New Brain Pathways Found in the Vocal Control System of a Songbird

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ABSTRACT
Songbirds use a complex network of discrete brain areas and connecting fiber tracts to sing their song, but our knowledge of this circuitry may be incomplete. The forebrain area, “caudal mesopallium” (CM), has received much attention recently for its song-related activities. HVC, a prominent song system nucleus, projects to a restricted area of the CM known as the avalanche nucleus (Av). However, the other connections of Av remain unknown. Here we used tract-tracing methods to examine the connections of Av to other song system nuclei. Injections of biotinylated dextran amine (BDA) into Av labeled both afferent terminals and neurons in HVC and the interfacial nucleus of the nidopallium (NIf), suggesting that there is complex feedforward and feedback communication between these nuclei (HVC→Av→NIf). Labeled neurons were also found in the uvaeform nucleus (Uva), which was substantiated by BDA injections into Uva that labeled terminals in Av. Double fluorescent tracing experiments confirm that both HVC and Uva project to Av. The present study adds complex new connections that expand the traditional song system circuitry into the caudal mesopallium. These new pathways are likely to have broad implications for deciphering how this intricate system works. J. Comp. Neurol. 518:3086–3100, 2010.

INDEXING TERMS: feedback; mesopallium; networks; complexity

The discovery of brain circuits for the control of bird-song opened a new era in the study of neural substrates and mechanisms of vocal learning in nonhuman animals (Nottebohm et al., 1976). The “song system” has also become an attractive subject for the study of such important but diverse issues as memory and learning (Scharff and Nottebohm, 1991; Doupe et al., 2004; Bolhuis and Gahr, 2006), plasticity (De Groof et al., 2008; Kao et al., 2008), brain sexual dimorphism (Nottebohm and Arnold, 1976), the birth of new neurons in the brain (Alvarez-Buylla et al., 1990), and neural network theory (Seung, 2009), to name a few. Because the song system is a discrete array of interconnected brain areas (Nottebohm et al., 1976), a complete knowledge of all of their connections is necessary for understanding how the different parts communicate with and therefore influence each other. Since its discovery, much of the song system circuitry has been characterized (Fig. 1A), but because its connections to other parts of the brain remain obscure, the current schemata are likely to be incomplete. One such area is the caudal part of the mesopallium (CM). Recent studies have implicated this area of the songbird brain in the storage of auditory memories (Gentner and Margoliash, 2003; Doupe et al., 2004), auditory song selectivity (Theunissen et al., 2004), feedback control of song (Theunissen et al., 2004; Bauer et al., 2008; Keller and Hahnloser, 2009), and as a site of entry of auditory information into the song system (Bauer et al., 2008). However, because CM encompasses a large part of the anterodorsal part of the brain (Stokes et al., 1974; Poirier et al., 2008) it is presently unclear exactly where in this vast expanse of neurons such relevant song-related activities are taking place. Even references to the “caudal ventral mesopallium” (CVM) are insufficient, given the large size of the lower mesopallium of the zebra finch brain. Our previous article (Akutagawa and Konishi, 2005) dealt mainly with the midbrain connections of the uvaeform nucleus (Uva), but our studies also generated preliminary data to suggest that there might be a separate projection from this thalamic nucleus toward the CM region in the telencephalon. Here we investigated this novel pathway...
obtained from a local vendor (Magnolia Bird Farm, Los Angeles, CA) or from our breeding colony. All experiments were conducted in accordance with protocols approved by the Office of Laboratory Animal Resources at the California Institute of Technology. Birds were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg Ketaject; Phoenix Pharmaceuticals, St. Joseph, MO) and xylazine (15 mg/kg; Lloyd Laboratories, Shenandoah, IA) and held in a stereotaxic device (Narishige, Tokyo, Japan). After a local injection of Marcaine analgesia (Hospira, Lake Forest, IL) into the scalp, a small incision was made and the skull exposed. All stereotaxic measurements were made with the head angle fixed at 45° (beak down 45° relative to the horizontal axis) and the “0” reference point as the midsagittal sinus intersection with the cerebellar bifurcations. Uva was located by a combination of stereotaxic coordinates (Konishi laboratory, unpubl. stereotaxic atlas of the male zebra finch), responses to light in the contralateral eye (Wild, 1994), and nonselective auditory responses (Coleman et al., 2007). HVC, NIf, and Av were located by our stereotaxic atlases in the adult male zebra finch brain displays some of the major brain divisions in a cresyl violet (left) and fiber-stained (right) section. The approximate locations of NIf and Av are shown on the right section, which is rostral to HVC to also include the location of Uva. Hp, hippocampus; M, mesopallium; N, nidopallium; St, striatum; LaM, mesopallial lamina; PSL, pallial-subpallial lamina; V, ventricle; Uva, uvaeform nucleus; SpM, medial spiniform nucleus; DA; dorsal arcopallial tract; OM, occiptomesencephalic tract; FA, fronto-arcopallial tract; CP, posterior commissure; TeO, optic tectum; NIf, interfacial nucleus of the nidopallium; Av, nucleus avalanche. Scale bar = 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2. A low-power, composite, coronal section of a whole male zebra finch brain displays some of the major brain divisions in a cresyl violet (left) and fiber-stained (right) section. The approximate locations of NIf and Av are shown on the right section, which is rostral to HVC to also include the location of Uva. Hp, hippocampus; M, mesopallium; N, nidopallium; St, striatum; LaM, mesopallial lamina; PSL, pallial-subpallial lamina; V, ventricle; Uva, uvaeform nucleus; SpM, medial spiniform nucleus; DA; dorsal arcopallial tract; OM, occiptomesencephalic tract; FA, fronto-arcopallial tract; CP, posterior commissure; TeO, optic tectum; NIf, interfacial nucleus of the nidopallium; Av, nucleus avalanche. Scale bar = 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 1. The old (A) and new (B) song system circuits are compared in the adult male zebra finch. Only the major forebrain song nuclei are shown and most of the middle and lower brain areas are left out for brevity. A: The old version represents the traditional song system schematic, where Av is usually left out. The magenta-dotted arrow represents the unknown connections of Av to other song system nuclei, which is the subject of this study. B: The new song system schematic incorporates the results reported here, which includes the added connections that Av has with HVC, NIf, and Uva (magenta arrows). The song system now extends beyond the traditional circuitry to include part of the caudal mesopallium. A future challenge is to determine the connections of the oval nucleus of the mesopallium (MO, magenta dotted arrow), which was discovered by singing-induced gene expression studies (see text). Lateral (L) or medial (M) magnocellular nucleus of the anterior nidopallium, MAN; X, Area X; RA, robust nucleus of the arcopallium; Cb, cerebel-lum; DLM, dorsal lateral nucleus of the medial thalamus; nXIIIs, hypoglossal nucleus; HVC, used as the letter-based proper name; NIf, interfacial nucleus of the nidopallium; Uva, uvaeform nucleus; Av, nucleus avalanche. With the exception of Figure 2, all subsequent figures of the song system are parasagittal sections with the dorsal side up and rostral to the right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in greater detail and ultimately focused on a small, inconspicuous area within the CVM, the avalanche nucleus (Av), which is located dorsally to the interfacial nucleus of the nidopallium (NIf) (Figs. 1, 2). Since its discovery in canaries (Nottebohm et al., 1982), subsequent studies of Av have demonstrated singing-driven gene expression of ZENK/egr 1 and c-fos in Av similar to that in other song nuclei (Jarvis and Nottebohm, 1997; Wada et al., 2006; Feenders et al., 2008). In addition to canaries and zebra finches, an analog of Av was also found in hummingbirds (Jarvis et al., 2000) and parrots (Jarvis and Mello, 2000). While these studies imply that Av is a part of the vocal system, the connections of Av to other song system areas have never been demonstrated in detail. In the present study we confirm the connection between Uva and Av and elucidate several new connections and circuits to Av that require changes in our current view of the neural mechanisms of song production and perception (Fig. 1B).

