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RESEARCH ARTICLE

Female zebra finches do not sing yet share neural pathways necessary for singing in males

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Abstract

Adult female zebra finches (*Taeniopygia guttata*), which do not produce learned songs, have long been thought to possess only vestiges of the forebrain network that supports learned song in males. This view ostensibly explains why females do not sing—many of the neural populations and pathways that make up the male song control network appear rudimentary or even missing in females. For example, classic studies of vocal-premotor cortex (HVC, acronym is name) in male zebra finches identified prominent efferent pathways from HVC to vocal-motor cortex (RA, robust nucleus of the arcopallium) and from HVC to the avian basal ganglia (Area X). In females, by comparison, the efferent targets of HVC were thought to be only partially innervated by HVC axons (RA) or absent (Area X). Here, using a novel visually guided surgical approach to target tracer injections with precision, we mapped the extrinsic connectivity of the adult female HVC. We find that female HVC shows a mostly male-typical pattern of afferent and efferent connectivity, including robust HVC innervation of RA and Area X. As noted by earlier investigators, we find large sex differences in the volume of many regions that control male singing (male > female). However, sex differences in volume were diminished in regions that convey ascending afferent input to HVC. Our findings do not support a vestigial interpretation of the song control network in females. Instead, our findings support the emerging view that the song control network may have an altogether different function in nonsinging females.

KEYWORDS

premotor cortex, RRID:SCR_001775, RRID:SCR_002798, sex difference, vocal learning, zebra finch

1 | INTRODUCTION

Zebra finches show a binary sex difference in vocal behavior (males sing, females do not). The discovery of a discrete network of brain regions that control the learning and production of male song (Nottebohm & Arnold, 1976, Nottebohm, Paton, & Kelley, 1982, Bottjer, Halsema, Brown, & Miesner, 1989) facilitated attempts to understand the neural origin of the striking sex difference in singing. Nottebohm and Arnold (1976) used Nissl staining to document dramatic morphological sex differences in several brain regions associated with male song—HVC and its two main efferent targets, RA and Area X, as well as nXIIts (the tracheosyringeal portion of the hypoglossal nucleus), the cranial nerve nucleus that innervates the vocal organ. All regions were smaller in females, except for Area X, which appeared to be missing in females. Sex dimorphism in the size of several singing-related brain regions—and the wholesale absence of others—provided a compelling account for the absence of singing by female zebra finches.

Konishi and Akutagawa (1985) added a synaptic explanation for the lack of female singing, reporting that the developmental formation of male-typical HVC-RA connectivity was incomplete in juvenile female zebra finches, a finding later supported by Mooney and Rao (1994) and Holloway and Clayton (2001). Because HVC and RA are part of an essential descending motor pathway for male singing (HVC-RA-nXIIts), the incomplete formation of HVC-RA connectivity would ostensibly explain the lack of singing by females. Whether HVC-RA

connectivity eventually forms and is present in adult females remains unclear. Although Williams (1985) reported that nXIIts nerve activity could not be driven by HVC stimulation in adult females, Gurney (1981) reported backfilling of HVC neurons in adult females following tracer injections in and around RA, and Wang, Sakaguchi, and Sokabe (1999) reported physiological evidence of HVC-RA connectivity in adult females. Nevertheless, HVC-RA connectivity in adult female zebra finches is described in the literature as substantially less robust (Wade, 2001; Wade & Arnold, 2004) or even absent (Beach, Tang, Kerver, & Wade, 2016; Condro & White, 2014).

The status of Area X, the second main efferent target of male HVC, also remains unclear in female zebra finches. Although a female Area X cannot be discerned in Nissl-stained tissue (Bottjer, Glaessner, & Arnold, 1985; Nixdorf-Bergweiler, 1996; Nottebohm & Arnold, 1976), Gahr (2007) backfilled HVC neurons with a tracer injected at the stereotaxic coordinates of male Area X, thereby providing evidence of male-typical HVC-Area X connectivity in females (Figure 1a). However, due to the lack of a labeling technique to quantify the precise location and dimensions of a putative female Area X, this region has been described in the literature as absent and indicative of an incomplete song network in female zebra finches (Beach et al., 2016; Condro & White, 2014; Grisham et al., 2007; Wade, 2001; Wade & Arnold, 2004).

Based on the foregoing narrative, the song network of adult female zebra finches has often been interpreted as vestigial, with a developmental trajectory that impairs capacity to produce male-typical song. However, recent data suggest an alternative view, where development shapes the female song network toward unique female-typical functionality. Benichov et al. (2016) demonstrated a sex difference in predictive call timing—a type of jamming avoidance behavior—where adult female zebra finches display a greater ability to adjust the timing of their calls than males. This ability was lost following ablation of a portion of the arcopallium that included female RA, suggesting a role for the female song network in this behavior. Moreover, call timing in males was disrupted by transection of HVC-RA axons, suggesting that even an apparently vestigial HVC-RA connection might be functional in females, although this possibility was not evaluated.

Given the discrepancies in the literature regarding the functional connectivity of "song" regions in the female, we revisited the connectivity of HVC in adult female zebra finches with a novel visually guided surgical approach to target injections of bidirectional tracers into the narrow confines of female HVC. A visually guided approach eliminates possible errors in interpretation due to slightly mislocated tracer injections, a difficulty associated with the use of stereotaxic coordinates to target a small region such as female HVC. Use of bidirectional tracers allowed us to map input as well as output connectivity of female HVC and make comparisons to the connectivity of male HVC. Defined hodologically, we find that female HVC shows the same general pattern of extrinsic connectivity as male HVC, save the relatively small male-typical RA-HVC connectivity (Roberts, Klein, Kubke,

