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Cortical inter-hemispheric circuits for multimodal vocal learning in songbirds

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Abstract

Vocal learning in songbirds and humans is strongly influenced by social interactions based on sensory inputs from several modalities. Songbird vocal learning is mediated by cortico-basal ganglia circuits that include the SHELL region of LMAN (lateral magnocellular nucleus of the anterior nidopallium), but little is known concerning neural pathways that could integrate multimodal sensory information with SHELL circuitry. In addition, cortical pathways that mediate the precise coordination between hemispheres required for song production have been little studied. In order to identify candidate mechanisms for multimodal sensory integration and bilateral coordination for vocal learning in zebra finches, we investigated the anatomical organization of two regions that receive input from SHELL: the dorsal caudolateral nidopallium (dNCL_{SHELL}) and a region within the ventral arcopallium (Av). Anterograde and retrograde tracing experiments revealed a topographically organized inter-hemispheric circuit: SHELL and dNCL_{SHELL}, as well as adjacent nidopallial areas, send axonal projections to ipsilateral Av; Av in turn projects to contralateral SHELL, dNCL_{SHELL}, and regions of nidopallium adjacent to each. Av on each side also projects directly to contralateral Av. dNCL_{SHELL} and Av each integrate inputs from ipsilateral SHELL with inputs from sensory regions in surrounding nidopallium, suggesting that they function to integrate multimodal sensory information with song-related responses within LMAN-SHELL during vocal learning. Av projections share this integrated information from the ipsilateral hemisphere with contralateral sensory and song-learning regions. Our results suggest that the inter-hemispheric pathway through Av may function to integrate multimodal sensory feedback with vocal-learning circuitry and coordinate bilateral vocal behavior.

Graphical Abstract

Anterograde and retrograde tracing experiments demonstrated inter-hemispheric circuits that integrate multi-sensory inputs with cortical pathways for vocal learning and behavior in songbirds.

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Conflict of interest statement

The authors declare no conflict of interest.

Role of Authors

AKP and SWB designed research; AKP performed research and analyzed data; AKP and SWB wrote the manuscript.

These novel circuits are well-suited to mediate social interactions and coordinate bilateral song-related neural activity.

Keywords

songbird; avian; LMAN SHELL; ventral arcopallium; dorsal caudal nidopallium; bilateral coordination; sensory integration; RRID:AB_10115846; RRID:AB_2336827; RRID:SCR_013672; RRID:SCR_002078; RRID:SCR_010279

Introduction

Vocal learning requires the evaluation of sensorimotor and auditory feedback of immature vocalizations in relation to a memory of sounds learned from an adult tutor. In both humans and songbirds, this process is strongly influenced by social interactions between juveniles and their tutors (Bolhuis, Okanoya, & Scharff, 2010; Goldstein, King, & West, 2003; Johnson, Sablan, & Bottjer, 1995; Jones & Slater, 1996; Kuhl, 2014; Morrison & Nottebohm, 1993; Woolley, 2012). During the learning process, sensory input from social cues - including visual, auditory, tactile, and olfactory interactions (Amy, Salvin, Naguib, & Leboucher, 2015; Jones & Slater, 1996; King, West, & Goldstein, 2005; Krause, Kruger, Kohlmeier, & Caspers, 2012; Mann, Slater, Eales, & Richards, 1991; Ota, Gahr, & Soma, 2015; West & King, 1988) - must be integrated with the memory of tutor sounds and with sensory feedback of current behavior. Vocal learning involving multimodal sensory interactions is more effective than that based on auditory experience alone; for example, juvenile birds who interact visually and acoustically with their tutor produce more accurate imitations of the tutor song than those who experience acoustic interactions only (Chen, Matheson, & Sakata, 2016; Deregnaucourt, Poirier, Kant, Linden, & Gahr, 2013; Eales, 1989; Hultsch, Schleuss, & Todt, 1999; Ljubić, Hyland Bruno, & Tchernichovski, 2016; Price, 1979).

Vocal learning in humans and birds involves complex forebrain circuitry including cortex and basal ganglia. We use the term “cortex” throughout this text in a generic sense as described by Reiner et al. (2004) as including that part of the telencephalon that is “pallial in nature and therefore homologous as a field to the brain region of mammals that includes the neocortex, claustrum, and pallial amygdala” (p. 395). In juvenile songbirds, the cortical region LMAN (lateral magnocellular nucleus of the anterior nidopallium) is required for vocal learning (Aronov, Andalman, & Fee, 2008; Bottjer, Miesner, & Arnold, 1984; Scharff

& Nottebohm, 1991). LMAN consists of CORE and surrounding SHELL regions which make parallel connections through the basal ganglia and thalamus (Fig. 1A) (Bottjer, 2004; Johnson et al., 1995; Luo, Ding, & Perkel, 2001). The LMAN-CORE pathway mediates vocal production in juvenile songbirds (Aronov et al., 2008; Elliott, Wu, Bertram, & Johnson, 2014; Scharff & Nottebohm, 1991), whereas the LMAN-SHELL pathway mediates vocal learning: lesions in the SHELL pathway of juvenile birds prevent the development of stable imitations of the tutor song (Bottjer & Altenau, 2010). The SHELL pathway may evaluate feedback about current vocal performance in relation to a memory of the tutor song encoded by SHELL neurons (Achiro & Bottjer, 2013; Bottjer, Alderete, & Chang, 2010; Bottjer & Altenau, 2010). SHELL circuitry may also process information regarding social cues for vocal learning. However, neural pathways which integrate multimodal sensory information with SHELL circuitry are unknown.

Neural circuits for song behavior, including CORE and SHELL pathways, are located in both hemispheres of the brain. Birds independently control and rapidly switch between left and right sides of the vocal organ during singing, but there has been little evidence for telencephalic pathways between song-control regions which could learn and mediate precise coordination between hemispheres (Ashmore, Bourjaily, & Schmidt, 2008; Johnson et al., 1995; Marc F. Schmidt, 2008; M. F. Schmidt & Wild, 2014; C. Z. Wang, Herbst, Keller, & Hahnloser, 2008; Wild, Williams, & Suthers, 2000).

Previous work from our lab demonstrated that LMAN-SHELL projects to three ipsilateral cortical regions (Fig. 1A-B). One population of SHELL neurons projects to the dorsal portion of the intermediate arcopallium (AId), a region which arches laterally and ventrally away from the lateral border of RA (robust nucleus of the arcopallium) (Bottjer & Altenau, 2010; Bottjer, Brady, & Cribbs, 2000; Johnson et al., 1995). A separate population of SHELL neurons projects to a region within dorsal caudolateral nidopallium (dNCL_{SHELL}) (Bottjer et al., 2000; Iyengar, Viswanathan, & Bottjer, 1999). In zebra finch, immediate early gene expression in dNCL_{SHELL} is greatest in juveniles engaged in both singing and listening to their tutor song, suggesting a role in multimodal learning (Bottjer et al., 2010). In other avian species, dNCL receives visual, auditory, and somatosensory information and is critical in pigeons for working memory and in chicks for filial imprinting, another type of polysensory developmentally-regulated learning (Braun, Bock, Metzger, Jiang, & Schnabel, 1999; Gunturkun, 1997; Gunturkun & Kroner, 1999; Helduser, Cheng, & Gunturkun, 2013; Kroner, Gottmann, Hatt, & Gunturkun, 2002; Letzner, Simon, & Gunturkun, 2016; Leutgeb, Husband, Ritters, Shimizu, & Bingman, 1996; Metzger, Jiang, & Braun, 1998; Shanahan, Bingman, Shimizu, Wild, & Gunturkun, 2013). These data suggest that the SHELL-recipient portion of dNCL may play a role in integrating multimodal information during vocal learning.

The third cortical region that receives input from LMAN-SHELL is located within the ventral arcopallium and is referred to as Av (see below). In zebra finch, Av receives inputs from ipsilateral SHELL and sends projections to contralateral SHELL, forming the only known cortical inter-hemispheric circuit to date between song-related regions (Johnson et al., 1995). This pathway could provide bilateral coordination between left and right SHELL for vocal learning. Studies in pigeons have also identified inter-hemispheric projections from

the arcopallium to sensory association areas in the contralateral hemisphere (Letzner et al., 2016; H. J. Zeier & Karten, 1973), suggesting that the circuit through zebra finch Av could be a component of a conserved pathway coordinating multi-sensory information between hemispheres.

Understanding the neural mechanisms mediating vocal learning and behavior requires precise anatomical knowledge of the circuits involved. This study investigated axonal connections of dNCL_{SHELL} and Av using anterograde and retrograde tracing techniques in order to advance understanding of the SHELL pathway. Our goal was to identify candidate regions and circuits for multimodal sensory integration and bilateral coordination in vocal learning.

Materials and Methods

Subjects

Zebra finches (*Taeniopygia guttata*) were bred and raised by their parents in group aviaries at the University of Southern California. All housing and handling procedures conformed with protocols approved by the University of Southern California Institutional Animal Care and Use Committee. Adult male birds (100-180 days post hatch) were anesthetized with 1.5% isoflurane via inhalation and placed in a stereotaxic apparatus with coordinates set to zero at the bifurcation of the mid-sagittal sinus. A consistent head angle was established by rotating the beak downward from the horizontal plane such that the absolute difference in depth at the skull surface between the bifurcation of the mid-sagittal sinus and a point 5 mm anterior to the bifurcation was 2.2 ± 0.05 mm. Small craniotomies were made above each location to be injected. Following surgery, birds were returned to single or paired cages in the aviary for 4-10 days to allow for tracer transport before being overdosed (Euthasol) and perfused (0.7% saline followed by 10% formalin). Brains were removed and postfixed for 20-50 hours before being cryoprotected in 30% sucrose solution overnight.

Neural Tracers

A total of 116 birds received 188 injections for all experiments described in this study (see Table 1 for injection numbers and stereotaxic coordinates). Cholera Toxin Subunit B (CTB) conjugated to Alexa-488 (green) or Alexa-555 (red/magenta) (Life Technologies, Cat. #C34775 and #C34776) was reconstituted to 1% in sterile 0.2M Phosphate Buffered Saline (PBS) and used to analyze patterns of retrograde label as well as short-range anterograde label. Dextran amines (10,000 molecular weight, 10k-DA) conjugated to either Alexa-488 or Alexa-555 (Life Technologies, Cat. #D22910 and #D34679) were reconstituted to 10% in sterile 0.2M PBS and used to analyze patterns of anterograde label. Tracers were delivered via iontophoresis using pulses of positive current (3-5 μ A, 6 second on/off pulses) for 3-8 minutes for CTB or 10-25 minutes for 10k-DA.

Seventeen birds received dual injections for double-label analysis: CTB conjugated to either Alexa-555 or Alexa-488 was injected into dNCL_{SHELL} and the other color was injected into Av to retrogradely label cells in ipsilateral LMAN-SHELL and in contralateral Av. Experiments were counterbalanced to ensure that no bias emerged due to tracer transport

properties. As a control, injections of a 50:50 mixed solution of Alexa-555- and Alexa-488-conjugated CTB were made into dNCL_{SHELL} (n=1 injection) or into Av (n=2 injections) to test the ability of both tracer colors to be retrogradely transported and produce double-labeled cells.

Histology

In order to maintain a consistent sectioning angle between brains, a video camera was mounted inside a cryostat and connected to a computer so that the brain could be viewed from the side on the computer monitor. Brains were blocked on their anterior edge and mounted inside the cryostat (anterior-side down) on a tilting platform. Several sections of the cerebellum and hindbrain were removed to establish a flat posterior surface. A protractor was used to measure the angle between the cut posterior surface and the dividing line between the cerebrum and optic lobe (Karten et al., 2013). The tilt of the brain was adjusted until the angle measured between 67-71 degrees. Cases in which this cutting angle was not able to be achieved were used only to supplement findings of brains cut within the required angle range (see Table 1). Coronal sections of 50 μ m were collected onto gelatin-coated glass slides in two alternate series. After 30 hours of air-drying, one series of slides was coverslipped with Prolong Diamond Antifade (LifeTechnologies, Cat. #P36965) and used for fluorescent analysis, and the alternate series was counterstained with thionin and coverslipped with Permount (Fisher Scientific, Cat. #SP15-500) for Nissl visualization of anatomical landmarks (Bottjer et al., 2000; Karten et al., 2013) (Figure 1D, F).