MATERIALS AND METHODS

We used a total of 46 adult (>120 days posthatching) zebra finches (Taeniopygia guttata) that were either

Figure 1. The old (A) and new (B) song system circuits are compared in the adult male zebra finch. Only the major forebrain song nuclei are shown and most of the middle and lower brain areas are left out for brevity. A: The old version represents the traditional song system schematic, where Av is usually left out. The magenta-dotted arrow represents the unknown connections of Av to other song system nuclei, which is the subject of this study. B: The new song system schematic incorporates the results reported here, which includes the added connections that Av has with HVC, NIf, and Uva (magenta arrows). The song system now extends beyond the traditional circuitry to include part of the caudal mesopallium. A future challenge is to determine the connections of the oval nucleus of the mesopallium (MO, magenta dotted arrow), which was discovered by singing-induced gene expression studies (see text). Lateral (L) or medial (M) magnocellular nucleus of the anterior nidopallium, MAN; X, Area X; RA, robust nucleus of the arcopallium; Cb, cerebel-lum; DLM, dorsal lateral nucleus of the medial thalamus; nXIIIs, hypoglossal nucleus; HVC, used as the letter-based proper name; NIf, interfacial nucleus of the nidopallium; Uva, uvaeform nucleus; Av, nucleus avalanche. With the exception of Figure 2, all subsequent figures of the song system are parasagittal sections with the dorsal side up and rostral to the right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Neurophysiological experiments were conducted in a sound-attenuating chamber and multiunit recordings were performed with finely pulled glass electrodes (WPI, Sarasota, FL; 5–7 μm i.d.) filled with either 10% w/v biotinylated dextran amine (BDA; 10,000 MW, Invitrogen, Carlsbad, CA) and 0.2% KCl, or 10% w/v tetramethylrhodamine or dextran fluorescein (3,000 MW, Invitrogen) in 25 mM phosphate buffer (PB), pH 7.4, containing 0.2% KCl. Individual bird songs were recorded in a soundproof box (Acoustic Systems, Cedar Park, TX) with an Aardvark Direct Pro microphone. These songs were analyzed with Song Analysis Pro (Tchernichovski et al., 2004). Song templates of either BOS or RBOS were prepared using Goldwave software (St. John's, NL). Songs were played back from a wide-band speaker (Madison Speaker Components, Madison, WI) after 10 kHz low-pass filtering through a 6-pole antialiasing filter (FT6; Tucker-Davis, Gainesville, FL). Sound stimuli were presented at ≈70 dB and monitored with a sound level meter (RadioShack, Ft. Worth, TX). Auditory and neural recordings were amplified (four channel differential AC amplifier; AM Systems, Everett, WA), bandpass-filtered between 400 Hz and 10 kHz (7-pole antialiasing filter FT6-7; Tucker-Davis Technologies) and digitized at 20 kHz with a 100-MHz, 16-bit DAQ board (PCI-MIO-16XE-10; National Instruments, Austin, TX). Data collection software was written by A. Leonardo for Labview (National Instruments, Austin TX) and the data were analyzed with MatLab (MathWorks, Natick, MA) using a PSTH analysis program of auditory responses written by B. Fischer (Caltech), where the PSTH was generated by thresholding just above the spontaneous firing rate. The auditory response rate for each bird was calculated in MatLab, where the average spikes/sec during the stimulus period was subtracted from the average background spikes/sec over a similar time period. The auditory response rates of all birds were averaged and line plots were generated with Origin 6.0 (MicrocalSoftware, Northampton, MA). Upon locating the desired brain area, either BDA or fluorescent tracers were iontophoresed with positive current at 5–7 μA pulses (7 sec on / 7 sec off) for 10 minutes using a custom-made iontophoresis delivery system (Caltech electronic shop, M. Walsh).