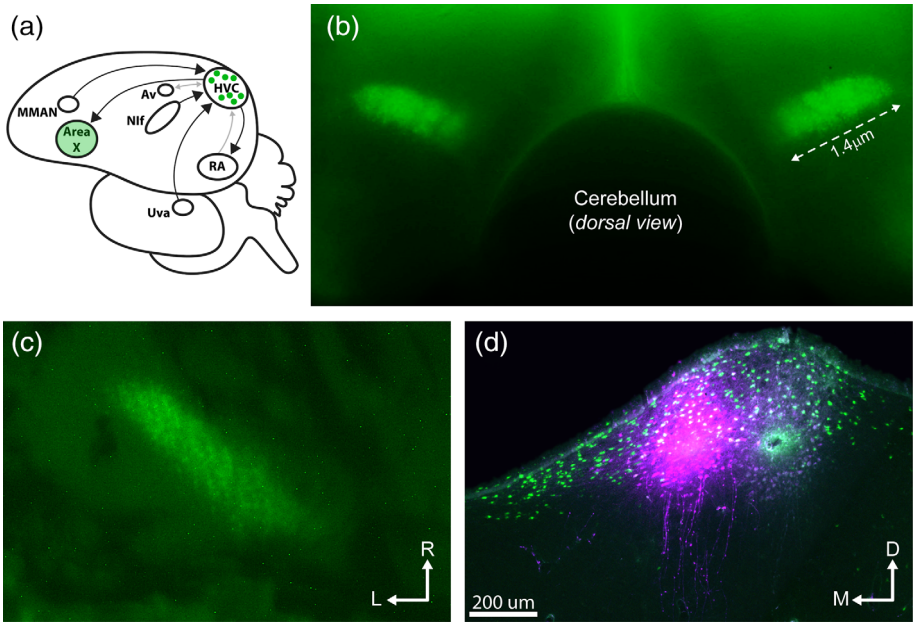

FIGURE 1 Summary of surgical approach for volumetric analysis of female HVC and visually guided tracer injections into female HVC. (a) Sagittal schematic of the extrinsic connectivity of male HVC shows that filling Area X with a green tracer backfills neurons within HVC (green dots). Male-typical pathways that were identified in females are shown in black. Pathways that could not be identified in females are shown in gray. Abbreviations: HVC, proper name; RA, Robust nucleus of the Arcopallium; Area X, Area X of the avian basal ganglia; MMAN, medial magnocellular nucleus of the anterior nidopallium; Av, nucleus avalanche; NIf, nucleus interfacialis; Uva, nucleus uvaeformis of the thalamus. (b) Low-power dorsal view of a postmortem adult female zebra finch brain under epifluorescence. Following the method of Gahr (2007), bilateral injections of a fluorescent tracer into Area X were used to backfill HVC, which appears bilaterally near the dorsal surface of the caudal telencephalon. The borders of tracer-labeled HVC were identified in serial sections and used to estimate the volume of female HVC. (c) in vivo dorsal view of labeled HVC neurons during surgery, which enabled visual guidance for placement of tracer injections into female HVC. (d) Coronal section of female HVC shows colocalization of backfilled female HVC neurons (green cells) with a visually guided tracer injection site (red labeling converted to magenta). Injection sites of this type were used to map the extrinsic connectivity of female HVC in this study

Wild, & Mooney, 2008) and HVC connectivity with nucleus avalanche (Av, Akutagawa & Konishi, 2010). Importantly, we find robust, well-defined fields of terminal labeling within female RA and Area X following HVC tracer injections. In general, song regions are smaller in females, but the sex difference is lessened in magnitude or absent in regions providing ascending afferent input to HVC. The findings suggest that HVC in both sexes may be driven by similar patterns of ascending input, with sex-specific functionality emerging through the interaction of HVC with its downstream efferent targets.

2 | METHODS

2.1 | Subjects

Adult zebra finches [*N* = 11 (male), *N* = 12 (female), >90 days post-hatch] raised in our breeding aviaries, resided in individual cages (28.50 × 21.00 × 38.00 cm) prior to surgery. Birds were housed in a room with a 14 hr/10 hr light/dark cycle, and an ambient temperature of 26 °C. Birds acclimated to solo-housing for a minimum of 1 week before tracer injections. Animals were provided water, millet-based assorted seed (400 Finch Blends; Jones Seed Company: Lawton, OK, USA) daily, as well as dietary supplements (Lafeber Finch Granules; Lafeber Company: Cornell, IL, USA) and grit weekly. All daily care procedures and experimental procedures were in accordance with the National Science Foundation and approved by the Florida State University Animal Care and Use Committee.

2.2 | Tracer injection surgeries

Birds were anesthetized via intramuscular injection of Equithesin (0.045 cc), then situated securely in a stereotaxic instrument (ASI Instruments, Inc, Warren, MI, USA). A central incision down the rostral-caudal axis of the birds' head exposed the skull, the bilateral scalp flaps were retracted laterally with forceps, and stereotaxic zero was set at the bifurcation of the midsagittal sinus, which can be visualized dorsally through the skull. Sterile phosphate buffered saline (PBS, 0.02 M) was used to clean and hydrate the surgical area.

2.2.1 | Area X injections in female birds

In *N* = 5 female birds, the method of Gahr (2007) was followed to visualize female HVC via retrograde labeling of HVC neurons that project to Area X (HVCx neurons). A rectangular craniotomy was opened above Area X using predetermined stereotaxic coordinates for male Area X (Basista et al., 2014). Glass micropipettes (1.2 mm outer diameter/0.68 mm inner diameter, World Precision Instruments: Sarasota, FL, USA) were pulled by a vertical pipette puller (model 720; David Kopf Instruments: Tujunga, CA, USA) to a tip diameter of 15–20 μm. Small quantities of green or red fluorescent tracer (DiO or DiI, 5% in DMSO, Thermo Fisher Scientific: Waltham, MA, USA) were loaded into glass micropipettes by vacuum pressure. A small amount of tracer (~200 nL) was deposited through the glass micropipette at the stereotaxic coordinates for male Area X using a controlled nitrogen gas pressure system (MPPI-3; Applied Scientific Instrumentation: Eugene, OR, USA). Tracer was allowed at least 7 days to transport in a retrograde fashion to HVC. Although Area X tracer injections were bilateral and produced symmetrical labeling across left and right HVC (Figure 1b), the statistical analysis included measurement of only one HVC (left or right) per bird.