In seven brains, the alternate series was stained for calbindin using established immuno-histochemical procedures. Calbindin expression specifically labels thalamic axons terminating in LMAN (Achiro & Bottjer, 2013; Pinaud, Saldanha, Wynne, Lovell, & Mello, 2007) and delineates the borders of LMAN-SHELL precisely (Fig. 1D, lower panel). Briefly, birds were perfused with 4% paraformaldehyde (PFA) containing 0.4% glutaraldehyde, and brains were post-fixed in 4% PFA for 30-50 hours before being immersed in 30% sucrose. During cryostat sectioning, one series was mounted directly onto slides and coverslipped with Prolong Diamond Antifade, and the alternate series was collected free-floating into 10% PBS. Floating sections were incubated with 1:5000 monoclonal mouse anti-bovine kidney calbindin 28K antibody (Sigma-Aldrich Cat#C948, St Louis, RRID:AB_10115846) overnight at room temperature before being processed with the VECTASTAIN Elite ABC-Peroxidase kit (Vector Laboratories, Cat# PK-7100, RRID:AB_2336827). Sections were stained with 3,3'-Diaminobenzidine (Sigma) using increasing concentrations (0.003% and 0.015%) of H₂O₂, mounted on gelatin-coated slides and coverslipped with Permount. In 3 brains, sections stained for calbindin were also counter-stained with thionin for dual visualization of LMAN-CORE and LMAN-SHELL.

Histological analysis

Slides were examined on a Zeiss LSM 510 confocal microscope and images were acquired using ZEN Digital Imaging software (www.zeiss.com/microscopy/en_us/products/microscope-software/zen.html, RRID:SCR_013672). In some cases, stacks of images were acquired at depth intervals of 1 μ m and combined as maximum-intensity images to visualize fine axon terminals in a range of focal depths. Comparison of fluorescent images with

alternate sections stained for Nissl or calbindin expression allowed anatomical identification of the location of injection sites and transported label, in reference to published canary and zebra finch brain atlases (Karten, Hodos, Nauta, & Revzin, 1973; Nixdorf-Bergweiler & Bischof, 2007; Stokes, Leonard, & Nottebohm, 1974) and nomenclature adopted in Reiner et al. (2004). Descriptions of control injection locations indicate the distance from the center of the injection site to the approximate border of the adjacent region. Image brightness, contrast, and color were adjusted using Adobe Photoshop CC (www.adobe.com/photoshop, RRID:SCR_002078), including converting Red-Green images to Magenta-Green images. In subsequent text and figures, Alexa-555 is referred to as magenta colored, while Alexa-488 is referred to as green. ZEN digital Imaging software and Adobe Illustrator CC (www.adobe.com/illustrator, RRID:SCR_010279) were used to annotate fluorescent images with anatomical boundaries identified on alternate sections stained for Nissl or calbindin and to create figure schematics.

Double-label analysis

For double-label experiments, images of both magenta and green fluorescence were acquired and merged to identify double-labeled cells in Av or in LMAN-SHELL. In some cases, serial images of double-labeled cells acquired at depth intervals of 1 μm were used to confirm the co-localization of both dyes within a focal plane in individual cells (i.e., to distinguish double-labeled from single-labeled cells that overlapped in different focal planes).

In LMAN-SHELL, the proportion of double-labeled somata out of all retrogradely labeled cells was quantified by counting fluorescent cell profiles using the Cell Counter plugin in ImageJ (<http://imagej.net/ImageJ>, RRID:SCR_003070). A labeled profile was counted if its size was at least one-third the diameter of an entire soma. Images were separated into magenta or green color channels, and all labeled profiles were manually marked and counted as magenta or green. Labeled profiles present in both color channels were counted as double-labeled. The proportion of double-labeled profiles was quantified using two counting methods. In one method ("TOTAL"), all fluorescently-labeled profiles in confocal images identified within Nissl-defined boundaries of LMAN-SHELL were tallied as magenta, green, or double-labeled. However, because the quantity of labeled profiles varied based on the size and exact location of each injection, images within SHELL often included subregions containing only magenta or only green labeled profiles in addition to subregions containing magenta, green, and double-labeled profiles. To assess the proportion of double-labeled cells specifically within regions of LMAN-SHELL well labeled by both individual injections, a second counting method ("BOX") was also carried out on the same images analyzed by the TOTAL method: 1-3 boxes of fixed area ($150 \times 150 \mu\text{m}$) were placed in areas of SHELL containing a high density of both magenta and green labeled cells. In this latter method, labeled profiles were counted only if more than half of the labeled area fell within the box boundary. Fluorescently-labeled profiles within the boundaries of these boxes were tallied to quantify the number of both single- and double-labeled cells within areas strongly labeled by both injections. Single-labeled cells were categorized as Av-labeled or dNCL_{SHELL}-labeled for comparison between birds, as injections of magenta- and green-labeled CTB into Av and dNCL_{SHELL} were counterbalanced. In order to control for

variations in the size and quality of injections, we calculated the proportion of double-labeled cells both as a percentage of the total population labeled by Av injections (Av-labeled) or by the total population labeled by dNCL_{SHELL} injections (dNCL_{SHELL}-labeled). A correction was applied in order to estimate cell number based on profile counts (Abercrombie, 1946): neuron number (N) was determined by multiplying the profile count (n) by a correction factor obtained by dividing the section thickness (T = 50) by the sum of section thickness plus the average soma diameter (d = 14.5): $N = n(T/(T+d))$. It should be noted that this correction factor did not change the percentages of double-labeled cells because the same correction was applied to all counts.

We assessed the topographic matching of our dual injections into dNCL_{SHELL} and Av by comparing terminal anterograde label in Av from dNCL_{SHELL} injections with the location of Av injections. Degree of overlap (representing how well the injections were topographically matched) was calculated as the percentage of the Av injection site which overlapped with the area of terminal label in Av from the dNCL_{SHELL} injection. Areas were measured using the “region of interest” tool in ImageJ. We compared the degree of overlap with the proportion of double-labeled SHELL neurons to assess whether one sub-population of SHELL neurons that project to dNCL_{SHELL} also send branches to Av while other sub-populations project to only dNCL_{SHELL} or Av (see below).

Results

Characterization of dNCL_{SHELL} and adjacent regions

We injected neural tracers into SHELL-recipient dNCL and adjacent control regions in order to characterize the complete axonal connections of this area. As described in detail below, injections of retrograde tracers revealed that dNCL_{SHELL} receives topographically organized inputs from 1) ipsilateral LMAN-SHELL and surrounding regions of anterior nidopallium, 2) contralateral ventral arcopallium (Av), and 3) local inputs from surrounding regions of dNCL. Injections of anterograde tracers showed that dNCL_{SHELL} sends topographically organized projections to ipsilateral AId and Av. Previous work has shown that LMAN-SHELL, like dNCL_{SHELL}, projects to ipsilateral AId and Av (Fig. 1A, B), and that Av projects in turn to contralateral LMAN-SHELL (Johnson et al., 1995). Thus, both LMAN-SHELL and dNCL_{SHELL} participate in a cortical inter-hemispheric circuit via Av (Fig. 1B, and see below). Control injections into areas of dNCL adjacent to dNCL_{SHELL} revealed that the SHELL→dNCL_{SHELL}→AId pathway carrying information important for vocal learning is part of a larger pattern of projections from the anterior nidopallium to dNCL to the arcopallium (Bottjer et al., 2000).

Topographic organization of inputs to dNCL_{SHELL} and adjacent regions

Inputs to dNCL_{SHELL} from LMAN-SHELL and surrounding anterior nidopallium

—The topographic pattern of retrograde label in LMAN-SHELL and the adjacent anterior nidopallium following injections of CTB into dNCL_{SHELL} is illustrated in Figure 2A-C. Injections into mid dNCL_{SHELL} (n=11) produced retrogradely labeled cell bodies concentrated within ventral and dorsal LMAN-SHELL, as well as dorsal to SHELL, with few cells labeled in medial or lateral SHELL (Fig. 2A). Injections into lateral dNCL_{SHELL}

(n=10, Fig. 1E) produced retrogradely labeled somata in lateral SHELL and dorsolateral to SHELL; a lower density of labeled cells was located lateral to SHELL (Fig. 2B). Injections into medial dNCL_{SHELL} (n=13) produced retrogradely labeled cells concentrated in medial SHELL as well as dorsal and medial to medial SHELL; the latter region was located between LMAN-SHELL and medial magnocellular nucleus of the anterior nidopallium (MMAN) (Foster & Bottjer, 2001; Foster, Mehta, & Bottjer, 1997; Reiner, Perkel, Bruce, et al., 2004). Figure 2C shows this topographic pattern of retrograde label in SHELL and adjacent regions following dual tracer injections into medial and mid dNCL_{SHELL}. In addition, we found that projections from SHELL to dNCL_{SHELL} are organized along the anterior-posterior axis (Fig. 2I). Injections into anterior dNCL_{SHELL} (n=6) produced retrogradely labeled cells in dorsal and anterior SHELL, dorsolateral to anterior SHELL, and in the nidopallium immediately anterior to SHELL. In contrast, injections into posterior dNCL_{SHELL} (n=4) labeled cells in posterior and ventral SHELL, and in the nidopallium directly posterior to SHELL. The patterns of retrograde label that we observed are consistent with previous work from our lab demonstrating that injections of anterograde tracers into medial versus lateral LMAN-SHELL produce topographic terminal label in medial and lateral dNCL_{SHELL}, respectively (Bottjer et al., 2000; Iyengar et al., 1999). However, the current results show that the SHELL-recipient region of dNCL receives topographically organized inputs not only from neurons within the borders of LMAN-SHELL, but also from regions of the anterior nidopallium surrounding SHELL.

The border between CORE and SHELL regions of LMAN can be distinguished based on the density of magnocellular somata (Fig. 1F), and the outer borders of SHELL can be distinguished from surrounding regions based on the intensity of neuropil staining. Some brains were stained immunohistochemically for calbindin, which specifically labels thalamic axons projecting to LMAN (Fig. 1D, lower panel) (Achiro & Bottjer, 2013; Pinaud et al., 2007). Calbindin expression defined the outer borders of LMAN-SHELL precisely, and revealed that even small injections into dNCL_{SHELL} produced retrograde label within regions immediately surrounding SHELL (n=4). Therefore, inputs to dNCL_{SHELL} include not only those from LMAN-SHELL, but also from adjacent regions of anterior nidopallium which convey visual, auditory, and trigeminal somatosensory information (Karten et al., 1973; Krutzfeldt & Wild, 2004; Letzner et al., 2016; Wild & Farabaugh, 1996; Wild & Gaede, 2016; Wild & Williams, 1999). These data suggest that dNCL_{SHELL} may integrate song-learning related information from LMAN-SHELL with multiple types of sensory information important for social vocal learning.

Inputs to dNCL_{SHELL} from contralateral Av—Injections of retrograde tracer into SHELL-recipient dNCL also produced labeled cells in the parvocellular-rich ventral portion of the contralateral arcopallium, within a central region along the ventral edge of the telencephalon (Fig. 2D, E). We designated this region as Av, following the terminology of Johnson et al. (1995). The anterior-posterior range of retrogradely labeled cells in Av contralateral to dNCL_{SHELL} injections extended from the level of posterior nucleus taeniae of the amygdala (TnA) rostrally to the level of posterior RA/AId caudally (Fig. 2G-H). Labeled cells were concentrated within the central portion of the ventral arcopallium, beneath the occipito-mesencephalic (OM) tract, with scattered labeled cells extending

dorsally into the OM tract at more anterior levels. The maximal extent of the region retrogradely labeled by dNCL_{SHELL} injections was ca. 950 μ m (medial-lateral) by 250 μ m (dorsal-ventral) by 850 μ m (anterior-posterior). Distinct borders of Av could not be distinguished from adjacent regions of ventral arcopallium based on differences in size or density of Nissl-stained somata, except at anterior levels where labeled cells were bounded medially by TnA and by the fronto-arcopallial tract (FA) laterally (Fig. 2H). Johnson et al. (1995) described a similar region of ventral arcopallium, bounded by TnA, FA, and OM, which receives axonal projections from ipsilateral SHELL and sends projections to contralateral SHELL. The labeled cells in Av resulting from our dNCL_{SHELL} injections overlapped with the region described by Johnson et al., but also extended farther posteriorly (Fig. 2G; and see below, Fig. 8).