Survival times after tracer injections varied from 4 days (NIf, Av) to 1 week (Uva, HVC). The birds were then sacrificed for histology by administering a lethal dose of Nembutal (Abbott Labs, Chicago, IL) and perfused through the left ventricle of the heart with 0.9% saline until clearing followed by 1 hour of 2% paraformaldehyde (PF) in 25 mM PB, pH 7.4. After removal from the skull the brain was postfixed for an additional day and put into (PF) in 25 mM PB, pH 7.4, containing 0.2% KCl. Individual bird songs were recorded in a soundproof box (Acoustic Systems, Cedar Park, TX) with an Aardvark Direct Pro microphone. These songs were analyzed with Song Analysis Pro (Tchernichovski et al., 2004). Song templates of either BOS or RBOS were prepared using Goldwave software (St. John’s, NL). Songs were played back from a wide-band speaker (Madison Speaker Components, Madison, WI) after 10 kHz low-pass filtering through a 6-pole antialiasing filter (FT6; Tucker-Davis, Gainesville, FL). Sound stimuli were presented at ≈70 dB and monitored with a sound level meter (RadioShack, Ft. Worth, TX). Auditory and neural recordings were amplified (four channel differential AC amplifier; AM Systems, Everett, WA), bandpass-filtered between 400 Hz and 10 kHz (7-pole antialiasing filter FT6-7; Tucker-Davis Technologies) and digitized at 20 kHz with a 100-MHz, 16-bit DAQ board (PCI-MIO-16XE-10; National Instruments, Austin, TX). Data collection software was written by A. Leonardo for Labview (National Instruments, Austin TX) and the data were analyzed with MatLab (MathWorks, Natick, MA) using a PSTH analysis program of auditory responses written by B. Fischer (Caltech), where the PSTH was generated by thresholding just above the spontaneous firing rate. The auditory response rate for each bird was calculated in MatLab, where the average spikes/sec during the stimulus period was subtracted from the average background spikes/sec over a similar time period. The auditory response rates of all birds were averaged and line plots were generated with Origin 6.0 (MicrocalSoftware, Northampton, MA). Upon locating the desired brain area, either BDA or fluorescent tracers were iontophoresed with positive current at 5–7 μA pulses (7 sec on / 7 sec off) for 10 minutes using a custom-made iontophoresis delivery system (Caltech electronic shop, M. Walsh).

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California Institute of Technology’s Biological Imaging Center provided the digital imaging microscopes and related computer software for both the light microscope (AxioCam and AXIOVISION 3.0.6, and the laser scanning confocal microscope (Zeiss Pascal, Thornwood, NY). Photomicrographs taken on the light microscope were adjusted for brightness, contrast, and sharpness. Fluorescent images were processed with the LSM software that came with the confocal microscope. None of the fluorescent images were z-stacked for enhancement but represent data from a single focal plane. The photographs were organized, lettered, and printed in Photoshop 7.0 (Adobe, San Jose, CA). All anatomical identifications conform to the revised avian brain nomenclature (Reiner et al., 2004).

RESULTS

Two populations of neurons in Uva are revealed by double-fluorescence labeling

Uva has known projections to two telencephalic song system areas, the interfascicular nucleus of the nidopallium (NIf) and HVC (used as the proper name) (Nottebohm et al., 1982). Recent neurophysiological studies have suggested that the Uva cells that project to NIf (Uva\textsubscript{NIf}) are distinct from the HVC projecting ones (Uva\textsubscript{HVC}) (Hahnloser et al., 2008). These conclusions were based on single-cell recordings of different Uva cells by observing antidromic spike collisions whenever either NIf or HVC cells
were electrically stimulated. We show these differences directly by iontophoresing the fluorescent tracer dextran rhodamine (see Materials and Methods) into NIf and delivering dextran fluorescein into the ipsilateral HVC (n = 4). The imaging results within Uva clearly show two distinct cells of either magenta or green fluorescence (Fig. 3). No double-labeled cells (white) were observed, and the differently labeled cell populations are approximately equal in size and distribution.