2.2.2 | Area X injections followed by HVC injections in female birds

In *N* = 7 female birds, Area X tracer injections (DiO) were followed 1 week later by HVC tracer injections to identify and/or quantify HVC input/output connections in females. Due to the small size of female HVC and individual differences in the position of HVC relative to stereotaxic zero, backfilling of HVC_X neurons with a fluorescent tracer provides a means to visualize female HVC in vivo and precisely target a tracer injection. To prepare for visualization of female HVC, a rectangular craniotomy was opened above the approximate bilateral location of male HVC—centered over the bifurcation of the midsagittal sinus. Following removal of the skull piece, the dura mater was excised from the entire exposed region of dorsal telencephalon. Using a dissecting microscope equipped with epifluorescence (Olympus SZX10, Olympus Corporation: Shinjuku, Tokyo, Japan) to image the green fluorescent labeling of backfilled HVCx neurons in vivo (Figure 1c), red fluorescent tracer injections (DiI or rhodamine-conjugated dextran, 3,000 molecular weight, Thermo Fisher Scientific) were visually guided into female HVC. Tracer was pressure-injected through micropipettes, as described above for Area X injections. Tracer volume was estimated by determining the pressure and duration settings necessary to eject 40 nL of PBS drawn into a glass micropipette.

In some female birds, the tracer injections into Area X were unilateral, so that measured coordinates of the unilaterally labeled HVC could be mirrored across the midline. HVC injections in a hemisphere undisturbed by a prior Area X injection provided the opportunity to clearly visualize the terminal labeling of HVCx neurons. In other birds, Area X injections were bilateral, and targeted bilateral HVC tracer injections followed. However, the statistical analysis included measurement of labeling in only one hemisphere (left or right) per bird.

2.2.3 | HVC injections in male birds

To compare the morphology of regions providing afferent input to male and female HVC, tissue sections from *N* = 6 birds included in Elliott, Wu, Bertram, Hyson, and Johnson (2017) were used. Briefly, these birds received visually guided tracer injections in HVC, using the heavy myelination that surrounds male HVC to precisely target the tracer injections. These injections backfilled neurons in five different regions that provide afferent input to male HVC: RA, MMAN (medial magnocellular nucleus of the anterior nidopallium), Av, NIf (nucleus interfacialis), Uva (nucleus uvaeformis of the thalamus). See Elliott et al. (2017) for additional details.

2.2.4 | Contralateral Area X/HVC injections in a male bird

We performed surgery on an additional (*N* = 1) male bird where an Area X tracer injection in one hemisphere and an HVC tracer injection in the other were used to confirm that retrograde labeling of HVCx neurons matches the Nissl-defined border of HVC in males, and that

anterograde labeling of HVC axon terminals matches the Nissl-defined borders of RA and Area X in males.

2.2.5 | No surgery

After confirming the accurate match between tracer labeling and the Nissl borders of HVC, RA, and Area X, we perfused (as described below) $N = 5$ male birds that did not receive surgery beforehand. The brains of these birds were prepared for volumetric estimation of HVC, Area X, and RA, where we used Nissl criteria to define the border of each region.

2.3 | Perfusion and sectioning

At 2 or 7 days after the final tracer injections (2 if dextran, 7 if DiI or DiO), birds were overdosed with Equithesin (0.15 cc) and perfused intracardially first with saline (20 mL), then with ice-cold 4% paraformaldehyde (40 mL). Following perfusion, the brain was dissected and placed overnight in 4% paraformaldehyde for further fixation. The paraformaldehyde was then replaced with PBS until the brain was sectioned. The brains were sectioned in parasagittal or coronal planes. For parasagittal sectioning, the brain was split down the midline with a razor blade and the two sides were separated from each other and sectioned. For coronal sectioning, a razor-blade cut was made orthogonal to the midline—directly above the rostral edge of the midbrain, splitting the brain into rostral and caudal halves. Regardless of the plane of sectioning, all brains were sliced into 50 μm sections using a vibratome (Leica VT1000S, Leica Biosystems: Wetzlar, Germany).

2.4 | Fluorescent imaging of labeled cells

Fluorescent images were taken with a digital camera (Optronics Microfire, Optronics: Goleta, CA, USA) attached to a Leica DM5000B microscope. Serial sections containing nuclei that send or receive input to HVC were mounted on glass slides and imaged with a 5×, 10×, or 20× objective. Using the imaging capabilities of Neurolucida 7.0 (www.mbfbioscience.com, RRID: SCR_001775), images of each brain section that contained retrograde or anterograde labeling were taken. Once all relevant fluorescent images had been acquired, all sections were Nissl stained with thionin, and cover slipped.

2.5 | Volume estimation of female HVC, RA, Area X, NIf, and Uva

For each bird, all unilateral images of a given retrogradely or anterogradely labeled region were loaded into Neurolucida. One by one, in serial sections, borders were drawn around each region of interest, outlining the shape of the observed fluorescent labeling. Information about the number of sections in which fluorescent labeling was observed, as well as the thickness of the sections, was entered into Neurolucida. In conjunction with the area values defined by the outlines drawn around fluorescent labeling, the software used section number and thickness information to calculate an estimated unilateral volume (in mm^3) for each region of interest.

2.6 | Volume estimation of male HVC, RA, Area X, NIf, and Uva

For HVC, RA, and Area X in male birds, serial brightfield images of each region were captured and loaded into Neurolucida. An estimated unilateral volume for each region was obtained by tracing the Nissl border of each region (HVC, RA, Area X) in serial sections. Although NIf and Uva are both visible in Nissl-stained tissue, the borders of these regions are not clearly defined in Nissl. Thus, in birds that received fluorescent tracer injections in HVC (Elliott et al., 2017), we estimated the unilateral volumes of these regions by tracing the outline of backfilled neurons in serial sections.

2.7 | Statistics

Two-tailed *t* tests were performed to compare the volumes of male and female song regions. All statistical analyses performed in GraphPad Prism version 5.03 for Windows (www.graphpad.com, RRID: SCR_002798).