The projection from Av to contralateral dNCL_{SHELL} exhibited topographic organization along the medial-lateral axis. Injections into medial dNCL_{SHELL} (n=13) produced retrogradely labeled cells in medial contralateral Av, while injections into lateral dNCL_{SHELL} (n=10) labeled cells in lateral contralateral Av. Figure 2D shows retrogradely labeled cells in Av following two adjacent, slightly overlapping injections of CTB conjugated to different color dyes into medial and mid dNCL_{SHELL} (Fig. 2C). The labeled cells in Av reflected a corresponding medial-to-lateral topography, with the middle region of labeled cells containing both single-labeled cells and some double-labeled cells. Even well separated, non-overlapping pairs of injections within dNCL_{SHELL} produced some degree of overlapping retrograde label in Av, indicating that small subregions of Av project to large areas of contralateral dNCL_{SHELL} (n=3 injection pairs).

Inputs to regions adjacent to dNCL_{SHELL}—We made control injections of retrograde tracer into regions of the caudal nidopallium adjacent to SHELL-recipient dNCL to investigate whether dNCL_{SHELL} is part of a larger pattern of connectivity. These control injections yielded a general pattern consisting of retrogradely labeled cells in regions adjacent to those labeled by dNCL_{SHELL} injections, maintaining a topographic organization. For example, injections directly adjacent to dNCL_{SHELL} produced retrogradely labeled cells in the anterior nidopallium adjacent to SHELL that overlapped partially with those labeled by dNCL_{SHELL} injections, whereas injections farther away from dNCL_{SHELL} produced labeled cells farther away from SHELL.

Figure 3A shows a control injection lateral to dNCL_{SHELL} that produced retrogradely labeled cells ipsilaterally in the far lateral anterior nidopallium, ventrolateral to LMAN-SHELL and extending anteriorly and posteriorly from the level of LMAN (n=3). The distribution of labeled cells extended to the lateral edge of the nidopallium; Figure 3B shows sparsely distributed labeled cells lateral to the Basorostral nucleus (Bas). Injections like this one, located >300-400 μ m lateral to dNCL_{SHELL}, produced retrogradely labeled cells that overlapped very little or not at all with those projecting to dNCL_{SHELL} (compare Figs. 2 and 3), whereas injections immediately lateral to dNCL_{SHELL} (within ca. 350 μ m) produced retrogradely labeled cells closer to LMAN-SHELL that partially overlapped with those projecting to dNCL_{SHELL}. However, the labeled cells we observed in the far lateral anterior nidopallium do overlap with cells that are part of the trigeminal sensorimotor circuitry involved in beak, jaw and tongue movements for feeding and articulatory behaviors (Ohms,

Snelderwaard, Ten Cate, & Beckers, 2010; Sadananda & Bischof, 2006; Suthers, Rothgerber, & Jensen, 2016; Wild, Arends, & Zeigler, 1985; Wild & Farabaugh, 1996). Control injections immediately lateral to dNCL_{SHELL} also produced retrogradely labeled cells in the contralateral ventral arcopallium, overlapping with lateral Av and extending further laterally. Control injections located farther lateral to dNCL_{SHELL} produced retrograde label in the ventral arcopallium lateral to contralateral Av, but not within Av itself (Fig. 3A).

Control injections posterior to dNCL_{SHELL} (n=3) produced labeled cells posterior to LMAN-SHELL (Fig. 3I), whereas injections anterior to dNCL_{SHELL} (n=4) produced labeled cells anterior to SHELL (Fig. 3J). This pattern extends the anterior-posterior organization of the projection from anterior nidopallium to caudal nidopallium shown in Figure 2I, although these control injections produced a lower density of retrogradely labeled cells distributed in smaller areas of the anterior nidopallium than did equivalently sized injections into dNCL_{SHELL}. Injections located <300 μ m anterior or posterior to dNCL_{SHELL} produced retrograde label that partially overlapped the area retrogradely labeled by dNCL_{SHELL} injections, while injections farther than 300 μ m anterior or posterior to dNCL_{SHELL} produced non-overlapping label anterior or posterior to SHELL, respectively. In addition, control injections within 300 μ m of the anterior or posterior borders of dNCL_{SHELL} yielded labeled cells contralaterally within the ventral arcopallium, overlapping with and extending slightly beyond anterior or posterior Av, respectively. Sparse retrograde label was also identified anterior to contralateral Av following control injections >300 μ m anterior to dNCL_{SHELL} (Fig. 3J, right panels). In contrast, no labeled cells were observed in the contralateral ventral arcopallium following control injections located >300 μ m posterior to dNCL_{SHELL}.

Control injections ventral to dNCL_{SHELL} (n=5) produced retrogradely labeled cells posterior, ventrolateral and anterolateral to LMAN-SHELL as shown in a caudal-to-rostral series in Figure 3C. In their posterior-most extent, labeled cells were concentrated caudal to LMAN-SHELL (Fig. 3D) and adjacent to Bas (Fig. 3E). At the level of LMAN, labeled cells were located ventrolateral to SHELL (Fig. 3F). Anterior to LMAN, scattered labeled cells were distributed in the lateral anterior nidopallium, concentrated near the Pallial-Subpallial Lamina (LPS) and the Mesopallial Lamina (LaM) (Fig. 3G). Labeled cells in the anterior nidopallium following injections located <300 μ m from dNCL_{SHELL} overlapped partially with cells labeled by dNCL_{SHELL} injections; injections >300 μ m from dNCL_{SHELL} produced non-overlapping retrograde label. In addition, all control injections ventral to dNCL_{SHELL}, including those up to 500 μ m away, produced retrogradely labeled cells in contralateral Av (Fig. 3H), overlapping with the region labeled by dNCL_{SHELL} injections and extending further dorsally though OM up to the ventral border of AId. All ventral control injections maintained medial-lateral topographic organization.

In summary, injections ventral, lateral, anterior and posterior to dNCL_{SHELL} produced retrogradely labeled cells in a topographic pattern within the anterior nidopallium, but no injection located more than 300-400 μ m away from dNCL_{SHELL} labeled cells within LMAN-SHELL or immediately surrounding regions. The pattern of labeling revealed a continuum such that control injections just outside of dNCL_{SHELL} produced retrogradely

labeled cells in the nidopallium immediately adjacent to LMAN (as did injections within dNCL_{SHELL}), whereas injections located farther away from dNCL_{SHELL} did not overlap with labeled cells surrounding SHELL. These data demonstrate that the projection from LMAN-SHELL to dNCL_{SHELL} is contained within a larger pattern of topographically organized projections from the anterior nidopallium to dNCL. In addition, regions immediately adjacent to dNCL_{SHELL} also receive input from the contralateral ventral arcopallium that overlaps with Av. The region ventral to dNCL_{SHELL} is more extensively connected with Av, since control injections up to 500 μ m away receive input from Av as well as the region dorsal to Av.

No retrogradely labeled cells were seen in primary or higher levels of auditory cortex (Field L and caudo-medial nidopallium (NCM)) (Bolhuis & Gahr, 2006; Fortune & Margoliash, 1992; Reiner, Perkel, Bruce, et al., 2004) following injections into dNCL_{SHELL} or adjacent regions of dNCL. Although auditory cortex does project to dNCL in pigeons and barn owls (Cohen, Miller, & Knudsen, 1998; Wild, Karten, & Frost, 1993), our results are in accordance with the findings of Vates et al. (1996) that injections into Field L did not produce anterograde label in dNCL of zebra finches.

dNCL_{SHELL} and adjacent regions participate in a local network—All injections of CTB into dNCL_{SHELL} produced retrogradely labeled cells surrounding the injection site. The highest density of labeled cells occurred within 250 μ m of the injection site, and decreased with increasing distance from the center of the injection site. Medial and lateral to the injection site, labeled cells were located at distances up to 300 μ m away from the borders of the injection site. In contrast, retrogradely labeled cells were located as far as 700 μ m anterior and 500 μ m posterior to the borders of the injection, indicating a greater range of local anterior-posterior connectivity compared to medial-lateral connectivity. Figure 2F shows an example of retrogradely labeled cells located 600 μ m anterior to the border of the injection site. This pattern of intrinsic labeling was seen following injections into dNCL_{SHELL} (n=44) as well as following control injections anterior (n=4), posterior (n=4), or lateral (n=3) to dNCL_{SHELL}, indicating the presence of a highly interconnected local network connecting anterior and posterior regions of dNCL, including dNCL_{SHELL}. Such a local network could provide integration throughout dNCL of sensory information from the anterior nidopallium, and thereby contribute to multimodal learning.

Topographic organization of efferent projections from dNCL_{SHELL} and surround

Topographic projections of dNCL_{SHELL} to ipsilateral Aid and Av—Anterograde projections from the SHELL-recipient portion of dNCL were analyzed using both 10k-DA and CTB. Although CTB is primarily a retrograde tracer, it also labels strong anterograde projections over short distances (Letzner et al., 2016; Shimizu, Cox, Karten, & Britto, 1994). Previous work from our lab showed that injections into Aid produce abundant retrogradely labeled cells in ipsilateral dNCL_{SHELL} as well as ipsilateral LMAN-SHELL (Bottjer et al., 2000; Iyengar et al., 1999). As expected, our injections of anterograde tracers into dNCL_{SHELL} (Fig. 4B) produced robust terminal label in ipsilateral Aid. Figure 4C shows labeled axons coursing ventrally following an injection into dNCL_{SHELL}; these axons crossed the dorsal archistriatal lamina (LAD) and terminated in Aid (Fig. 4A,D-F).

A summary schematic of the topographic arrangement of efferent projections of dNCL_{SHELL} is shown in Figure 4A. Injections into lateral dNCL_{SHELL} (n=13) produced dense terminal label within lateral AId (Fig. 4F), and injections into the center of SHELL-recipient dNCL_{SHELL} (n=14) produced terminal label in the middle region of AId (Fig. 4E). Injections into medial dNCL_{SHELL} labeled axons that terminated in a narrow portion of medial AId immediately lateral to RA, which we refer to as the “neck” region (Fig. 4D) (n=17). Terminal label stemming from medial injections also extended dorsally and ventrally beyond this neck region, wrapping around the lateral borders of RA. This region of label overlaps partially with a region that surrounds RA and receives input from auditory cortex, known as RA cup (Kelley & Nottebohm, 1979; Mello, Vates, Okuhata, & Nottebohm, 1998; Reiner, Perkel, Mello, & Jarvis, 2004).

Injections of anterograde tracers into dNCL_{SHELL} also labeled axons which continued ventrally below the terminal field in AId to terminate within ipsilateral Av (Fig. 4A, D, G-H). We were unable to distinguish whether the axons terminating in Av represented branches of dNCL_{SHELL} axons projecting to AId or originated from a separate population. Projections from dNCL_{SHELL} to Av were topographically organized: injections into lateral dNCL_{SHELL} produced anterograde label in lateral Av whereas injections into medial dNCL_{SHELL} produced label in medial Av. Non-overlapping dual injections into mid and lateral dNCL_{SHELL} produced terminal label in mid and lateral AId and Av, respectively. In Av, but not AId, a middle region contained overlapping terminal label of both colors (Fig. 4H, n=3 injection pairs). This pattern indicates that large areas of dNCL_{SHELL} converge into overlapping, smaller regions of Av.

The topography of the pathways described here, from LMAN-SHELL to dNCL_{SHELL} and from dNCL_{SHELL} to AId, matches the topographic organization of a separate population of SHELL neurons projecting directly to AId (Bottjer et al., 2000). LMAN-SHELL thus sends information with conserved topography to ipsilateral AId both directly and indirectly.

Efferent projections of regions adjacent to dNCL_{SHELL}—Anterograde label produced by control injections into regions adjacent to dNCL_{SHELL} demonstrated that efferent projections from dNCL_{SHELL} to ipsilateral AId and Av are contained within a larger pattern of projections from dNCL to the arcopallium.