Uva sends afferents to the caudal ventral mesopallium

Injections of BDA into Uva (Fig. 4A; n = 6) revealed a new projection pathway into the telencephalon. Branching off rostrally from the dorsal portion of NIf (Figs. 4B, 6A) the labeled fibers crossed the mesopallial lamina (LaM) (Fig. 4C,D) and converged in an area of the ventral part of the caudal mesopallium (Fig. 4B). This terminal area from Uva occupies an area of CVM just dorsal to the characteristic "bend" of the LaM and at approximately the same parasagittal location as NIf (Fig. 4B). Within CVM the labeled fibers appeared highly branched, with many swellings and varicosities, which are indicative of synaptic endings (Fig. 4D). Higher magnification shows in greater detail how the labeled fibers from Uva ramify away from NIf and terminate in a discrete area just dorsal to the LaM bend (Fig. 4C,D). Neutral red counterstaining, however, does not show this area to be distinguishable from its CVM surround by any obvious differences in cell size or density (Fig. 4C). No retrogradely labeled cells were found in either Av or CM as a result of the BDA injections into Uva (Fig. 4C,D). Control injections that were made close to, but outside of, Uva failed to show this new projection (data not shown).

Uva afferents in CVM may be in the same location as the avalanche area

An earlier study involving horseradish peroxidase (HRP) injections into the canary HVC briefly described an anterogradely labeled area in the CVM called the "avalanche" area (Nottebohm et al., 1982). However, because the results of our tracer injection studies in Uva showed a similar projection site, we wondered whether the two areas are coincident. Iontophoretic injections of BDA in the HVC of adult male zebra finches (n = 6) showed a discretely labeled area in the CVM that appeared very similar to the canary Av (Fig. 5). Best seen in uncounterstained sections, the location of this terminal field from HVC is similar to our earlier Uva tracer results in approximate size and shape, apposition to NIf, and location just above the LaM bend (compare Figs. 4B,D, 5B,C). Neutral red counterstaining further revealed this similarity (compare Figs. 4B, 5B). Like our previous tracer results from Uva, the labeled terminals in Av from the HVC injections are also highly branched and varicose (Fig. 5D), suggesting that the two inputs may share a common innervation target. An important difference between our HVC and Uva injections, however, was the presence of retrogradely labeled cells in Av after HVC injections (Figs. 5D, 6B), while we did not see labeled cells after Uva injections (Fig. 4D). Injections that either missed HVC or resulted in only small injections within HVC failed to label Av (data not shown).

Both Uva and HVC project to Av

Our previous results with BDA injections into either HVC or Uva suggested that both nuclei might send afferents to the same location within CVM. However, due to the relatively large extent of CM the exact coincidence of these two different terminal fields could be in question. To ascertain this possible overlap we injected dextran rhodamine into HVC and dextran fluorescein into Uva (Fig. 6C; n = 4). The results confirm that axons from both HVC and Uva nuclei project to Av, yielding both magenta and green labeled terminals within Av (Fig. 6A,B). Complementing our earlier results with BDA injections made into Uva, the results here also showed green fibers from Uva ascending past the dorsal NIf area and traveling forward and upward to reach Av (Fig. 6A). Also evident were a few rhodamine-labeled cell bodies in Av (Fig. 6B, arrows). Therefore, two separate experiments using different

Figure 3. Two populations of neurons are visualized in Uva following fluorescent dextran rhodamine tracer injections into NIf (magenta) and dextran fluorescein into HVC (green). Scale bar = 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Tracers demonstrated that Av has both anterograde and retrograde projections from HVC.

**Neurophysiological identification of Av within the caudal mesopallium**

To begin to characterize the inputs and outputs of Av, we needed a way to discriminate this area from the rest of CM. We therefore probed this area of the brain with glass multiunit electrodes (see Materials and Methods) with the hope of finding some distinguishing response properties in Av so that we could later inject tracers there reliably and accurately. We predicted that, like other HVC-linked areas, Av cells would display song-selective response properties (Doupe and Konishi, 1991; Leonardi, 2004). For each bird (n = 5), 20 repetitions of either the BOS, the RBOS, or WN were presented to the anesthetized bird while recording from different locations in CM. As expected, our results showed a discrete area in CVM that responded more vigorously to playback of BOS over either RBOS or WN. Figure 7A shows a schematic diagram of some of the recording sites in CM, including Av (dotted oval). Multiunit activity was quantified as the average auditory rate (AR) (spikes/sec) generated over 20 trials during the stimulus period minus the average rate produced during a similar time period of spontaneous activity (see Materials and Methods). The AR of the different recording sites for each region was grouped, averaged, and is plotted in Figure 7B. Recordings made anterior to Av (O symbol, dotted line; n = 5) were generally much more responsive to WN than to either BOS or RBOS. For BOS the AR in this region was 208.4 spikes/sec, with a standard error (SE) of 144.6 spikes/sec. For