3 | RESULTS

3.1 | Retrograde labeling of female HVC for measurement and visually guided tracer injections

Following the method of Gahr (2007, see Figure 1a), we fluorescently backfilled HVC neurons that project to Area X (HVC_X) to visualize HVC and measure HVC volume in female birds. We then compared female and male HVC to assess sex similarities and differences in HVC morphology. Viewed from a dorsal perspective (Figure 1b), female HVC takes a similar shape and position as male HVC, although the dimensions of female HVC are smaller across both medial–lateral and rostral–caudal axes. Similarly, when viewed in sagittal tissue sections, female HVC appears smaller across dorsal–ventral and rostral–caudal axes. Although this sex difference can be somewhat difficult to discern in Nissl-stained tissue, due mainly to the ambiguous ventral border of HVC in female tissue (compare Figure 2a,c), the sex difference in HVC dimensions is apparent following fluorescent backfilling of HVC_X neurons (compare Figure 2b,d).

We estimated the volume of female HVC by tracing the outline of HVC_X neurons filled with fluorescent tracer in sagittal tissue sections. In thionin-stained tissue sections, the Nissl border of female HVC appears to match the border observed in fluorescently labeled tissue (compare Figure 2a,b). In males, the Nissl border of HVC is visually apparent and matches the border observed in fluorescently labeled tissue (compare Figure 2c,d). Therefore, we estimated the volume of male HVC by tracing the Nissl border of HVC in sagittal tissue sections. Note that in all female song regions, whether the region is identifiable by Nissl criteria or not, neurons are smaller, at greater density, and less distinct from the surrounding neuronal population than the corresponding neurons in male song regions. HVC is no exception. Based on measures made in fluorescently labeled tissue sections, Figure 3a shows that the volume of female HVC (mean ± *SEM* = 0.109 ± 0.013 mm^3, $n = 5$) was consistently smaller than male HVC (mean ± *SEM* = 0.470 ± 0.069 mm^3, $n = 6$). This difference was statistically

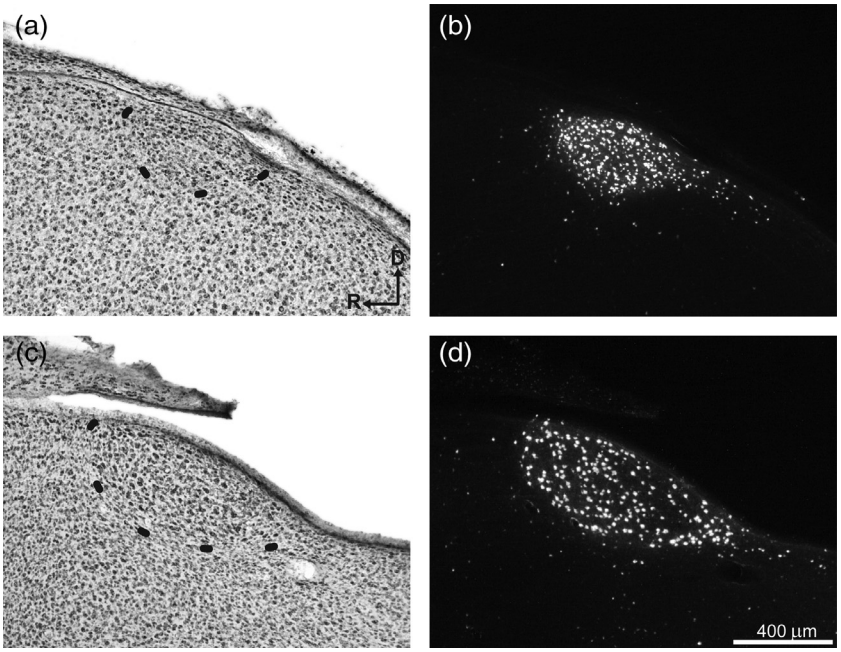

FIGURE 2 Sagittal views compare Nissl-defined and hodologically defined HVC in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the cell labeling produced by injection of a fluorescent tracer into Area X. The borders defined by backfilled HVC neurons map onto Nissl-defined borders in both sexes (white dashes in a, c). However, viewed in Nissl-stained tissue the ventral border of female HVC (a) is less distinguishable from the surrounding nidopallium than is the ventral border of male HVC (c). See Figure 3a for volumetric comparison of female and male HVC

significant (t_9 = 4.72, p = 0.001) and confirms prior measurements of the sex difference in HVC volume that were made in Nissl-stained tissue (Bottjer et al., 1985; Bottjer, Miesner, & Arnold, 1986; Nixdorf-Bergweiler, 1996; Nordeen & Nordeen, 1988) or in tissue labeled with neuropeptide (vasoactive intestinal polypeptide, enkephalin) immunocytochemistry (Ball, Absil, & Balthazart, 1995a).

We mapped the extrinsic connectivity of female HVC using a variant of the fluorescent-labeling procedure used to identify and measure female HVC. That is, as described in the Section 2, another group of female birds received an initial tracer injection to backfill HVC_X neurons (tracer targeted stereotaxically to Area X). One week later, we conducted a second surgery where we imaged the backfilled HVC under in vivo epifluorescence (Figure 1c) and made a second tracer injection (tracer targeted visually to HVC). Visualizing HVC during the second surgery enabled highly targeted injections of a bidirectional tracer (Figure 1d). All retrograde and anterograde labeling observed following HVC tracer injections remained ipsilateral to the injection site, and all descriptions that follow refer to ipsilateral patterns of labeling.

3.2 | Anterograde Labeling of female RA

HVC tracer injections were used to anterogradely label HVC neurons that project to RA (HVC_{RA}). We then visualized the terminal labeling of female HVC_{RA} neurons in sagittal tissue sections. Earlier findings suggested that the HVC projection into RA may never fully develop in female zebra finches (Holloway & Clayton, 2001; Konishi & Akutagawa, 1985; Williams, 1985).

However, we found a robust field of fluorescent terminal labeling filling out the Nissl-defined border of RA in adult female zebra finches (Figure 4a,b), anatomical data that confirm the physiological findings of Wang et al. (1999). Thus, in both sexes the terminals of HVC_{RA} neurons form a dense field of labeling that permeates RA and fills out the Nissl border.