A schematic in Figure 5A and photomicrograph in Figure 5D depict the results of a control injection of anterograde tracer lateral to dNCL_{SHELL}. Terminal label following lateral control injections (n=5) was located lateral to ipsilateral AId, but not within AId itself. Injections immediately adjacent to dNCL_{SHELL} (<400 µm) produced terminal label at the lateral edge of AId that extended further laterally. This pattern, along with the retrograde label in anterior nidopallium described above (Figs. 2, 3), corresponds with previous findings that lateral caudal nidopallium (lateral to dNCL_{SHELL}) receives input from lateral anterior nidopallium (lateral to LMAN-SHELL) and projects to lateral arcopallium; this pathway is part of a trigeminal sensorimotor circuit (Sadananda & Bischof, 2006; Wild & Farabaugh, 1996; Wild & Krutzfeldt, 2012). Control injections <400 µm lateral to dNCL_{SHELL} also produced ipsilateral terminal label within lateral Av as well as farther laterally within the ventral arcopallium; injections >400 µm lateral to dNCL_{SHELL} produced

terminal label only lateral to Av. This result complements the pattern of label in the contralateral ventral arcopallium following control injections of retrograde tracer lateral to dNCL_{SHELL} (see above and Fig. 3A) and suggests that a medial-lateral continuum exists in the connections between dNCL_{SHELL}-to-Av and lateral dNCL-to-lateral ventral arcopallium in both hemispheres.

Additional control injections were located anterior (n=4) or posterior (n=4) to dNCL_{SHELL}; labeled axons from injections >300 µm away from dNCL_{SHELL} crossed LAD to terminate in the arcopallium directly anterior or posterior, respectively, to AId but not within AId itself (Fig. 5E-F). Anterior and posterior control injections <300 µm from dNCL_{SHELL} produced a similar pattern of label that also encompassed the anterior and posterior borders of AId, respectively. In comparison with the bright, densely concentrated terminal label in AId following dNCL_{SHELL} injections of equivalent sizes, anterior and posterior control injections produced terminal label that was weaker in intensity and distributed over larger areas. In addition, sparse terminal label extended into the ventral arcopallium following anterior and posterior control injections <300 µm from dNCL_{SHELL}, partially overlapping with Av and extending either anteriorly or posteriorly. Injections located >300 µm anterior to dNCL_{SHELL} produced sparse terminal label in the ventral arcopallium anterior to Av. No terminal label was observed in the ventral arcopallium following control injections at least 300 µm posterior to dNCL_{SHELL}.

Control injections located ventral to dNCL_{SHELL} (n=5) produced terminal label in the ipsilateral arcopallium both dorsal and ventral to AId, as previously described (Bottjer et al., 2000), organized in a medial-to-lateral topography (Fig. 5A-C). The arrow in Figure 5B indicates labeled fibers passing through AId to terminate ventral to AId, indicating the absence of terminal label within AId itself for control injections >300 µm ventral to dNCL_{SHELL}. All control injections ventral to dNCL_{SHELL} produced terminal label within Av and dorsal to Av; following more ventral injections (300-500 µm away), terminal label tended to encompass the entire area from the ventral border of AId to the ventral edge of the telencephalon, overlapping with Av (Fig. 5B-C). In contrast, injections within dNCL_{SHELL} produced terminal label within AId (and directly adjacent to the neck of AId in cases of medial injections) and within Av, but not within the region between AId and Av (Fig. 4D, arrowheads).

In summary, control injections more than 300-400 µm ventral, lateral, anterior and posterior to dNCL_{SHELL} produced anterograde label in regions of ipsilateral arcopallium adjacent to AId, maintaining overall medial-lateral and anterior-posterior topography, while injections within dNCL_{SHELL} produced strong terminal label specifically within AId. Injections both within and ventral to dNCL_{SHELL} also produced strong anterograde label in ipsilateral Av. Injections into all regions immediately adjacent to dNCL_{SHELL} tended to produce label overlapping with Av and extending slightly into surrounding ventral arcopallium. Injections at least 300-400 µm lateral or anterior to dNCL_{SHELL} produced terminal label lateral or anterior to Av, whereas injections more than 300 pm posterior to dNCL_{SHELL} did not produce terminal label anywhere in the ventral arcopallium. These data, along with results of the retrograde studies described above, indicate that the song-learning circuitry that includes LMAN-SHELL, dNCL_{SHELL}, and AId/Av is a component of a general pattern of

connectivity connecting the anterior nidopallium (LMAN and surround) to the caudal nidopallium (dNCL) to the arcopallium. However, connections within the song-learning circuit are more robust than those connecting adjacent regions.

Inter-hemispheric circuits connecting song regions in both hemispheres—To confirm that the results of our anterograde and retrograde studies of dNCL_{SHELL} indicated the presence of an inter-hemispheric circuit through Av, we made dual injections of differently colored tracers into left and right dNCL_{SHELL} (Fig. 6A). When injections were topographically well matched (n=3 injection pairs), the anterograde projection from dNCL_{SHELL} to ipsilateral Av strongly overlapped the area containing retrogradely labeled cells of the alternate color (Fig. 6B, C). This pattern confirms the presence of a novel inter-hemispheric circuit, shown in blue in Figure 6D: left dNCL_{SHELL} projects to left Av, which projects to right dNCL_{SHELL}, which projects to right Av, which returns to left dNCL_{SHELL}. In addition, small areas of Av receive inputs from large areas including ipsilateral dNCL_{SHELL} and the region ventral to it before projecting contralaterally. Furthermore, Av is known to receive inputs from ipsilateral LMAN-SHELL and project to contralateral SHELL, shown in red in Figure 6D (Johnson et al., 1995). These data suggest that Av serves an integrative role in two inter-hemispheric circuits directly connecting cortical song learning regions.

Characterization of Av and adjacent regions

To further investigate patterns of axonal connectivity of Av with LMAN-SHELL and dNCL_{SHELL}, we injected anterograde and retrograde tracers into Av and adjacent regions. As described in detail below and depicted in Figure 1B, the results revealed that Av receives ipsilateral input from both dNCL_{SHELL} and LMAN-SHELL as well as surrounding regions. Dual injections of differently colored tracers into dNCL_{SHELL} and Av revealed a subpopulation of double-labeled neurons in LMAN-SHELL that send axonal branches to both of these post-synaptic targets. In turn, Av sends contralateral projections to dNCL_{SHELL} and LMAN-SHELL, as well as areas of nidopallium adjacent to each. Av also projects directly to contralateral Av, and some single Av neurons send collateral branches to both contralateral Av and dNCL_{SHELL}.

Topographic organization of inputs to Av

Inputs to Av from ipsilateral dNCL—Injections of CTB into Av produced a high density of intensely labeled somata in ipsilateral dNCL_{SHELL}, confirming that dNCL_{SHELL} sends a strong anterograde projection to Av (Fig. 4D,G-H). Retrograde label following even small Av injections was not restricted to dNCL_{SHELL}, but extended into immediately adjacent regions (up to 300-400 μ m away), as well as the area ventral to dNCL_{SHELL} up to 500-600 μ m away, indicating that small areas of Av integrate inputs from large parts of the caudal nidopallium (Fig. 7A). Figure 7A depicts the medial-lateral topography of afferent inputs to Av from ipsilateral dNCL. Injections into medial Av (n=8) (green, Fig. 7B) produced retrogradely labeled cells in medial dNCL_{SHELL} (Fig. 7D) as well as ventral to medial dNCL_{SHELL}. Following injections into lateral Av (n=4) (magenta, Fig. 7B), retrogradely labeled cells were located in lateral dNCL_{SHELL} (Fig. 7E) and in adjacent regions ventral and immediately lateral to dNCL_{SHELL}.

Extremely sparse retrograde label was also located within the dorsal-intermediate arcopallium following injections into Av (n=27), primarily located near the ventral, dorsal, and lateral borders of AId and anterior to AId, at distances 200 μ m or further from the track left by the injection electrode. Following 10 of 47 injections into Av, 3-6 brightly labeled cells were located within AId as well as in adjacent arcopallium (Fig. 7C). This may indicate a sparse projection from AId and adjacent arcopallium into Av; alternatively, as the arcopallium is known to send efferent projections through the OM fiber tract (Bottjer et al., 2000; Letzner et al., 2016; H. J. Zeier & Karten, 1973), it is possible this sparse label resulted from tracer absorbed by axons of arcopallial neurons passing through OM adjacent to the injection site.

Inputs to Av from ipsilateral LMAN-SHELL and surrounding anterior

nidopallium—Injections of CTB into Av also produced retrograde label in the ipsilateral anterior nidopallium in and around LMAN-SHELL, organized with medial-lateral topography (Fig. 7A, F-H). Medial Av injections produced retrogradely labeled cells in medial SHELL (Fig. 7G) and adjacent regions in the medial, anterior, and posterior directions (n=8, Fig. 7F). Injections into lateral Av produced labeled cells in lateral SHELL which extended into adjacent regions of anterior nidopallium in the dorsolateral, ventrolateral, anterior, and posterior directions (n=4, Fig. 7A, H). Dual adjacent injections of different colors into medial and lateral Av (Fig. 7A, B) produced topographically organized retrograde label in SHELL and adjacent regions of nidopallium, with a small number of double-labeled cells in areas of topographic overlap, as seen in the nidopallium posterior to SHELL in Figure 7F (double-labeled cells indicated by arrowheads). Injections of CTB into Av also produced sparse retrograde label within ipsilateral LMAN-CORE, consistent with the findings of Johnson et al. (1995).

Direct inter-hemispheric pathway from Av to contralateral Av—Injections of retrograde tracer into Av also produced a small cluster of labeled cells in contralateral Av as shown in Figure 7I-J. Injections located in medial Av (n=8) produced weakly labeled cell bodies in contralateral medial Av, whereas injections into lateral Av (n=4) produced larger numbers of more intensely labeled somata in contralateral lateral Av. Av therefore appears to participate in a direct Av-to-Av inter-hemispheric circuit, as described in pigeon (Letzner et al., 2016; H. J. Zeier & Karten, 1973), that is topographically organized and is stronger in lateral than medial Av.

Inputs to regions adjacent to Av—Control injections of CTB into the ventral arcopallium lateral to Av (n = 4) produced labeled cells primarily lateral and ventrolateral to ipsilateral dNCL_{SHELL}. Figure 8F,G shows labeled somata lateral to dNCL_{SHELL} following an injection ca. 500 μ m lateral to Av and ventrolateral to the lateral border of AId (Fig. 8G). This result confirms the terminal label in lateral ventral arcopallium resulting from control injections lateral to dNCL_{SHELL} (Fig. 5A,D). Somata in the anterior nidopallium lateral to LMAN-SHELL were also retrogradely labeled by control injections lateral to Av (Fig. 8H). In addition, lateral control injections produced a high density of intensely labeled cells in the contralateral ventral arcopallium lateral to Av (Fig. 8I; cf. Figs. 2, 7). This result supports the idea that the direct inter-hemispheric projection between left and right ventral arcopallium is

arranged in a gradient such that medial Av projects only weakly to contralateral medial Av, whereas lateral Av and regions lateral to Av project more strongly to lateral portions of contralateral ventral arcopallium.

Control injections dorsal to Av, between the ventral border of AId and OM as shown in Figure 8E, produced retrogradely labeled cells as far as 500 μ m ventral to ipsilateral dNCL_{SHELL} (n=6), but not within dNCL_{SHELL} itself, confirming the terminal label in this region following injections of anterograde tracer ventral to dNCL_{SHELL} (Fig. 5B,C) (Bottjer et al., 2000). Sparsely distributed labeled cells were also observed in the corresponding region of contralateral arcopallium between Av and AId. We did not observe retrograde label in or adjacent to LMAN-SHELL following control injections dorsal to Av. Control injections overlapping with either anterior or posterior Av also produced retrogradely labeled cells in contralateral Av, with the exception of one injection located almost completely posterior to Av; no retrograde label in contralateral ventral arcopallium was found following this posterior injection.

Our attempts to make control injections anterior and posterior to Av led to an interesting discovery. Injections anterior to the region showing strong terminal label following dNCL_{SHELL} injections (n = 5) produced sparse labeled cells anterior to dNCL_{SHELL}, as expected, but dense retrograde label in LMAN-SHELL and adjacent regions (Fig. 8B-C; cf. Johnson et al. 1995). In contrast, more posterior injections that encompassed part of dNCL_{SHELL}-recipient Av and adjacent posterior ventral arcopallium (n=4), produced retrograde label in posterior dNCL_{SHELL} and regions ventral and immediately posterior to dNCL_{SHELL}, but did not label neurons in SHELL or SHELL-adjacent nidopallium (Fig. 8A). As diagrammed in Figure 8D, these data indicate that dNCL_{SHELL} (and adjacent regions) send axonal projections to posterior regions of Av, whereas LMAN-SHELL (and surrounding areas) project to anterior regions; the intermediate region of Av receives inputs from both. This partially overlapping distribution indicates that the inter-hemispheric pathways connecting left and right dNCL_{SHELL} and LMAN-SHELL via Av are likely to be integrated with each other.

