of the canary forebrain before, during, and after song learning. Proc. Natl. Acad. Sci. USA *85:*8722–8726.

Ball, G.F., P. Absil, and J. Balthazart (1995) Assessment of volumetric sex differences in the song control nuclei HVC and RA in zebra finches by immunocytochemistry for methionine enkephalin and vasoactive intestinal polypeptide. Brain Res. *699:*83–96.

Bernard, D.J. and G.F. Ball (1995) Two histological markers reveal a similar photoperiodic difference in the volume of the high vocal center in male European starlings. J. Comp. Neurol. *360:*726–734.

Bernard, D.J., J.M. Casto, and G.F. Ball (1993) Sexual dimorphism in the volume of song control nuclei in European starlings: Assessment by a Nissl stain and autoradiography for muscarinic cholinergic receptors. J. Comp. Neurol. *334:*559–570.

Bernard, J.-F., M. Alden, and J.-M. Besson (1993) The organization of the efferent projection from the pontine parabrachial area to the amygdaloid complex: A *Phaseolus vulgaris*-leucoagglutinin (PHA-L) study in rat. J. Comp. Neurol. *329:*201–229.

Bottjer, S.W. and A.P. Arnold (1997) Developmental plasticity in neural circuits for a learned behavior. Annu. Rev. Neurosci. *20:*459–481.

Bottjer, S.W., E.A. Miesner, and A.P. Arnold (1984) Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science *224:*901–903.

Bottjer, S.W., S.L. Glaessner, and A.P. Arnold (1985) Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J. Neurosci. *5:*1556–1562.

Bottjer, S.W., K.A. Halsema, S.A. Brown, and E.A. Miesner (1989) Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J. Comp. Neurol. *279:*312–326.

Chew, S.J., D.S. Vicario, and F. Nottebohm (1995) A large-capacity memory system that recognizes the calls and songs of individual birds. Proc. Natl. Acad. Sci. USA *93:*1950–1955.

Chew, S.J., D.S. Vicario, and F. Nottebohm (1996) Quantal duration of auditory memories. Science *274:*1909–1914.

Durand, S.E., J.M. Tepper, and M.F. Cheng (1992) The shell region of the nucleus ovoidalis: a subdivision of the avian auditory thalamus. J. Comp. Neurol. *323:*495–518.

Fortune, E.S. and D. Margoliash (1995) Parallel pathways and convergence onto HVc and adjacent neostriatum of adult zebra finches (*Taeniopygia guttata*). J. Comp. Neurol. *360:*413–441.

Foster, E.F., R.P. Mehta, and S.W. Bottjer (1997) Axonal connections of the medial magnocellular nucleus of the anterior neostriatum in zebra finches. J. Comp. Neurol. *382:*364–381.

Gahr, M. (1990) Delineation of a brain nucleus: comparisons of cytochemical, hodological, and cytoarchitectural views of the song control nucleus HVc of the adult canary. J. Comp. Neurol. *294:*30–36.

Gahr, M. (1997) How should brain nuclei be delineated? Consequences for developmental mechanisms and for correlations of area size, neuron numbers and functions of brain nuclei. Trends Neurosci. *20:*58–62.

Gahr, M., G. Flügge, and H.-R. Güttinger (1987) Immunocytochemical localization of estrogen binding neurons in the songbird brain. Brain Res. *402:*173–177.

Jarvis, E.D. and F. Nottebohm (1997) Motor-driven gene expression. Proc. Natl. Acad. Sci. USA *94:*4097–4102.

Johnson, F. and S.W. Bottjer (1992) Growth and regression of thalamic efferents in the song-control system of male zebra finches J. Comp. Neurol. *326*:442–450.

Johnson, F. and S.W. Bottjer (1995) Differential estrogen accumulation among populations of projection neurons in the higher vocal center of male canaries. J. Neurobiol. *26:*87–108.

Johnson, F., M.M. Sablan, and S.W. Bottjer (1995) Topographic organization of a forebrain pathway involved with vocal learning in zebra finches. J. Comp. Neurol. *358:*260–278.

Kelley, D.B. and F. Nottebohm (1979) Projections of a telencephalic auditory nucleus—Field L—in the canary. J. Comp. Neurol. *183:*455–470.

Konishi, M. and E. Akutagawa (1985) Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. Nature *315:*145–147.