We estimated the volume of female RA by tracing the outline of fluorescently labeled HVC axon terminals that filled RA. When sections are stained with thionin, the border of female RA can be discerned using Nissl criteria, and this border generally matches the border observed in fluorescently labeled tissue (compare Figure 4a,b). In males, the Nissl border of RA is visually apparent and matches the border observed in fluorescently labeled tissue (compare Figure 4c,d). Here, we estimated the volume of male RA by tracing the Nissl border of RA. Consistent with the sex difference in the neuronal morphology of HVC, female RA neurons are smaller and less distinct from the surrounding neuronal population than the corresponding neurons in male RA. Figure 3b shows that the volume of female RA (mean $\pm$ *SEM* = 0.038 $\pm$ 0.007 mm^3, n = 6) is considerably smaller than male RA (mean $\pm$ *SEM* = 0.288 $\pm$ 0.031 mm^3; n = 6). This difference was statistically significant (t_{10} = 7.84, p < 0.0001) and confirms prior measurements of the sex difference in RA volume that were made in Nissl-stained tissue (Bottjer et al., 1985; Bottjer et al., 1986; Nixdorf-Bergweiler, 1996; Nordeen & Nordeen, 1988), or in tissue labeled with neuropeptide (vasoactive intestinal polypeptide, enkephalin) immunocytochemistry (Ball et al., 1995a).

In males, a small subpopulation of neurons in dorsal–caudal RA receives and returns connectivity with HVC (Roberts et al., 2008). Injecting HVC with a bidirectional tracer backfills these neurons in males, where due to the intensity of terminal labeling they are most clearly seen at portions of the dorsal–caudal margin of RA where the intensity of terminal labeling is reduced (Basista et al., 2014; Elliott

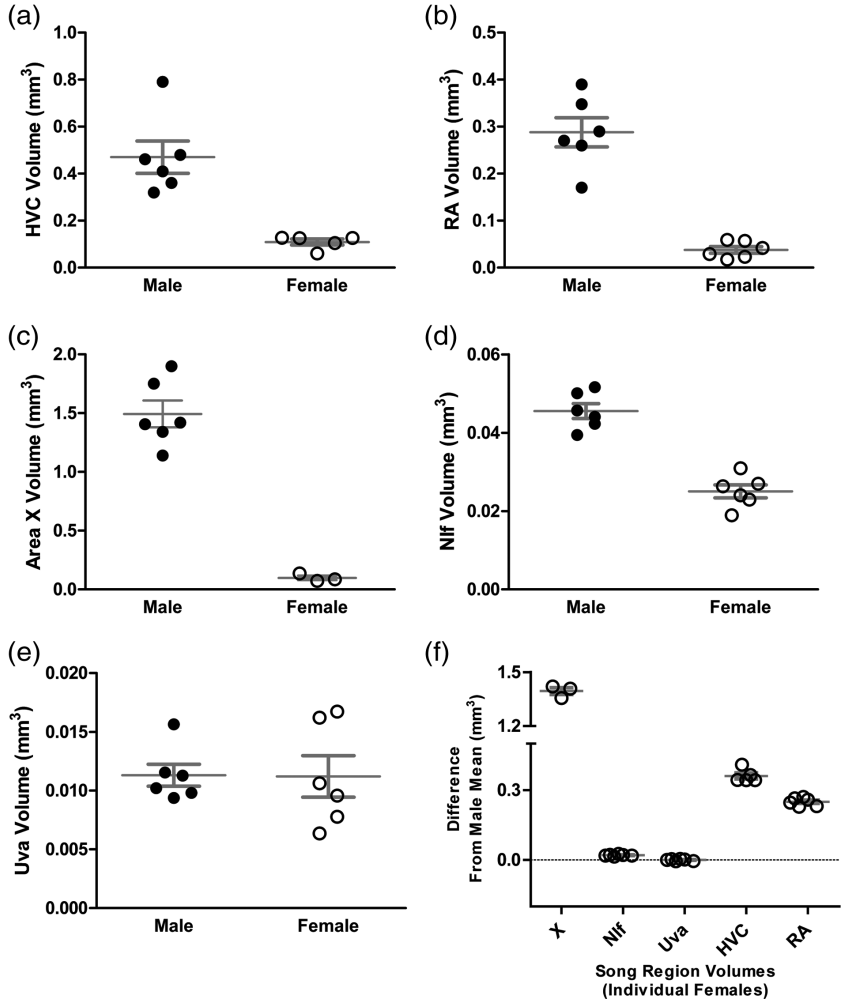

FIGURE 3 Sex differences and a similarity in the unilateral volumes of five brain regions involved in the control of male song. Individual male and female data points shown as filled and open circles, respectively. Mean ± *SEM* shown for all comparisons, with $p < 0.01$ indicated by dual asterisks. (a, b, c) The volumes of HVC and its two main efferent targets, RA and Area X, are significantly larger in males than in females. In magnitude, these differences are considerable, with female HVC, RA, and Area X less than one-quarter the volume of the corresponding male regions (f). (d) The volume of female NIf, which relays ascending input to HVC, is significantly smaller than that of males, although female NIf is about one-half the volume of male NIf (f). (e) The volume of female Uva, the primary source of ascending thalamic input to HVC, is not significantly different from that of male Uva. However, female Uva shows substantial individual variation in volume relative to the average volume of male Uva (f)

et al., 2017). We did not observe a distinct population of backfilled cells at the dorsal–caudal margin of female RA, which may indicate a sex difference in RA–HVC connectivity. However, we do not rule out the possibility that some RA–HVC projecting neurons were present within our sections of female RA but obscured by the intensity of the terminal labeling. HVC injections with the primarily retrograde tracers used by Roberts et al. (2008) may be necessary to determine the presence or absence of RA–HVC connectivity in female zebra finches.