Individual Av neurons send axonal branches to contralateral dNCL_{SHELL} and contralateral Av—The retrograde patterns of label described above reveal novel efferent projections from Av to both contralateral Av and contralateral dNCL_{SHELL}. Information conveyed from Av to these two contralateral targets could be processed in parallel, by separate populations of Av neurons sending axons to only one efferent target. Alternatively, individual Av neurons could send collateral branches of axons to both contralateral targets, conveying identical information to both. To distinguish between these possibilities, we injected differently colored retrograde tracers into dNCL_{SHELL} and Av in the same hemisphere and looked for double-labeled cells in Av in the contralateral hemisphere, as diagrammed in Figure 9A, B. Because the projections from Av to both contralateral dNCL_{SHELL} and Av are organized with medial-lateral topography, we made large injections into dNCL_{SHELL} to label cells in a large topographic area in 10 of our 17 paired injections. This increased the likelihood of the Av injection topographically corresponding with a portion of the dNCL_{SHELL} injection, and thus the chance of finding double-labeled cells in areas labeled by both injections.

Double-labeled cells were identified in mid and lateral Av following 8 paired injections into the contralateral hemisphere. The arrow in Figure 9D-F indicates an individual Av cell containing fluorescent label in both color channels. Figure 9C and G (arrows) show examples of double-labeled cells in Av of two birds that received topographically matched pairs of injections into contralateral dNCL_{SHELL} and Av. As described above, injections of CTB into medial Av labeled cells in contralateral medial Av only weakly, while injections into lateral Av produced more abundant and darkly labeled cells in contralateral lateral Av. Thus, even after injections that produced some clearly double-labeled cells (as shown in Fig. 9C-G), it was often difficult to determine the quantity of labeled cells in medial Av labeled by our Av injections. We were therefore unable to quantify the proportion of double-labeled cells in Av; we estimate that the proportion of double-labeled cells ranged from 30-70% within areas of Av well labeled by topographically matched injection pairs. These data indicate that a population of individual Av neurons send axonal branches carrying identical information to terminate in both contralateral Av and dNCL_{SHELL}, providing a mechanism for coordinating activity between hemispheres. Triple-label studies will be required to test whether single Av neurons project to dNCL_{SHELL}, Av and LMAN-SHELL. In addition, it is likely that some Av neurons project to individual post-synaptic targets.

Topographic organization of efferent projections from Av

Av projections to contralateral caudal nidopallium and contralateral Av—

Injections of 10k-DA into Av produced terminal label in broad areas of contralateral dNCL_{SHELL} and extended into regions immediately lateral, anterior, and posterior to dNCL_{SHELL} as well as the region ventral to dNCL_{SHELL} (n=20). These results confirm the experiments described above in which labeled cells were found within Av (and extending beyond it) following injections of retrograde tracers into contralateral dNCL_{SHELL} (Fig. 2D-E, G-H) and adjacent regions (Fig. 3H-J).

The anterograde label resulting from an injection covering most of Av in the right hemisphere is shown in a caudal to rostral series of schematics in Figure 10 and corresponding photomicrographs in Figure 11. Labeled processes extended rostrally from the Av injection site (Fig. 10A) within the OM tract (Fig. 10B-C, 11C) and arched ventrally in OM (Fig. 10D) to enter the anterior commissure (Fig. 10E, 11D), where they crossed to the opposite hemisphere. Contralateral to the injection site, labeled axons entered OM (Fig. 10D) and extended caudally (Fig. 10C). Terminal label was located in Av contralateral to the injection site (Fig. 10A); other labeled processes traveled dorsally to terminate in contralateral dNCL_{SHELL} and regions of caudal nidopallium adjacent to dNCL_{SHELL} (Fig. 10A-B, 11A-B), as described above.

The projection from Av to contralateral dNCL_{SHELL} and surrounding regions demonstrated medial-to-lateral topography. Injections into medial Av (n=7) produced terminal label in medial dNCL_{SHELL} and areas ventral to medial dNCL_{SHELL}, whereas injections into lateral Av (n=4) labeled lateral dNCL_{SHELL} as well as areas ventral and lateral to dNCL_{SHELL}. This organization corresponds with the topography we identified in the pattern of retrograde labeling in Av after injections into medial or lateral contralateral dNCL_{SHELL} (Fig. 2D).

Av projections to contralateral anterior nidopallium—Injections of 10k-DA into Av also produced terminal label in the contralateral anterior nidopallium, within both LMAN-SHELL and immediately adjacent regions. After crossing into the contralateral hemisphere via the anterior commissure, labeled processes entered the lateral forebrain bundle (FPL) and traveled in a rostro-lateral direction, crossing the striatum and forming a terminal field in LMAN-SHELL and surrounding medial, lateral, dorsal and posterior regions (Figs. 10F-H, 11E-G). Efferent projections from Av to contralateral SHELL and adjacent nidopallium maintained a medial-to-lateral topography: injections into medial Av (n=7) produced terminal label within medial SHELL and medial to SHELL (Fig. 11G), whereas lateral Av injections (n=4) produced terminal label in lateral SHELL as well as lateral and dorsal to SHELL (Fig. 11F).

Sparse Av projections to ipsilateral anterior nidopallium—Large injections of 10k-DA which covered most of Av (n=4) produced a small amount of terminal label in ipsilateral LMAN-SHELL and regions immediately posterior and dorsal to the boundaries of ipsilateral SHELL (Fig. 10G, H). In five additional cases from smaller injections restricted to small portions of Av, extremely sparse labeled processes were located posterior and dorsal to SHELL, but terminal fields could not be identified. These data correspond with previous findings that Av sends a weak reciprocal projection to ipsilateral SHELL (Johnson et al., 1995).

Efferent projections of regions adjacent to Av—Control injections of 10k-DA lateral to Av produced terminal label in the contralateral hemisphere 400-700 μ m lateral to dNCL_{SHELL} (n=2). This confirms our finding that control injections of retrograde tracer >400 μ m lateral to dNCL_{SHELL} labeled cell bodies lateral to contralateral Av (Fig. 3A). Lateral control injections also produced terminal label in contralateral ventral arcopallium (lateral to Av), and in the contralateral anterior nidopallium lateral to LMAN-SHELL. These results provide further evidence for a medial-lateral continuum in the inter-hemispheric organization of dNCL_{SHELL}-Av and lateral dNCL-lateral arcopallium pathways, and suggest the same pattern may exist in the circuit from LMAN-SHELL to Av. A control injection located dorsal (n=1) to Av produced terminal label in the contralateral hemisphere ventral to dNCL_{SHELL}, confirming our finding that tracer injections located ventral to dNCL_{SHELL} retrogradely labeled cells within contralateral Av and extending beyond Av in a dorsal direction (Fig. 3H,J). Sparse terminal label was also observed in the corresponding region of ventral arcopallium between Av and AId, consistent with the reciprocal projection between left and right ventral arcopallium. No terminal label was observed in the anterior nidopallium in either hemisphere following this dorsal control injection.

Anterior injections encompassed both anterior Av and the immediately adjacent area of ventral arcopallium (n = 2); these injections yielded robust terminal label in contralateral LMAN-SHELL and weak terminal label in ipsilateral LMAN-SHELL, in accordance with the results above (Fig. 10) and the description of the SHELL circuit through Av described by Johnson et. al (1995). These anterior Av injections also produced anterograde label contralaterally in anterior Av and anterior to contralateral dNCL_{SHELL} but not within dNCL_{SHELL} itself, as expected from Figure 8D. An injection that was concentrated posterior

to Av, but slightly overlapped it ($n = 1$) produced some anterograde label in posterior contralateral dNCL_{SHELL} as well as immediately posterior to it, as well as sparse label in contralateral Av, but no label in or around contralateral SHELL.

Av contributes to inter-hemispheric circuits between both dNCL_{SHELL} and LMAN-SHELL—In summary, Av receives information from two ipsilateral regions that are part of a circuit important for vocal learning, dNCL_{SHELL} and LMAN-SHELL, as well as from adjacent nidopallial regions. Av relays information to corresponding contralateral regions via efferent projections to dNCL_{SHELL} and LMAN-SHELL and adjacent regions. These inter-hemispheric circuits are organized in a medial-lateral continuum, such that medial dNCL_{SHELL} and medial LMAN-SHELL interconnect bilaterally with medial Av, whereas lateral dNCL_{SHELL} and lateral LMAN-SHELL interconnect bilaterally with lateral Av; regions immediately lateral to dNCL_{SHELL} and LMAN-SHELL interconnect with both lateral Av and regions lateral to Av in both hemispheres. In addition, we identified a population of individual Av neurons which send collateral branches to both contralateral Av and contralateral dNCL, providing a neural pathway for coordinating activity in multiple areas in both hemispheres. We do not know whether some single Av neurons project contralaterally to both Av and LMAN-SHELL, or whether some Av neurons might project to all three contralateral targets (Av, dNCL_{SHELL} and LMAN-SHELL); double- and triple labeling studies are needed to resolve these questions.

Individual LMAN-SHELL neurons send axonal branches to ipsilateral dNCL_{SHELL} and Av

As diagrammed in Figure 1A and B, LMAN-SHELL sends ipsilateral projections to three cortical targets: AId, dNCL_{SHELL} and Av (Bottjer et al., 2000; Johnson et al., 1995). Two separate populations of SHELL neurons project to either dNCL_{SHELL} or to AId (Bottjer et al., 2000). The projections from SHELL to dNCL_{SHELL} and Av could also arise from separate populations of neurons that process information independently and project in parallel to only one target or the other. Alternatively, single SHELL neurons could send identical information through collateral axon branches to dNCL_{SHELL} and Av. We distinguished between these two modes of processing using the same dual injections described above (Fig. 9A): different colors of retrograde tracer were injected into dNCL_{SHELL} and Av in the same hemisphere (Fig. 12A,C) to retrogradely label cell bodies of ipsilateral SHELL projection neurons.

We identified single somata containing both colors of tracer (Fig. 12B,F,G) in LMAN-SHELL and immediately adjacent regions ($n=9$ paired injections), demonstrating that some individual neurons project to both dNCL_{SHELL} and Av. Double-labeled cells (marked by arrows in Fig. 12) were located in areas topographically matched for both injection sites. Double-labeled cells were not clustered together or restricted to a specific region of SHELL or adjacent nidopallium, but were interspersed among single-labeled cells of both colors. In cases where dNCL_{SHELL} and Av injections were topographically mismatched ($n=8$), retrogradely labeled cells in SHELL from each injection site were topographically separated and no double-labeled cells were observed.

The incidence of single- versus double-labeled cells was quantified using two methods for the nine well-matched injections: by counting all labeled cells identified within SHELL (“TOTAL”), and by counting labeled cells within $150 \times 150 \mu\text{m}^2$ boxes (“BOX”) within subregions of SHELL containing a high density of retrogradely labeled cells of both colors (such as the blue squares in Fig. 12G). Subregions of SHELL often contained labeled cells of only one color – for example, if a green injection into dNCL_{SHELL} was larger than a magenta injection into Av, then SHELL contained a higher number and wider distribution of green than magenta cells. Counts using the TOTAL method thus resulted in a low percentage of double-labeled cells. The BOX method more accurately quantified the proportion of double-labeled cells by targeting subregions well-labeled by both injection sites.

In addition, because the quantity of labeled cells from each injection depended on the size and quality of the injection, we calculated the proportion of double-labeled cells both as a percentage of the total population labeled by the Av injection (“Av-labeled”) or by the total population labeled by the dNCL_{SHELL} injection (“dNCL_{SHELL}-labeled”). Equal percentages of double-labeled cells out of Av-labeled and dNCL_{SHELL}-labeled populations, as seen in Or168 in Figure 13A,C indicate approximately equivalent injection sites. In contrast, divergent proportions indicate that one injection produced more retrogradely labeled cells in SHELL than the other; for example, Or48 had a higher total number of cells labeled by a dNCL_{SHELL} injection and therefore a lower percentage of double-labeled dNCL_{SHELL}-projecting cells (Fig. 13).

An average of 20% of dNCL_{SHELL}-labeled cells and 17% of Av-labeled cells contained both colors of tracer when counted with the TOTAL method, whereas 36% of dNCL_{SHELL}-labeled cells and 31% of Av-labeled cells were double-labeled when counted with the BOX method (Fig. 13, Table 2). In no case did the percentage of double-labeled cells exceed 60% of either population of labeled cells. Control injections were made by injecting a mixture of both colors of tracer (n=2 into Av and n=1 into dNCL_{SHELL}); these produced 78-98% (BOX) double-labeled cells (Table 2), demonstrating that the two tracers could be co-transported by single cells with high efficiency.