![Figure 4](https://www.interscience.wiley.com)
RBOS, the AR was $502.9 \pm 179.5$ SE spikes/sec. WN presentations here produced a much higher AR of $2953.8 \pm 895.8$ SE spikes/sec. Posterior to Av we noticed a significant increase in auditory responsiveness to all stimuli, but still found no selectivity for BOS (▲ symbol, dashed line; $n = 4$). The AR for this area to BOS was $2002.0 \pm 1191.8$ SE spikes/sec. RBOS produced a similar AR of $2467.3 \pm 776$ SE spikes/sec, while the AR to WN was slightly higher at $3175.8 \pm 969.4$ SE spikes/sec. We also made a couple of recordings dorsal to Av (● symbol, solid black line), which were generally only weakly auditory and not selective for BOS. The AR here for BOS was $300.5 \pm 81.3$ SE spikes/sec. For RBOS, the AR was higher at $679.1 \pm 282.2$ SE spikes/sec. Presentations of WN produced slightly higher responses, with an AR of $910.4 \pm 134.5$ SE spikes/sec. However, within Av (magenta square symbol, solid magenta line; $n = 5$) we noticed a statistically significant increase in auditory selectivity for BOS over both RBOS ($P = 0.01$, Student’s paired t-test) and WN ($P = 0.003$). For BOS the AR in Av was $1621.7 \pm 563.7$ SE spikes/sec. Responses to RBOS were much weaker, with an AR of $608.0 \pm 78.9$ SE spikes/sec. WN presentations in Av resulted in much lower auditory responses here than in any of the other areas, with the AR of $168.9 \pm 157.5$ SE spikes/sec. A representative example of a multiunit Av recording is shown in Figure 7C. Panel 1 shows the raw auditory stimulus patterns, while panel 2 displays the corresponding raster plots resulting from the 20 iterations of the stimuli presented over each 10-second time frame. “Fr” is the firing rate of the neurons (spikes/sec) accumulated over 20 trials each. Panel 3 shows the resulting MatLab-generated PSTHs and the stimulus response windows are expanded in panel 4.

Figure 5. Av is revealed by BDA injections into HVC. A: Uncounterstained section shows Av (arrows) and its relative position to Nif. B: Neutral red counterstained section indicates the location of Av within the ventral CM. C: Medium magnification shows the location of Av just above the LaM bend, and its apposition to Nif. D: Higher magnification shows varicosities and branching of terminals in Av (arrows). Triangles indicate retrogradely labeled cell bodies. Scale bars = 200 µm in A,B; 50 µm in C; 25 µm in D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
for added detail. A common pattern noticed in the PSTHs was the presence of an initial spike regardless of stimulus type (Fig. 7C, arrow in panel 3). This seems to indicate that all of the test sounds elicited an onset response but that continued neuronal firing was maintained mainly for BOS, while RBOS and especially WN responses were significantly suppressed very soon thereafter (Fig. 7C, panels 3 and 4). Similar recordings in either HVC or NIf, using the same type of electrodes, anesthesia, and equipment did not produce this onset spike (data not shown).

New connections and circuits are revealed by tracer injections in Av

The song-selective properties of Av allowed us to accurately target this nucleus for tracer injections. In the original canary HRP experiment, several retrogradely labeled cells in Av were seen to mingle with the terminals emanating from HVC (Nottebohm et al., 1982). This suggested to us that Av might have reciprocal connections to HVC. We therefore investigated this issue further in the male zebra finch by iontophoresing BDA into Av (n = 6) and found a strong projection back to HVC (Fig. 8A and B). We did not find any retrogradely labeled cells in the medial magnocellular nucleus of the anterior nidopallium (MMAN), or anterograde labeling in Area X, thus obviating any concerns about inadvertently leaking tracer into the fibers of passage between HVC and these two anterior nuclei. These results also confirmed our earlier findings that showed retrogradely labeled cells in Av from injections of either BDA (Fig. 5D) or dextran rhodamine (Fig. 6B) into HVC. While we do not yet know

Figure 6. Double fluorescent labeling demonstrates that Uva (injected with dextran fluorescein) and HVC (injected with dextran rhodamine) both project to Av. A: Composite panel shows the trajectory of the Uva labeled axons (green) branching off the top portion of NIf to enter Av (arrows). B: Higher magnification shows how both terminals from HVC (magenta) and Uva (green) colocalize in Av. Some retrogradely labeled cells that project to HVC are also seen (arrows). C: Schematic drawing of the injection sites and resultant terminations in Av. The pathways indicated by the magenta and green arrows are for simplification purposes and do not indicate the actual trajectories. Scale bars = 50 μm in A; 25 μm in B. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
whether the same NIf neurons project to both HVC and Av, we can see that the labeled fibers from the BDA injections in Av traveled in a dorsal–caudal direction along the LaM and splayed out dorsally into HVC (Fig. 9A,B). We also do not see a significant amount of labeled fibers from NIf to HVC (Fig. 9A), suggesting that there is a direct reciprocal connection between HVC and Av that does not depend on a common NIf intermediate. Additionally, tracer injections into HVC showed labeled fibers going toward Av by closely following the LaM and sharply turning upwards to innervate Av right at or near the LaM bend (Fig. 5A,C). This suggests that LaM may serve as a common conduit for both incoming and outgoing fibers traveling between Av and HVC. BDA injections made into Av also result in retrogradely labeled cells specifically within Uva (Fig. 8D), thereby confirming our earlier results of anterograde transport of BDA from Uva injections to Av (Fig. 4). We also find several retrogradely labeled cells within HVC, which is consistent with this nucleus being an input source for the terminal field seen in Av (Fig. 8B). While the 10,000 MW BDA that we used in this study is a good tracer, it is especially useful for anterograde labeling and studies have shown that it is not as sensitive as a retrograde marker (Köbbert et al., 2000; Reiner et al., 2000). As a testament to this fact, Figure