3.3 | Anterograde labeling of female area X

The backfilling of female HVC neurons following tracer injections into the dorsal striatum indicates that female HVC contains neurons that project to a region that corresponds to Area X in males (Gahr, 2007, present results). However, it has long been recognized that the dorsal striatum of adult female zebra finches does not contain a Nissl-defined region that corresponds to Nissl-defined Area X in adult males (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996; Nottebohm & Arnold, 1976). Therefore, we sought to establish the dimensions of female Area X using anterograde labeling of the terminal arborizations of HVC_X neurons. For this procedure, HVC was backfilled with a unilateral tracer injection into the dorsal striatum. One week later, we visualized the unilaterally labeled HVC under in vivo epifluorescence and stereotaxic coordinates for the backfilled HVC were then mirrored to the unlabeled (contralateral) hemisphere for HVC tracer injections. The goal was to provide a clear view of the terminal labeling of HVC_X neurons, without the visual impediment of a large injection of tracer in the dorsal striatum. This procedure was successful in three of six birds—in the other birds, the tracer injection missed because the injection site was below the ventral border of HVC, a consequence of the difficulty in targeting a tracer injection within the thin dorsal–ventral axis of female HVC (~200 μm).

As defined by the terminal arborizations of HVC_X neurons, female Area X presents as a distinct, small region of labeling in the dorsal

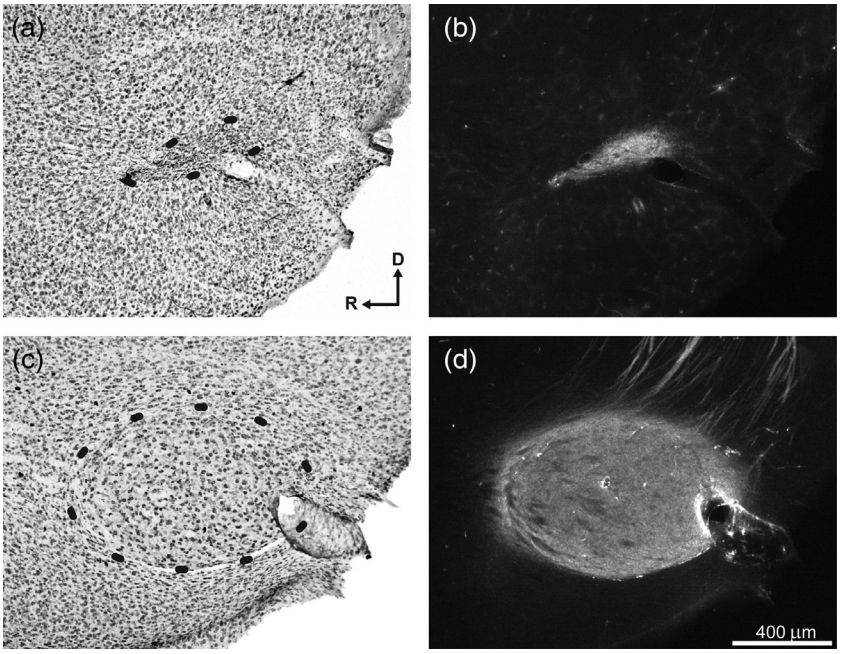

FIGURE 4 Sagittal views compare Nissl-defined and hodologically defined RA in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the terminal labeling produced by injection of a fluorescent tracer into HVC. In both sexes, terminal labeling fills the Nissl-defined borders of RA (white dashes in a, c). See Figure 3b for volumetric comparison of female and male RA

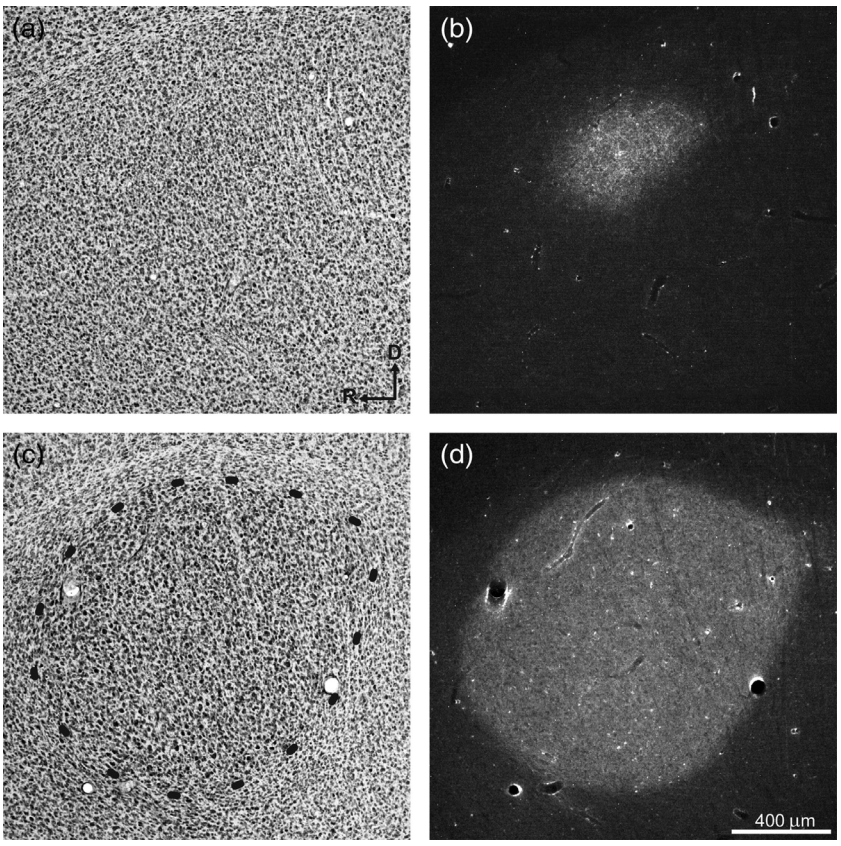

FIGURE 5 Sagittal views compare Nissl staining with hodologically defined Area X in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the terminal labeling produced by injection of a fluorescent tracer into HVC. Area X is clearly visible by anterograde labeling from HVC in females (b) but cannot be identified in Nissl-stained tissue (a). It may be helpful for the reader to block panel (b) from view when inspecting the Nissl-stained panel (a) to prevent negative afterimages that can cause false perception of a Nissl-defined female Area X. In males, the Nissl-defined borders of male Area X can be distinguished from the surrounding striatum (white dashes in c) and the terminal labeling in (d) maps onto these borders. See Figure 3c for volumetric comparison of female and male Area X