If all SHELL neurons which project to dNCL_{SHELL} also send collateral branches to Av, then we would expect to find a higher proportion of double-labeled cells for injection pairs with a greater extent of topographic overlap; the number of double-labeled cells within areas well-labeled by both injections would increase while single-labeled cells would decrease in correspondence to better topographic matching. In contrast, if SHELL contains separate neuronal sub-populations that project to only dNCL_{SHELL} or Av as well as neurons that project to both targets, then better topographic overlap of injection sites would be unlikely to correlate strongly with the proportion of double-labeled cells in areas well-labeled by both injections. Well-matched injections would produce a higher incidence of both single- and double-labeled cells, and therefore would not significantly increase the relative proportion of double-labeled cells. We defined the degree of topographic overlap as the percentage of the area of the injection site in Av that overlapped with terminal label in Av from the dNCL_{SHELL} injection site (outlined in yellow in Fig. 12D,E). This analysis yielded no evidence that the proportion of double-labeled cells depended on how well matched the injections were. Figure 13B,D shows that a higher degree of topographic overlap did not

correlate with a higher percentage of double-labeled cells. For example, results of the BOX counting method for the population of cells labeled by dNCL_{SHELL} injections (Fig. 13C-D, Table 2) show that injection sites in R167 overlapped by 44% and produced 52% double-labeled cells, whereas injection sites in Db160 overlapped by 74% and produced 50% double-labeled cells. This pattern indicates that not all dNCL_{SHELL}-projecting SHELL neurons send collaterals to Av. Rather, it is likely that LMAN-SHELL contains one sub-population of neurons that sends collateral branches to both targets distributed among separate sub-populations that project to only dNCL_{SHELL} or Av.

Discussion

This study reveals novel recurrent and inter-hemispheric circuits within a cortico-basal ganglia pathway necessary for vocal learning. LMAN-SHELL sends topographically organized projections to dNCL_{SHELL}, Aid, and Av (Bottjer et al., 2000; Iyengar et al., 1999; Johnson et al., 1995). dNCL_{SHELL} projects in turn to Aid and Av with topography overlapping the direct projection from LMAN-SHELL, such that Aid and Av receive input from SHELL both directly (SHELL→Aid, SHELL→Av) and indirectly (SHELL→dNCL_{SHELL}→Aid, SHELL→dNCL_{SHELL}→Av) (Fig. 1A,B). A similar pattern of direct and indirect projections occurs in the songbird trigeminal pathway located lateral to the SHELL pathway (Wild & Farabaugh, 1996), the pigeon trigeminal system (Wild et al., 1985), the chick visual system (Ahumada-Galleguillos, Fernandez, Marin, Letelier, & Mpodozis, 2015), and in the auditory system for sound localization in barn owls (Cohen et al., 1998). For example, a region of anterior nidopallium lateral to LMAN-SHELL projects directly to the lateral arcopallium in zebra finches, as well as indirectly via dNCL (Wild and Farabaugh, 1996; and see Figs. 3, 5 above); this circuitry conveys afferent information from the tongue and beak as well as from auditory brainstem regions. All major sensory systems in avian brain seem to make direct projections from anterior nidopallium to the arcopallium as well as indirect projections to the arcopallium via a multi-synaptic circuit from anterior nidopallium to caudal nidopallium to arcopallium. This parallel organization of dual inputs to arcopallium may contribute to processes of sensorimotor integration as well as to integration of different streams of information processing (cf. Bottjer et al., 2000).

Our results also show that a sub-population of SHELL neurons send axonal branches to both dNCL_{SHELL} and Av. Thus, some single SHELL neurons convey identical information into both the direct and the indirect pathways to Av. Additional sub-populations within LMAN-SHELL likely project to either dNCL_{SHELL} or Av, and may convey different information to each target. Because separate populations of SHELL neurons project to either dNCL_{SHELL} or Aid (Bottjer et al., 2000), it is possible that the Av-only projections we observed represent collaterals of Aid-projecting SHELL neurons. In addition, single SHELL-recipient dNCL neurons may send collateral branches to both Aid and Av. This circuitry thus contains multiple loci of possible integration.

Gale et al. (2008) and Mandelblat-Cerf et al. (2014) reported that an area of intermediate arcopallium in close proximity to RA projects to the ventral tegmental area/substantia nigra (VTA/SNc); Gale et al. described this region as “lateral, ventral and anterior to RA”. This region corresponds to an area referred to as RA-cup, described by Kelley and Nottebohm

(1979), Vates et al. (1996), and Mello et al. (1998) as surrounding RA on all sides and receiving inputs from auditory cortical regions (including Field L, HVC-shelf, and CM). RA-cup overlaps partially with the medial neck region of AId and adjacent regions surrounding the lateral border of RA that receive input from dNCL_{SHELL} (Fig. 4). In addition to the projection from RA-cup to VTA/SNc reported by Gale et al. and Mandelblat-Cerf et al., Bottjer et al. (2000) reported a projection from AId to VTA. Together, these papers suggest a broad projection from intermediate arcopallium to VTA/SNc. Thus, the medial neck region of AId plus immediately adjacent arcopallium surrounding the lateral border of RA is part of RA-cup, suggesting that this region may receive inputs from auditory cortex as well as from dNCL_{SHELL}, and project to VTA/SNc. In contrast, Av, defined here in terms of its inter-hemispheric connections with LMAN-SHELL and dNCL_{SHELL}, was located along the ventral edge of the telencephalon, lateral to the lateral-most extent of RA, and thus is apparently located ventro-lateral to the area of arcopallium described by Mandelblat-Cerf et al. (2014; but see their Fig. 1F). In accord with this idea, our injections into Av did not produce anterograde label in VTA/SNc or retrograde label in cortical auditory regions.

Multimodal Integration

The SHELL→dNCL_{SHELL}→AId/Av pathways are a component of a larger pattern of topographic projections from anterior nidopallium to caudal nidopallium to arcopallium. Both dNCL_{SHELL} and Av integrate inputs from SHELL with inputs from anterior nidopallial areas surrounding SHELL. Av additionally integrates inputs from dNCL_{SHELL} with inputs from dNCL_{SHELL}-adjacent caudal nidopallium. dNCL_{SHELL} and adjacent regions of nidopallium contain a network of local connectivity, primarily oriented in the anterior-posterior direction; this connectivity is similar to the rostrocaudal organization of the vocal premotor nucleus HVC, which is located anterior and medial to dNCL_{SHELL} (Day, Terleski, Nykamp, & Nick, 2013; Nottebohm, Paton, & Kelley, 1982; Stauffer et al., 2012). Such a local network would allow neurons to integrate information across regions of dNCL, which themselves integrate information from the anterior nidopallium. In contrast to dNCL_{SHELL} and Av, AId receives inputs only from LMAN-SHELL and not from surrounding regions (Bottjer et al., 2000). This pattern is consistent with the idea that an important function of the circuit from SHELL to dNCL_{SHELL} and Av is to integrate multiple sources of information. Av is a point of further convergence and integration of information from anterior and caudal nidopallium, which is then shared with the contralateral hemisphere.

The topographic organization of projections both within and adjacent to SHELL pathways could allow parallel processing of song-related responses and other auditory, visual and somatosensory information to points of convergence and integration. Many neurons in SHELL of juvenile birds respond selectively to either the bird's own current song or the memorized tutor song (Achiro & Bottjer, 2013). Nidopallial regions posterior to LMAN receive visual input via a tectofugal pathway (Krutzfeldt & Wild, 2004; Wild & Gaede, 2016), while neurons located lateral to LMAN receive auditory information and beak and tongue somatosensory inputs from the Basorostral nucleus (Wild & Farabaugh, 1996; Wild & Williams, 1999). Areas surrounding LMAN also receive visual and somatosensory input from rostral hyperpallium (HA) (Wild, 1994; Wild & Williams, 1999), and the anterior

nidopallium in pigeon and chick receives inputs from auditory, visual, and somatosensory cortical areas (Ahumada-Galleguillos et al., 2015; Dubbeldam, den Boer-Visser, & Bout, 1997; Gunturkun & Kroner, 1999; Helduser et al., 2013; Korzeniewska & Gunturkun, 1990; Kroner & Gunturkun, 1999; Shimizu, Cox, & Karten, 1995; Wild, 1994; Wild et al., 1985). Thus, multimodal information from SHELL-adjacent regions and song-selective information from LMAN-SHELL likely converges in ipsilateral dNCL_{SHELL} and Av.

The pattern of anterograde and retrograde label we observed following injections into Av indicates that this region corresponds with the ventral-most extent of sensorimotor intermediate arcopallium described in pigeon by Zeier and Karten (1971; 1973), and not the limbic division of the posterior/medial ventral arcopallium. Limbic arcopallium, including the posterior pallial amygdala, is defined by its efferent projections to the hypothalamus and other limbic structures (Atoji, Saito, & Wild, 2006; Davies, Csillag, Szekely, & Kabai, 1997; Dubbeldam et al., 1997; Kuenzel, Medina, Csillag, Perkel, & Reiner, 2011; Reiner, Perkel, Bruce, et al., 2004; Veenman, Wild, & Reiner, 1995; H. J. Zeier & Karten, 1973). Injections into Av did not produce anterograde or retrograde label in the hypothalamus, striatum, hippocampus, olfactory sensory areas, or other known limbic or amygdalar targets (Atoji et al., 2006; Atoji & Wild, 2014; Patzke, Manns, & Gunturkun, 2011; Swanson & Petrovich, 1998). Therefore, a role for Av in social vocal learning is likely to involve integration of multiple sensory inputs, including those from social interactions, rather than direct interactions with the limbic system.

The identification of dNCL_{SHELL} and Av as points of multimodal convergence supports a growing body of research demonstrating that multimodal sensory inputs are important behavioral mediators in both juvenile and adult zebra finches. For example, although adult males can use visual cues alone to select between two female birds shown in a live video feed, the addition of auditory cues induces stronger courtship responses than a silent video (Galoch & Bischof, 2006, 2007). Sensory cues conveying social context also modulate adult vocal behavior: males sing differently when directing their song to a female or to juveniles than when alone or with other adult males, and the rate of singing is affected by the number and familiarity of nearby birds (Adar, Lotem, & Barnea, 2008; Bischof, Böhner, & Sossinka, 1981; Chen et al., 2016; Dunn & Zann, 1997; Jesse & Riebel, 2012; Morris, 1954; Toccalino, Sun, & Sakata, 2016; Williams, 2001). Multimodal sensory integration in adults could therefore enable males to produce socially appropriate female- or juvenile-directed song. Song production as a courtship behavior itself also requires multimodal integration, as birds coordinate song production with dance movements oriented towards a visually-perceived female (Cooper & Goller, 2004; Dalziel et al., 2013; Ota et al., 2015; Price, 1979; Ullrich, Norton, & Scharff, 2016). In juveniles, visual isolation from an adult tutor strongly impairs vocal learning, whereas pairing auditory tutoring with a visual stimulus enhances learning (Chen et al., 2016; Eales, 1989; Hultsch et al., 1999; Morrison & Nottebohm, 1993; Price, 1979). Visual cues from adult females, such as wing strokes in response to song elements, provide feedback which influences juvenile sensorimotor song learning (King et al., 2005; West & King, 1988). Non-auditory cues also impact the juvenile's choice of which tutor to imitate: juveniles selectively imitate a tutor whose coloring matches their foster parent over a tutor of unfamiliar coloring, and preferentially learn from aggressive over non-aggressive tutors (Jones & Slater, 1996; Ljubić et al., 2016; Mann et al., 1991).

Multimodal and social signals therefore may enhance perception and learning of auditory signals in juveniles, perhaps in part by modulating attention as proposed for human speech learning (Chen et al., 2016; Deregnaucourt et al., 2013; Kuhl, 2007; Ljubić et al., 2016; Macroy-Higgins & Montemarano, 2016).