Figure 7. Av neurons are selective for BOS. A: A schematic sagittal section shows the approximate recording sites in and around Av (dotted oval) in CM. The data are grouped into four general regions and displayed in both (A,B): ▲ for the part of CM posterior to Av; ● represents the CM region above Av; magenta square for recordings made in Av; and ○ for the CM area mostly anterior to Av. B: The auditory rate for each group was plotted for each of the three auditory stimuli. Contrary to other areas of CM, recordings in Av (magenta line) show selectivity for BOS over either RBOS or WN. C: Representative example of multiunit song selective responses in Av. (1) Song stimuli. (2) Raster plots for twenty auditory presentations over 10-second intervals. (3) PSTHs for the three different stimuli show greater responses to BOS over RBOS or WN. Fr is the mean firing rate, calculated from 20 trials. (4) Expanded windows of the stimulus response area timeframe reveal greater detail of the different auditory responses. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
9C,D shows examples of many more retrogradely labeled cells in HVC and Uva, respectively, following 3,000 MW dextran rhodamine injections that were made into Av. However, we chose to use BDA for the bulk of our tracing studies for its reliability, ease of use (both technically and photographically), and the fact that it does travel in both directions. Closer inspection of Figure 8B also shows a dense accumulation of labeled terminals circumscribing HVC that may extend into the shelf region that has been previously described (Kelley and Nottebohm, 1979; Mello et al., 1998). However, we have not yet confirmed this by placing restricted injections of tracer into the shelf region to check for retrogradely labeled cells in Av. Also evident, in both the Av and NIF BDA injection experiments, is a significant amount of both retro- and anterograde labeling outside of NIF (Figs. 8A,C, 10A,C). While not as dense or well-defined as the labeling seen in NIF, the importance of these and other unconfirmed labeling patterns that were noticed will be pursued in a later study. It is possible that tracer leakage out of Av resulted in nonspecific labeling of these areas that are outside of the song system. Control injections of BDA that were made outside of Av but within CM resulted in few, if any, labeled terminals in HVC and no retrogradely labeled cells there. We also found that Av is strongly interconnected with NIF (Fig. 8A,C), with many retrogradely labeled cells within this nucleus. Similar to the Av→HVC projections, these results demonstrate another reciprocal feedback pathway originating from Av (Fig. 8C).

To confirm this finding, we iontophoresed BDA into the dorsal part of NIF and found that the resulting pattern of

Figure 8. Tracer injections into Av reveal new connections to the song system. A: An uncounterstained section shows the BDA injection within Av and the resulting label in both HVC and NIF. B: Higher magnification shows many labeled terminals and some retrogradely labeled cells within HVC. C: Close-up of NIF reveals a dense accumulation of afferents and many retrogradely labeled cells within the nucleus. Also evident is labeling that extends outside of NIF, but still needs to be confirmed whether or not it is a specific projection from Av (see text). D: Neutral red counterstain shows retrogradely labeled cells within Uva. Scale bars = 200 μm in A; 50 μm in B,D; 25 μm in C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
retrogradely labeled cells now exposed the Av area more clearly (Fig. 10A,C). The ovoid-shaped mass of labeled cells sits right in the same LaM bend area that defined Av in our earlier experiments (compare Fig. 10B with both Figs. 4B, 5B). Under higher magnification we also found highly branched terminals in Av with the swellings and varicosities characteristic of synaptic endings (Fig. 10D), demonstrating that NIf also projects back to Av.

DISCUSSION

The avalanche nucleus was first discovered in 1982 when Nottebohm et al. (1982) injected HRP into the ca-
1997; Wada et al., 2006; Jarvis, 2007). These authors were the first to include Av in their song system schematics, and Av was also included in the revised avian brain nomenclature diagrams (Reiner et al., 2004). However, Av itself remained relatively obscure, due in part to the absence of tracing studies to show whether or even how this nucleus connects to song system areas other than HVC. As a result, few studies of the song system included Av in their figures or discussed Av as a possible participating member of the song system connection network.

To clarify how Av fits into the song system circuit, our present study is aimed at describing some of the major connections of this nucleus and begins to characterize some of its basic electrophysiological response properties to relevant auditory stimuli. We show that Uva sends afferents to Av, but we do not yet know which subpopulation of Uva cells is specifically involved. We also provide evidence that Av has reciprocal connections to both HVC and NIf. In addition, the label from Av injections to the HVC shelf area as well as a relatively large but indistinct area in the caudal nidopallium might reflect additional, as yet unconfirmed projections, or could reflect projections from the areas immediately around Av to areas around the other song nuclei. If the latter is true, these results would be consistent with past studies that showed that there is a parallel topography of functional gene activation in the HVC shelf region and also in the area between the ventral mesopallium and underlying nidopallium, including both Av and NIf (Feenders et al., 2008). The