striatum (Figure 5b). Examination of thionin-stained tissue sections failed to reveal a Nissl-defined region for female Area X (Figure 5a and Bottjer et al., 1985; Nottebohm & Arnold, 1976). Multiple attempts to discern and measure a Nissl-defined Area X in females—particularly under conditions when we were blind to the fluorescent pattern of terminal labeling—were fruitless. We note that when examining Figure 5a,b, care should be taken not to visually fixate on Figure 5b before looking over to Figure 5a, lest a negative afterimage cause the viewer to momentarily perceive what appears to be a Nissl-defined Area X in Figure 5a.

As defined by the terminal labeling of HVC_X neurons, female Area X takes a similar shape as male Area X (compare Figure 5b,d). Female Area X sits in the dorsal striatum, nestled just under the pallial-subpallial lamina (PSL) as it does in males. In males, Area X is robust and identifiable in Nissl-stained sections and the Nissl-defined borders match the area defined by the terminal arborizations of HVC_X neurons (Figure 5c,d). With the caveat that our female sample is small, tracings of fluorescent labeling were used to estimate the volume of female Area X, which we compared to Nissl tracings of Area X in males. Figure 3c shows Area X to be considerably smaller in females (mean $\pm$ *SEM* = 0.098 $\pm$ 0.019 mm^3; n = 3) than in males (mean $\pm$ *SEM* = 1.493 $\pm$ 0.114 mm^3; n = 6). This difference was statistically significant (t_7 = 8.305, p < 0.0001).

3.4 | Retrograde labeling of female NIf

In males, HVC receives afferent input from three regions of avian cortex: NIf, Av, and MMAN (Akutagawa & Konishi, 2010; Bottjer et al., 1989; Elliott et al., 2017; Fortune & Margoliash, 1995; Foster, Mehta, & Bottjer, 1997; Nottebohm et al., 1982; Vates, Vicario, & Nottebohm, 1997). None of these regions have borders that are readily identified by Nissl criteria in male zebra finches (let alone in female zebra finches) and all have often been identified through tracer labeling or through immunohistochemical labeling. Relative to HVC, the physically closest of these afferent inputs is NIf, which when labeled in the retrograde direction from HVC presents sagittally as an oblong grouping of neurons, extending from the caudal apex of the PSL toward the LaM, and angled so that the dorsal tip of NIf points towards HVC (Bottjer et al., 1989; Elliott et al., 2017; Nottebohm et al., 1982). In Nissl staining, NIf neurons tend to align in orientation and so a distinct region can be discerned. However, they do not form an obvious border defined by darkness of staining or neuron size and density (Fortune & Margoliash, 1992). Therefore, to our knowledge, NIf has never been traced by Nissl staining in males (or females) due to the ambiguity of its border (Figure 6a,c). As in males, when female NIf is labeled in the retrograde direction from HVC, it also presents sagittally as an oblong grouping of neurons that emerges from the caudal apex of the PSL and is angled toward HVC (compare Figure 6b,d). As with male NIf, female NIf is discernable in Nissl-stained sections, although neurons within female NIf are smaller and more densely organized than those within male NIf (compare Figure 6a,c).

We found the border of NIf in males and females to be clear and traceable when backfilled by a tracer injection into HVC, similar to the neuropeptide (vasoactive intestinal polypeptide, enkephalin) immunocytochemical labeling used by Ball, Absil, and Balthazart (1995b) to identify the border of NIf and measure volume. Thus, we measured the volume of male and female NIf by tracing the border of the fluorescent-backfilled neurons populating the region, finding that female NIf (mean $\pm$ *SEM* = 0.025 $\pm$ 0.002 mm^3; n = 6) was smaller than male NIf (mean $\pm$ *SEM* = 0.046 $\pm$ 0.002 mm^3; n = 6). This sex

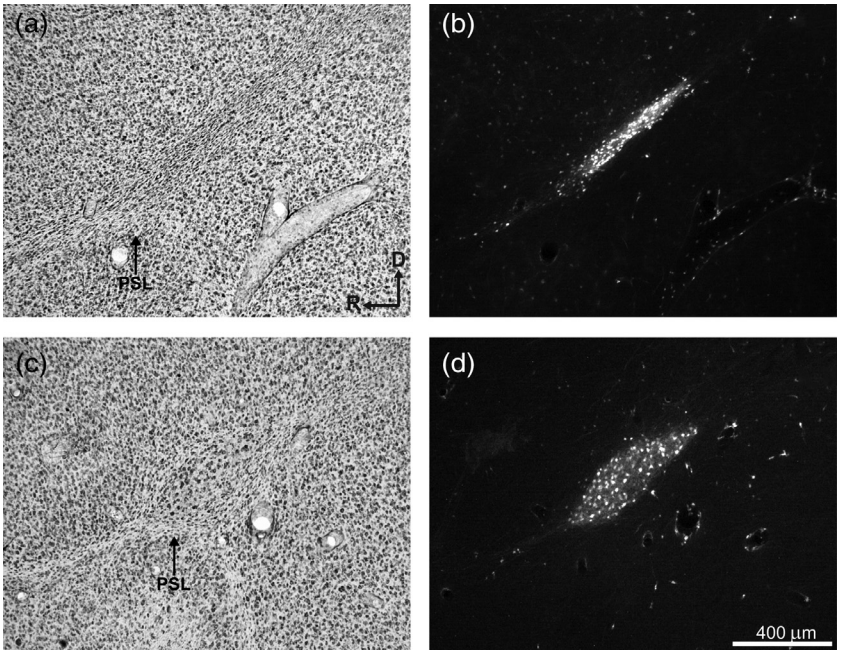

FIGURE 6 Sagittal views compare Nissl staining with hodologically defined NIf in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the retrograde labeling produced by injection of a fluorescent tracer into HVC. NIf is clearly visible by retrograde labeling in females (b) and males (d) but the borders of NIf are ambiguous in Nissl-stained tissue from both sexes (a, c). As a landmark, the position of the pallial-subpallial lamina (PSL) is noted with an arrow in (a) and (c)

difference in the volume of NIf was statistically significant (t_{10} = 8.119, p < 0.0001, see Figure 3d) and confirms the earlier findings of Ball et al. (1995b).