Mello, C.V. and D.F. Clayton (1994) Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J. Neurosci. *14:*6652–6666.

Mello, C.V., D.S. Vicario, and D.F. Clayton (1992) Song presentation induces gene expression in the songbird forebrain. Proc. Natl. Acad. Sci. USA *89:*6818–6822.

Mello, C.V., F. Nottebohm, and D.F. Clayton (1995) Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. J. Neurosci. *15:*6919–6925.

Mooney, R. (1992) Synaptic basis for developmental plasticity in a birdsong nucleus. J. Neurosci. *12:*2464–2477.

Mooney, R. and M. Rao (1994) Waiting periods versus early innervation: The development of axonal connections in the zebra finch song system. J. Neurosci. *14:*6532–6543.

Nastiuk, K.L., C.V. Mello, J.M. George, and D.F. Clayton (1994) Immediate-early gene responses in the avian song control system: Cloning and expression analysis of the canary c-*jun* cDNA. Mol. Brain Res. *27:* 299–309.

Nordeen, E.J. and K.W. Nordeen (1988a) Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J. Neurosci. *8:*2869–2874.

Nordeen, K.W. and E.J. Nordeen (1988b) Projection neurons within a vocal motor pathway are born during song learning in zebra finches. Nature *334:*149–151.

Nordeen, K.W. and E.J. Nordeen (1992) Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. Behav. Neural Biol. *57:*58–66.

Nordeen, K.W., E.J. Nordeen, and A.P. Arnold (1987) Estrogen accumulation in zebra finch song control nuclei: Implications for sexual differentiation and adult activation of song behavior. J. Neurobiol. *18:*569–582.

Nottebohm, F., T.M. Stokes, and C.M. Leonard (1976) Central control of song in the canary, *Serinus canarius.* J. Comp. Neurol. *165:*457–486.

Nottebohm, N., D.B. Kelley, and J.A. Paton (1982) Connections of vocal control nuclei in the canary telencephalon. J. Comp. Neurol. *207:*344–357.

Okuhata, S. and N. Saito (1987) Synaptic connections of thalamo-cerebral vocal nuclei of the canary. Brain Res. Bull. *18:*35–44.

Price, P.H. (1979) Developmental determinants of structure in zebra finch song. J. Comp. Physiol. Psych. *93:*260–277.

Scharff, C. and F. Nottebohm (1991) A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J. Neurosci. *11:*2896–2913.

Simpson, H.B. and D.S. Vicario (1990) Brain pathways for learned and unlearned vocalizations differ in zebra finches. J. Neurosci. *10:*1541–1556.

Smith, G.T., E.A. Brenowitz, and J.C. Wingfield (1997) Seasonal changes in the size of the avian song control nucleus HVC defined by multiple histological markers. J. Comp. Neurol. *381:*253–261.

Sohrabji, F., E.J. Nordeen, and K.W. Nordeen (1990) Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav. Neural Biol. *53:*51–63.

Stripling, R., S.F. Volman, and D.F. Clayton (1997) Response modulation in the zebra finch neostriatum: Relationship to nuclear gene expression. J. Neurosci. *17:*3883–3893.

Vates, G.E. and F. Nottebohm (1995) Feedback circuitry within a song-learning pathway. Proc. Natl. Acad. Sci. USA *92:*5139–5143.

Vates, G.E., B.M. Broome, C.V. Mello, and F. Nottebohm (1996) Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches (*Taenopygia guttata*). J. Comp. Neurol. *366:*613–642.

Veenman, C.L., J.M. Wild, and A. Reiner (1995). Organization of the avian "corticostriatal" projection system: a retrograde and anterograde pathway tracing study in pigeons. J. Comp. Neurol. *354:*87–126.

Wild, J.M. (1994) Visual and somatosensory inputs to the avian song system via nucleus uvaeformis (Uva) and a comparison with the projections of a similar thalamic nucleus in a nonsongbird, *Columbia livia.* J. Comp. Neurol. *349:*512–535.

Wild, J.M., H.J. Karten, and B.J. Frost (1993) Connections of the auditory forebrain in the pigeon (*Columbia livia*). J. Comp. Neurol. *337:*32–62.

Williams, H. and D.S. Vicario (1993) Temporal patterning of song production: Participation of nucleus uvaeformis of the thalamus. J. Neurobiol. *24:*903–912.