**Figure 10.** Tracer injection into NIf exposes reciprocal connections to Av. A: Uncounterstained section shows the BDA injection into NIf and the resulting label in HVC, Av (arrows), and also in areas outside of NIf that have yet to be confirmed. B: Neutral red counterstaining shows that the labeling in CM is in the same location of Av (see Fig. 5B). C: Closer view clearly shows Av as a distinct oval area of terminals and many retrogradely labeled cells. D: Higher magnification shows the highly branched terminals and varicosities (arrowheads) comingle with retrogradely labeled cells. Scale bars = 200 μm in A,B; 50 μm in C; 25 μm in D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
authors hypothesized that this could mean that Av and NIf evolved to be specialized for song from the surrounding brain subdivisions, while maintaining the basic functional properties of these subdivisions. Multiunit recordings in Av display BOS selectivity, in contrast to the CM immediately surrounding Av, which is auditory but nonsong-selective. Our finding of segregated auditory activity in the CM region (Fig. 7A) is similar to the pattern of differential immediate early gene activation discovered in this same area (Feenders et al., 2008). Together with their results, our neurophysiological data suggest that there are several functionally distinct domains of the CM that may play different roles in sensorimotor integration. Specifically, the posterior lateral nidopallium (PLN), which shows both hearing and moving-induced gene expression, overlaps with the area of high but nonsong-selective auditory activity (Fig. 7A, ▲ symbol); Av, which shows vocalization-driven and weak hearing-driven gene expression coincides with the area of BOS-selective auditory responses that we found (Fig. 7A, magenta square symbol); the caudal medial mesopallium (CMM), which shows only hearing-induced gene expression overlaps with the area that responds mainly to broadband noise (Fig. 7A, 〇 symbol).

Since the description of the vocal control nuclei in the canary (Nottebohm et al., 1976), several additions have been made to the song system circuitry (Reiner et al., 2004; Theunissen et al., 2004; Ashmore et al., 2008; Roberts et al., 2008). The new connections to Av, NIf, and HVC now allow us to update the current song system schematic (Fig. 1B) and expand the song system to include part of the mesopallium, an area of the songbird brain that was only recently referred to as being “outside” of the traditional song system (Keller and Hahnloser, 2009). Although we do not yet know the function of Av, the results reported here prompt us to discuss why these new connections may have broad implications for furthering our knowledge of how the song system works.

Uva’s increased importance in the song system
One conclusion of our results is that Uva’s role in the song system is more complex than we once thought. Uva is a small, ovoid nucleus in the thalamus that projects to both HVC and NIf (Nottebohm et al., 1982). By differential fluorescent retrograde labeling, we show that there are two separate classes of projection neurons in Uva, as suggested by Hahnloser et al. (2008). However, even this picture is incomplete. By now showing that Uva also projects to Av, either one (or both) of its neuron populations sends collaterals to Av, or there is a possible third projection neuron type. In any case, the new connections show that Uva’s role in the song system is likely to be more extensive than assumed from its small size and peripheral location within the song system. The reciprocal connections that Av has with HVC and NIf mean that Uva’s influence on the song system also becomes more complicated. Uva neurons respond to a wide variety of auditory stimuli and are thought to affect auditory gating in HVC (Coleman et al., 2007). Cardin and Schmidt (2004) demonstrated that auditory responses in HVC are dependent on the behavioral state of the bird and our previous work has suggested that Uva’s input sources could provide the substrate for both gating and neuromodulatory control (Akutagawa and Konishi, 2005). Bilateral lesions of Uva have also been shown to quickly and irrevocably degrade both song and long calls in the adult male zebra finch (Coleman and Vu, 2005). These results all show that Uva is an essential part of the song system.

New connections to Av provide alternative pathways to HVC
Characterizing the inputs and outputs of Av reveals several new alternative pathways to HVC, a preeminent nucleus that sits atop the song system hierarchy (Doupe and Konishi, 1991; Mooney, 2000; Theunissen et al., 2004). HVC is a sensorimotor nucleus that is essential for song learning and production (Nottebohm et al., 1976). In addition, HVC neurons respond selectively to BOS, and convey this property to other song system areas (Mooney, 2000). Therefore, knowledge of all of the connections to this nucleus is crucial to our understanding of how the song control system works. Our results suggest that Av could be strategically positioned to provide feedforward and feedback control and coordination to two key members of the song system, HVC and NIf. Also, the loop HVC→Av→NIf→HVC adds a previously unconsidered recurrent circuit. Hahnloser and Fee (2007) suggested that NIf could have an alternative route to HVC, based on the longer latency of signal arrival within HVC in response to electrical stimulation of one of the two subpopulations of NIf neurons. Av’s reciprocal connections to both NIf and HVC provide the anatomical basis to support their findings. Thus, it is now no longer as simple as Uva sending signals only to HVC or NIf, or NIf only directly to HVC. Instead, the connections of both Uva and NIf to Av add another set of indirect pathways to HVC. This means that any study that involves signal transmission from Uva up to and including HVC should now take into account this new set of intervening pathways. Such studies include, for example, the bilateral lesion effects of Uva on adult song (Coleman and Vu, 2005); the temporal effects on song structure by cooling HVC (Long and Fee, 2008); thalamic gating of auditory responses in HVC by Uva (Coleman et al., 2007); NIf lesions that affect auditory responses in HVC (Janata and Margoliash, 1999; Cardin and Schmidt, 2004; Coleman and Mooney, 2004);
and intrinsic HVC burst sequences (Jin et al., 2007), among others. Introducing alternative pathways to HVC means that the flow of information into this nucleus becomes more complex.