The caudal nidopallium, including songbird dNCL_{SHELL}, may have a conserved role in multimodal learning. In chick, the caudal nidopallium includes auditory, visual and somatosensory processing regions interconnected by strong local projections similar to the local network we identified in songbird dNCL (Metzger et al., 1998). In addition, NMDAR-mediated currents within this region are required for auditory and visual imprinting, another type of developmentally regulated multimodal learning (Bock & Braun, 1999; Bock, Schnabel, & Braun, 1997; Braun et al., 1999; Van Kampen & Bolhuis, 1993). Pigeon caudal nidopallium also receives visual, auditory, and somatosensory inputs, and blocking NMDA receptors in this region interferes with a pigeon's ability to learn new tasks and remember previously learned behaviors (Diekamp, Kalt, & Gunturkun, 2002; Gunturkun, 1997; Herold et al., 2011; Kroner & Gunturkun, 1999; Lengersdorf, Marks, Uengoer, Stuttgen, & Gunturkun, 2015; Shanahan et al., 2013). In zebra finch, NMDA receptors are known to mediate over 90% of synaptic transmission from the thalamus (DLM) to LMAN and from LMAN-CORE to vocal motor cortex (RA) (Bottjer, 2005; Stark & Perkel, 1999; J. Wang & Hessler, 2006; White, Livingston, & Mooney, 1999). Because NMDA receptors act as non-linear amplifiers, this pattern suggests that LMAN circuitry is well suited for integrating coincident inputs from multiple sources. Thus, as in pigeon and chick, NMDAR-mediated currents in dNCL_{SHELL} may mediate multimodal learning.

Inter-hemispheric integration

Our results also demonstrated the presence of a cortical inter-hemispheric pathway via Av: both SHELL and dNCL_{SHELL}, and nidopallial areas adjacent to each, send axonal projections to ipsilateral Av, which in turn sends inter-hemispheric projections to contralateral SHELL, dNCL_{SHELL}, and adjacent nidopallial regions. Although an overall medial-lateral topography is maintained throughout this inter-hemispheric circuit, the topography is compressed within Av; this small region of ventral arcopallium receives inputs from broad areas of ipsilateral anterior nidopallium (LMAN-SHELL and surround) as well as caudal nidopallium (dNCL_{SHELL} and surround), and projects in turn to corresponding contralateral targets. Neurons in Av also project directly to contralateral Av. Some single Av neurons send axonal branches to both contralateral Av and dNCL_{SHELL}, creating a reciprocal Av-Av pathway with direct links to dNCL_{SHELL}. Future double-labeling studies are needed to test whether this is also true for LMAN-SHELL.

Inter-hemispheric pathways through Av suggest that LMAN-SHELL circuitry may play a major role in hemispheric coordination of singing-related activity. Inter-hemispheric coordination of motor production has been thought to involve bilateral ascending projections from brainstem motor nuclei to the thalamic nucleus Uva, which projects ipsilaterally to vocal premotor cortex (HVC); the HVC→RA motor pathway is thought to control the ipsilateral vocal organ (Ashmore, Renk, & Schmidt, 2008; Goller & Cooper, 2004; Marc F. Schmidt, 2008; M. F. Schmidt, Ashmore, & Vu, 2004; Suthers & Zollinger, 2004; Wild,

2004). However, there has been little evidence for a cortical circuit that could coordinate regions involved in learning the precise bilateral patterns of song production (Johnson et al., 1995). In avian species that do not learn their vocalizations (including pigeon, chick, and mallard), inter-hemispheric projections have been identified from anterior and ventral arcopallium to the contralateral arcopallium and nidopallium (Davies et al., 1997; Dubbeldam et al., 1997; Letzner et al., 2016; Metzger et al., 1998; H. J. Zeier & Karten, 1973). The inter-hemispheric circuit described in this study via axons of Av neurons crossing in the anterior commissure is thus highly similar to inter-hemispheric connections identified in other avian species. This telencephalic circuitry provides a mechanism for coordination of left and right hemispheres that could complement the ascending bilateral brainstem pathway for coordination of premotor vocal regions during singing. Interestingly, AId projects to deep layers of tectum (Bottjer et al., 2000), which project in turn to Uva (Wild, 1994), thus creating a potential mechanism for integrating bilateral control of vocal behavior between these two pathways. In addition, AId projects to a dorsal thalamic zone that projects in turn to the medial magnocellular nucleus of the anterior nidopallium (MMAN) which projects in turn to HVC (Foster et al., 1997)). In general, information processed in the inter-hemispheric connections between SHELL and dNCL_{SHELL} is conveyed to AId (Fig. 1), which has multiple post-synaptic targets (Bottjer et al., 2000) including striatum, dorsal thalamus, lateral hypothalamus, tectum, AVT/SNC, reticular formation, and the medial spiriform nucleus (SpM, which projects to cerebellum). These crossed pathways would thus integrate the circuitry described here more broadly with circuits for limbic influences, premotor control, and other functions.

The goal of this study was to expand our knowledge of the anatomical organization of dNCL_{SHELL} and Av within the LMAN-SHELL pathway in order to suggest possible mechanisms for inter-hemispheric coordination and multimodal sensory integration. dNCL_{SHELL} and Av may function to integrate multimodal sensory information with song-selective responses in LMAN-SHELL encoding neural representations of self-produced vocal sounds and tutor-song memory during vocal learning. Av receives information from both SHELL and dNCL_{SHELL} and adjacent areas of anterior and caudal nidopallium, containing both song-selective responses for vocal learning and input from additional sensory modalities, including those encoding social interactions. Av then sends information contralaterally to anterior and caudal nidopallium, providing a mechanism to coordinate multimodal vocal learning in both hemispheres.

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List of Abbreviations

AC	anterior commissure
AId	dorsal intermediate arcopallium

Av	region of ventral arcopallium that receives input from LMAN-SHELL and dNCL _{SHELL}
Bas	basorostral nucleus
dNCL	dorsal caudolateral nidopallium
dNCL_{SHELL}	region of dNCL that receives input from LMAN-SHELL
FA	fronto-arcopallial tract
FPL	lateral forebrain bundle
HP	hippocampus
HVC	vocal premotor cortex (proper name)
LAD	dorsal arcopallial lamina
LMAN	lateral magnocellular nucleus of the anterior nidopallium
LMAN-SHELL (SHELL)	external shell region of LMAN
LMAN-CORE (CORE)	central core region of LMAN
MMAN	medial magnocellular nucleus of the anterior nidopallium
NCM	caudo-medial nidopallium
LAD	dorsal arcopallial lamina
LaM	mesopallial lamina
LPS	pallial-subpallial lamina
OM	occipitomesencephalic tract
RA	robust nucleus of the arcopallium (vocal motor cortex)
TnA	nucleus Taenae of the amygdala
Str	striatum
Uva	nucleus uvaeformis
X	Area X (song-control region within an area of basal ganglia containing both pallidal and striatal neuron types)

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Figure 1.

Parallel cortico-basal ganglia circuits for vocal learning and behavior in songbirds. **A.** The cortico-basal ganglia circuit traversing LMAN-CORE (dark gray) projects to vocal motor cortex (RA) and mediates vocal production in juvenile songbirds. A parallel circuit through LMAN-SHELL (light gray) is critical for vocal learning during development. LMAN-SHELL sends axonal projections to dNCL_{SHELL}, Ald, and Av. Based on Bottjer et al. (2000), Johnson et al. (1995), and current results. **B.** Summary schematic of inter-hemispheric connections of dNCL_{SHELL}, LMAN-SHELL, and Av. **C-D.** Schematics depicting song-related regions and accompanying Nissl-stained sections (coronal); RA, Ald, and Av are located within arcopallium; dNCL_{SHELL} is located within caudal nidopallium; SHELL and CORE regions of LMAN are located in anterior nidopallium; Area X is situated within medial striatum, which contains both striatal and pallidal neurons. Calbindin

expression clearly labels the external borders of LMAN-SHELL. **E.** Example of a large CTB injection into lateral dNCL_{SHELL}. Dashed line indicates dorsal border of telencephalon. **F.** Magnocellular CORE of LMAN can be distinguished from the surrounding parvocellular SHELL in 50- μ m Nissl-stained sections. See list of abbreviations for full names.

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Figure 2.

Topographic pattern of retrograde label following injections into dNCL_{SHELL}. Diagrams indicate location of injection sites and resultant pattern of retrograde label; boxes indicate position of photomicrographs. **A.** An injection of CTB into mid dNCL_{SHELL} produced retrograde label in mid (dorsal and ventral) SHELL, and in anterior nidopallium dorsal to SHELL. **B.** An injection into lateral dNCL_{SHELL} (shown in Fig. 1E) produced retrograde label in dorsal and lateral SHELL, as well as dorso-lateral and lateral to SHELL. **C.** Adjacent injections of CTB conjugated to different dyes into medial and mid dNCL_{SHELL} produced topographically organized label in and around medial and mid SHELL. **D.** The injections depicted in C also produced a topographic pattern of retrograde label in contralateral Av. **E.** Retrogradely labeled cells in contralateral Av following a large injection into mid dNCL_{SHELL}. **F.** An injection into dNCL_{SHELL} produced retrogradely labeled cells 600 μ m anterior to the injection site border, indicating the presence of a local network of

connectivity. **G-H.** Rostral-caudal distribution of retrograde label in Av is shown in comparison to Nissl-stained tissue. **I.** Sagittal illustration of the anterior-posterior organization of retrograde label within and adjacent to LMAN-SHELL following injections into anterior (blue) and posterior (purple) dNCL_{SHELL}. Red dashed line indicates the plane of section shown in F. In A-H and in all subsequent figures, sections are coronal and dorsal is to the top. Medial is left in A-C and right in D-H (in contralateral hemisphere). A and P indicate anterior or posterior coordinates of the section shown, corresponding to stereotaxic coordinates in the canary atlas of Stokes et al. (1974). Anatomical borders drawn onto photomicrographs were taken from alternate Nissl-stained sections. Dotted lines in photomicrographs mark ventral edge of telencephalon. Scale bars in this and subsequent figures represent 200 μ m unless otherwise noted.

Figure 3.

Retrograde label following control injections adjacent to dNCL_{SHELL}. **A-B.** An injection ca. 700 μ m lateral to dNCL_{SHELL} produced retrograde label in the lateral contralateral ventral arcopallium, and in the ipsilateral anterior nidopallium extending ventrolateral from SHELL and lateral to Bas. **C-G.** A rostral-caudal series of the pattern of retrograde label found in anterior nidopallium following an injection ca. 400 μ m ventral to dNCL_{SHELL}. Label was concentrated in more posterior sections directly caudal to SHELL (**D**) and dorsal to medial Bas (**E**), and was situated ventral and lateral to SHELL in more anterior sections (**F** and **G**). **H.** Injections ventral to dNCL_{SHELL} also produced retrogradely labeled cells in contralateral Av, overlapping and extending dorsal to the area retrogradely labeled by dNCL_{SHELL} injections. **I.** Retrogradely labeled cells in anterior nidopallium directly posterior to SHELL following an injection ca. 350 μ m posterior to dNCL_{SHELL}. **J.** Retrogradely labeled cells in anterior nidopallium directly anterior to SHELL and in contralateral ventral arcopallium,

anterior to Av, following an injection ca. 400 μm anterior to dNCL_{SHELL}. Medial is left in all panels except the right and lower panels of H and the photomicrograph in J, where medial is to the right. Measurements indicate the distance from the center of the injection site to the approximate border of the region in this and subsequent figures.

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Figure 4.

Anterograde label produced by injections into dNCL_{SHELL}. Medial is left in all panels. **A.** Summary diagram of topographic arrangement of axonal and terminal label following injections into medial, mid, and lateral dNCL_{SHELL}. Boxes indicate location of photomicrographs in C-H. **B.** Example 10k-DA injection site in central dNCL_{SHELL}. Dotted line indicates dorsal border of telencephalon. **C.** Labeled axon fascicles stream ventrally from the injection site in dNCL_{SHELL}. **D.** Terminal label from an injection restricted to medial dNCL_{SHELL} was located within medial “neck” region of AId and extended beyond borders of AId (see text). Labeled processes (indicated by arrows) continued ventral to AId to terminate in Av. **E.** Terminal label in central AId resulting from an injection into mid dNCL_{SHELL}. **F.** Terminal label located in lateral AId following an injection into lateral dNCL_{SHELL}. **G.** Terminal field in Av following an injection into dNCL_{SHELL}. **H.** Topographically arranged, overlapping terminal fields in Av produced by adjacent, non-overlapping injections of differently colored tracers into mid and lateral dNCL_{SHELL}.

Figure 5.