**Av is a unique subregion of CM**

Our results demonstrate that Av is a specialized area of the mesopallium. Recordings made within Av are song-selective, in contrast to the relatively indiscriminate auditory response patterns displayed in its immediate CM surround. Tracer injections within Av result in both retro- and anterograde staining in several other song system nuclei, while control injections made outside of Av do not. In support of this, an earlier study that made random tracer injections in the CM region also resulted in very few, if any, labeled terminals in HVC or any other song system nuclei, which we now interpret to mean that their injections probably missed Av (Vates et al., 1996). Since we did not explore the entire extent of the CM region, it is possible that other areas could exhibit Av-like properties. However, if song-relevant functions are taking place in CM but outside of Av, it is presently unclear how these areas connect to and therefore communicate with the rest of the vocal control nuclei. We have also provided evidence for complex feedback innervation of both HVC and NIf by Av. What functions could these feedback connections have? Most models of cortical processing focus mainly on serial feedforward excitation. However, recent studies in other species are now acknowledging the importance of feedback control as an effective way to modulate such varied tasks as keeping neuronal discharges proportional to stimulus strength (Douglas et al., 1995), selectively amplifying specific feedforward neural activity patterns (Douglas et al., 1995; Goldberg et al., 2004; Ganguli et al., 2008), and maintaining short-term memory (Goldman, 2009). Av could function to orchestrate the movement of information to and from NIf and HVC, thereby subserving either memory or auditory feedback. Our findings also push the field from the “nucleus to nucleus” formulation of circuitry toward a consideration of a more complex computational interaction between reciprocally interconnected neurons. The new model is more consistent with a formulation of the circuitry operating between complex layers of cortex, rather than the prevailing nuclear model. A recent study demonstrated that CM is important for providing auditory input to both NIf and HVC (Bauer et al., 2008). They also found that a part of CM was active both during song playback and singing, leading them to conclude that this area is important for song perception and processing of auditory feedback. Their tracer injections not only appear to be in the same approximate location as Av (just above the LaM), but the resulting anterograde transport to both HVC and NIf also somewhat resembles the results reported here. Although not specifically targeted, it is possible that their studies were actually based in Av. However, because their tracer results did not include some of the new Av connections reported in this study, either their lentiviral tracer is not as sensitive as BDA, travels primarily in the anterograde direction, or they inadvertently injected only a part of Av, especially since their tracer injections relied solely on stereotaxic coordinates. If they were located in Av, then the findings of Bauer et al. (2008) would be consistent with the singing-driven induced gene expression findings of Av (Jarvis and Nottebohm, 1997; Feenders et al., 2008) and the connectivity and neurophysiological BOS findings of the current study. However, although Av displays singing-driven induced gene expression in adult finches, it is not known whether an intact Av is necessary for normal song. In adult males, for example, bilateral lesions of LMAN (Bottjer et al., 1984), Area X (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), or NIf (Cardin et al., 2005) either do not affect normal song or show small changes in song variability for LMAN lesions (Kao et al., 2005), even though strong singing-induced gene expression and neural activity occurs in all of these nuclei in adults (McClosland, 1987; Jarvis and Nottebohm, 1997; Hessler and Doupe, 1999; Wada et al., 2006; Feenders et al., 2008). However, lesions of LMAN in young birds do affect the development of normal song (Bottjer et al., 1984), so it remains to be seen whether or not Av is more important during the acquisition phase of song development than in the maintenance of adult crystallized song.

**Tracing experiments can reveal hidden brain areas**

The neural circuits for complex vertebrate behavior are seldom recognizable without elaborate processes of reconstruction from stained or marked histological sections. Many of the forebrain areas that control birdsong are unique in this respect, because they and the connecting nerve fibers between them stand out so distinctly in histological sections that the major areas and fiber tracts are visible even to the unaided eye. However, not all functionally distinct brain areas are neatly compartmentalized into well-demarcated nuclei. There are many examples of this even within the zebra finch brain, including the auditory cup around RA (Kelley and Nottebohm, 1979; Mello and Clayton, 1994), field-L and its several subdivisions (Fortune and Margoliash, 1992), and the caudal medial nidopallium (Mello et al., 1992), to name a few. In addition, another relatively obscure nucleus in the forebrain of songbirds, the oval nucleus of the mesopallium (MO) was also discovered via singing-driven gene activation studies (Jarvis et al., 1998; Feenders et al., 2008) and,
similar to Av, has analogs in hummingbirds (Jarvis, 2000) and parrots (Jarvis and Mello, 2000). It is presently unclear how MO connects to other parts of the song system (Fig. 1B, dashed magenta arrow), but the analogous MO nucleus in parrots projects to the Area X and HVC analogs (Striedter, 1994; Durand et al., 1997; Feenders et al., 2008). Av was originally discovered over 30 years ago, but its connections to other parts of the brain have only now been described in detail. Part of the reason may be that Av cannot be distinguished from the surrounding CM by any obvious cytoarchitectural criteria, including cell size, density, or myelinated boundaries. Tract tracing, along with molecular (ZENK, c-fos), immunohistochemical (anti-aromatase), and other indirect methods like magnetic resonance imaging (MRI) studies (Boumans et al., 2008) can effectively expose such hidden brain areas.

Our results here provide the foundation for further research to determine the function of Av in song production or perception. We point out new pathways that increase the complexity of the telencephalic song system circuitry, but also open a new window that allows us to look at the way the song system works with a different perspective. Most studies recently have concentrated mainly on neurophysiological approaches to study the song system. While a powerful tool in its own right, it is difficult for this method alone to discover new brain areas and connections. Other studies concentrated on molecular approaches with activity-dependent genes, which can help identify new brain areas and some functions. But they too leave a gap. Neuroanatomical tracing studies can fill this gap, but they have the limitation that they can only go so far in determining function. Together, all three disciplines can act in synergy to uncover other new areas and connections in the song system that may still lie undiscovered.

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LITERATURE CITED