Anterograde label following control injections adjacent to dNCL_{SHELL}. Medial is left in all panels. **A.** Summary schematic depicting locations of control injections ventral and lateral to dNCL_{SHELL} and their terminal fields in arcopallium outside of Ald. Boxes indicate locations of photomicrographs. **B.** An injection ca. 400 μ m ventral to medial dNCL_{SHELL} produced anterograde terminal label in arcopallium dorsal and ventral to Ald. Arrow indicates labeled processes crossing Ald. **C.** Terminal label ventral to Ald following an injection ca. 400 μ m ventral to mid dNCL_{SHELL} extends from the ventral border of Ald into dorsal Av. **D.** Terminal label in lateral arcopallium lateral to Ald and Av following an injection ca. 700 μ m lateral to dNCL_{SHELL}. Dotted line indicates ventral edge of telencephalon. **E.** Diagram of injection site ca. 400 μ m anterior to dNCL_{SHELL} and resulting terminal label in arcopallium anterior to Ald and in dorsal portion of anterior ventral arcopallium. **F.** Diagram of injection site ca. 350 μ m posterior to dNCL_{SHELL} and resulting terminal label in arcopallium posterior to Ald.

Figure 6.

Cortical inter-hemispheric circuits for vocal behavior. **A.** Schematic of injections of differently colored tracers into left and right dNCL_{SHELL}, with resultant anterograde projections to ipsilateral AId and Av, and retrograde labeled cell bodies in contralateral Av. Boxes indicate field of view in B and C. **B,C.** Retrogradely labeled cell bodies following injections into contralateral dNCL_{SHELL} overlap with anterograde terminal label from injections into ipsilateral dNCL_{SHELL}. **D.** Av receives input from ipsilateral dNCL_{SHELL} and projects to contralateral dNCL_{SHELL}, which projects in turn to Av on the same side (blue). In a parallel circuit (red) identified by Johnson et al. (1995), LMAN_{SHELL} projects to ipsilateral Av, which sends information to contralateral SHELL. This pattern suggests Av as a site of integration of these two inter-hemispheric circuits.

Figure 7.

Retrograde label produced by injections into Av. **A.** Summary of topographic pattern of retrograde label following injections of CTB into Av. Boxes indicate location of photomicrographs. **B.** Injections of CTB conjugated to different dyes into medial and lateral Av. **C.** Extremely sparse retrograde label in arcopallium, including Aid, following an injection into Av. **D.** Labeled cell bodies in medial dNCL_{SHELL} and ventral to dNCL_{SHELL} following an injection into medial Av. **E.** Retrograde label in lateral dNCL_{SHELL} and ventral to lateral dNCL_{SHELL} following an injection into lateral Av. **F.** Topographically organized retrograde label in nidopallium directly posterior to LMAN-SHELL following dual injection shown in B; some double-labeled cells are present in the middle, indicated by arrowheads. **G.** Labeled cells in medial SHELL following injection into medial Av. **H.** Labeled cells in ventrolateral SHELL and lateral to SHELL following injection into lateral Av. **I.** Diagram of topographic retrograde label in Av contralateral to injection sites in medial and lateral Av. **J.**

shown in B. **J.** Retrogradely labeled cells in contralateral Av after injection into lateral Av. Medial is left in A-H, and right in I-J.

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Figure 8.

Retrograde label following injections into Av and adjacent arcopallium. **A.** Injections into posterior Av (including region anterogradely labeled by injections into posterior dNCL_{SHELL} and extending 400 μ m further posteriorly) produced retrograde label in posterior dNCL_{SHELL} and regions ventral and immediately posterior to dNCL_{SHELL}, but did not label cells in LMAN-SHELL. **B-C.** Injections into anterior Av (400 μ m anterior to region anterogradely labeled by injections into anterior dNCL_{SHELL}) produced retrograde label in LMAN-SHELL and adjacent anterior nidopallium, and sparse retrograde label anterior to dNCL_{SHELL}, but did not label cells in dNCL_{SHELL}. **D.** Diagram of anterior-posterior organization of Av; dNCL_{SHELL} projects to posterior regions, and SHELL to anterior regions; only the overlapping intermediate region receives inputs from both. **E.** Retrogradely labeled cells ventral to dNCL_{SHELL} following an injection ca. 200 μ m dorsal to

Av. **F.** Schematic of retrograde label produced by an injection ca. 500 μm lateral to Av. Boxes indicate the location of G-I. **G.** Retrograde label in ipsilateral caudal nidopallium, ventral and lateral to dNCL_{SHELL}. **H.** Labeled cells ventrolateral to LMAN-SHELL in ipsilateral anterior nidopallium. **I.** Retrograde label in contralateral ventral arcopallium, lateral to Av. Dotted line indicates ventral border of telencephalon. Medial is left in all panels except I.

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Figure 9.

Dual injections of CTB into dNCL_{SHELL} and Av in one hemisphere produced double-labeled cells in contralateral Av. **A.** Diagram of locations of injection sites and retrograde label; box indicates location of photomicrographs C-G in contralateral Av. **B.** Schematic of experiment; dual injections into dNCL_{SHELL} and Av produced double-labeled cells in contralateral Av, indicating that some individual Av axons branch to both targets. **C-G.** Arrows indicate double labeled cells in Av. Individual color channels are shown both separated and merged in D-G to demonstrate co-localization of neural tracers within individual cells. Medial is left.

Figure 10.

Posterior-to-anterior series of anterograde projections following injection of 10k-DA into right Av. Boxes indicate location of photomicrographs in Figure 11. Arrow indicates injection site in Av in A. Fine lines indicate regions of terminal label; thick lines indicate labeled processes.

Figure 11.

Representative photomicrographs of anterograde label resulting from injection of 10k-DA into Av. Locations indicated by boxes in Figure 10. **A.** Terminal label in lateral dNCL_{SHELL} also extends ventral and immediately lateral. **B.** Terminal label directly anterior to dNCL_{SHELL}. **C.** Labeled processes in OM. Dotted line indicates ventral edge of telencephalon. **D.** Labeled processes crossing the midline (dashed line) via the anterior commissure (AC). **E.** Terminal label in ventrolateral SHELL. **F.** Terminal label in dorsal and lateral SHELL and in nidopallium dorsal to SHELL. **G.** Terminal label in medial SHELL and in nidopallium medial and dorsal to SHELL. Medial is right in A-B and E-G, and to the left in C.

Figure 12.

Dual injections of retrograde tracers into dNCL_{SHELL} and Av in one hemisphere produce double-labeled cells in ipsilateral LMAN-SHELL and adjacent nidopallium. **A.** Schematic of experiment; dual injections of CTB conjugated to different dyes into dNCL_{SHELL} and Av retrogradely labeled individual cell bodies in SHELL and adjacent regions with both colors of tracers. **B.** Top panels show a cell labeled in both color channels, which are merged in bottom left panel. A series of images at intervals of 1 μ m focal depth provide a z-plane cross section view (corresponding to pink vertical line in bottom left panel) of the localization of the tracers, shown in bottom right panel. The two colors completely overlap, verifying that the tracers co-localized in this cell. **C-E.** Schematic and photomicrograph of method used to measure topographic overlap between injection sites in dNCL_{SHELL} and Av. Degree of overlap was calculated as percentage of the green injection site in Av that was covered by terminal label following a magenta injection into dNCL_{SHELL}. In E, the overlapping area (yellow dashed oval) measured 74% of the area of the Av injection site in this example. **F.**

Arrows indicate double-labeled neurons in SHELL distributed between single-labeled neurons. **G.** Green, magenta, and merged channels indicate both single and double-labeled neurons in SHELL following a large green CTB injection into dNCL_{SHELL} and a small magenta CTB injection into Av, which produced a larger number and wider distribution of green cells than magenta cells. Double-labeled cells were counted as percentages of both the total population of labeled cells in all of SHELL (TOTAL count) or within smaller regions, outlined in blue, containing high densities of both colors of cells (BOX count); see text.

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Figure 13.

Proportion of double-labeled cells in LMAN-SHELL following injections of retrograde tracer into dNCL_{SHELL} and Av in one hemisphere. Cells counted within the entire identified area of SHELL (TOTAL count) are shown in A and B; cells counted only within regions containing a high density of label (BOX count) are shown in C and D. Percentages of double-labeled cells were quantified as out of either the total dNCL_{SHELL}-projecting population or total Av-projecting population (labeled black and gray respectively). All data listed in Table 2. **A,C.** Graphs depicting the percentage of double-labeled cells in SHELL for each animal, average value across birds, and control injections. The latter represent single injections of mixed tracers to test whether both colors of tracer could be co-transported with high efficiency. The X axis includes bird ID and degree of overlap between injections in parentheses. **B,D.** Percentage of double-labeled cells plotted as a function of degree of overlap between injections; see text.

Table 1.

Stereotaxic coordinates used to target specific brain regions and the total number of injections into each region for all results described in this article.

Target	Coordinates (mm)			# Injections	Supplemental supporting injections
	<u>Lateral</u>	<u>Posterior</u>	<u>Depth</u>		
dNCL_{SHELL}	3.4-4.6	1.2-1.6	0.5-0.8	92	9
Control Locations					
anterior to dNCL _{SHELL}	3.6-4.4	0.9-1.1	0.5-0.8	3	1
posterior to dNCL _{SHELL}	3.6-4.4	1.7-1.8	0.5-0.8	2	2
lateral to dNCL _{SHELL}	4.65-4.8	1.3-1.5	0.5-0.8	3	2
ventral to dNCL _{SHELL}	3.6-4.4	1.3-1.5	1.2-1.6	5	0
Av	2.6-3.0	0.8-1.0	3.1-3.3	39	8
Control Locations					
anterior Av	2.6-2.9	0.5-0.7	3.1-3.4	4	3
posterior Av	2.6-2.9	1.1-1.3	2.9-3.2	2	2
lateral to Av	3.0-3.2	0.8-1.0	3.1-3.4	3	2
dorsal to Av	2.7-2.9	0.8-1.0	2.7-3.0	4	2
Total injections				157	31

Overlapping injections of the same color and tracer were considered individual injections. Lateral refers to the distance lateral from the midline; Posterior refers to the distance posterior from the bifurcation of the midsagittal sinus; Depth refers to the depth from the brain surface. Supplemental supporting injections were sectioned with a cutting angle outside the required angle range.

Table 2.

Proportion of retrogradely double-labeled (DL) cells in LMAN-SHELL after injections into dNCL_{SHELL} and Av by TOTAL count and BOX count.

Bird ID	Degree overlap (%)	TOTAL Count				BOX Count			
		%DL / dNCL _{SHELL} -labeled	%DL / Av-labeled	Profiles counted	Corrected Cell count	%DL / dNCL _{SHELL} -labeled	%DL / Av-labeled	Profiles counted	Corrected Cell count
Gy5	20.0	34.92	11.76	228	176.8	54.55	32.43	47	36.7
R167	43.8	21.21	27.86	409	317.2	52.31	44.16	108	83.8
R145	51.1	7.02	4.74	1168	905.9	24.39	19.23	166	128.7
Or168	52.6	10.78	10.78	702	544.5	34.72	31.25	127	98.5
Or48	55.6	4.58	30.00	145	114.8	6.98	23.08	53	41.1
Or47	62.9	23.26	16.34	471	365.3	36.70	39.60	170	131.9
Bk140	63.7	8.06	18.87	167	129.5	11.69	28.13	100	77.6
Bk54	67.1	34.50	16.34	473	366.9	55.71	32.23	152	117.9
Db160	73.8	36.00	16.36	71	55.1	50.00	27.78	21	16.3
Average	53.4	20.04	17.01	426	330.7	36.34	30.88	104.89	70.8

Controls	Injection Location	%DL/ magenta	%DL/ green	Profiles counted	Corrected Cell count	%DL/ magenta	%DL/ green	Profiles counted	Corrected Cell count
Or185	dNCL _{SHELL}	87.23	84.54	109	84.5	97.87	92.00	51	39.6
Or185b	Av	91.03	78.89	97	75.2	94.87	86.05	45	34.9
Or20	Av	95.16	79.73	82	63.6	97.56	78.43	56	43.4
Control Average		91.14	81.05	96	74.5	96.77	85.49	50.67	39.3

Corrected cell counts indicate Abercrombie corrected profile counts; cell profiles were counted if a portion of the soma was labeled (see Methods). Degree overlap represents the percentage of the injection site into Av which overlapped with the terminal label in Av from the dNCL_{SHELL} injection, by area. Controls received injections of a mixed solution of both colors of tracer.