3.5 | Absence of a tracer-defined female Av

Viewed in the sagittal plane, Av in male zebra finches is a small triangular or ovoid region within the caudal mesopallium (CM), immediately dorsal to the LaM, with NIf located below. Av has bidirectional connectivity with HVC, projecting to HVC and receiving input from a distinct population of HVC projection neurons (Akutagawa & Konishi, 2010; Elliott et al., 2017; Nottebohm et al., 1982; Roberts et al., 2017). However, following tracer injections into male HVC, the observed Av labeling—both in terms of the number of Av neurons projecting to HVC and the quantity of terminals received from HVC neurons—is quite sparse (Akutagawa & Konishi, 2010; Elliott et al., 2017; Nottebohm et al., 1982). Indeed, Av was originally considered a "field," with no differences in cell size or orientation to distinguish Av from the surrounding CM in Nissl-stained tissue (Nottebohm et al., 1982).

Unsurprisingly, a region that corresponds to male Av cannot be distinguished in Nissl-stained tissue from females. Therefore, we sought to use HVC tracer injections to identify female Av, as done previously in males (Akutagawa & Konishi, 2010, Elliott et al., 2017, Nottebohm et al., 1982). We employed the same bidirectional fluorescent tracers that successfully labeled male Av neurons in Elliott et al. (2017), and observed CM to be largely bereft of labeling of any kind. Careful visual inspection within CM failed to find retrograde or anterograde labeling in the region where male Av is found, immediately dorsal to LaM with NIf located below. In the same sagittal tissue sections where we were unable to discern female Av, we could determine that NIf labeling was robust (a positive control) and that the HVC injection was accurate and well-targeted.

3.6 | Retrograde labeling of female MMAN

Viewed in the sagittal plane, MMAN is located within the small, nidopallial space defined by the convergence of the LaM (above) and the PSL (below) near the medial edge of the telencephalon (Figure 7a,c). In zebra finches, the canary-based name of MMAN is a misnomer—in males, the neurons of MMAN are not larger, nor otherwise visibly distinct, from surrounding cells in Nissl-stained tissue (Bottjer et al., 1989; Ryan & Arnold, 1981). Thus, the backfilling of MMAN neurons following injection of a retrograde tracer into HVC has proven to be the most reliable method to identify MMAN in male zebra finches (Bottjer et al., 1989; Foster et al., 1997; Vates et al., 1997). Following backfilling from HVC, male MMAN resembles a loose federation of cells, which is perhaps the reason why it cannot be identified using Nissl criteria (Figure 7d).

Following injections of tracer into female HVC, backfilled neurons are present in the nidopallial location of male MMAN (Figure 7b). Like males, careful analysis of tissue sections following Nissl staining reveals the lack of a Nissl border around female MMAN (Figure 7a). Visual inspection suggests that female MMAN is not as large, or as robustly filled by HVC tracer injections as it is in males (compare Figure 7b,d). While we did not attempt volumetric reconstruction because female MMAN could only be clearly identified in 1 or 2 sagittal sections in individual birds, we note that male MMAN typically

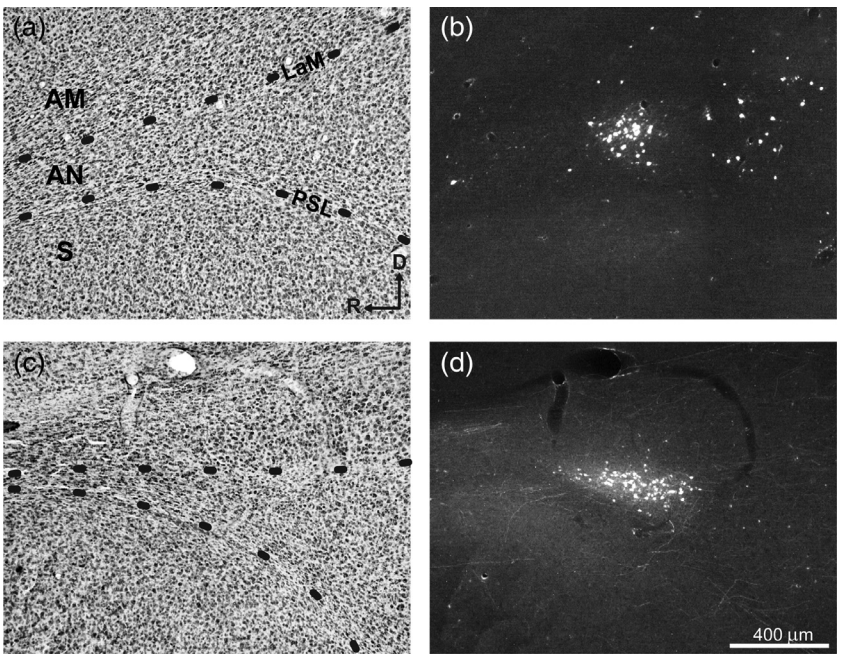

FIGURE 7 Sagittal views compare Nissl staining with hodologically defined MMAN in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the retrograde labeling produced by injection of a fluorescent tracer into HVC. The borders of MMAN but cannot be discerned in Nissl-stained tissue in either sex (a, c). However, under epifluorescence MMAN is visible as a distinct cluster of labeled neurons in the medial portion of the anterior nidopallium (AN) in females (b) and males (d). The anterior mesopallium (AM) and striatum (S) flank the anterior nidopallium dorsally and ventrally, respectively. The position of the mesopallial lamina (LaM) and pallial-subpallial lamina (PSL) are indicated by dotted lines in (a) and (c)

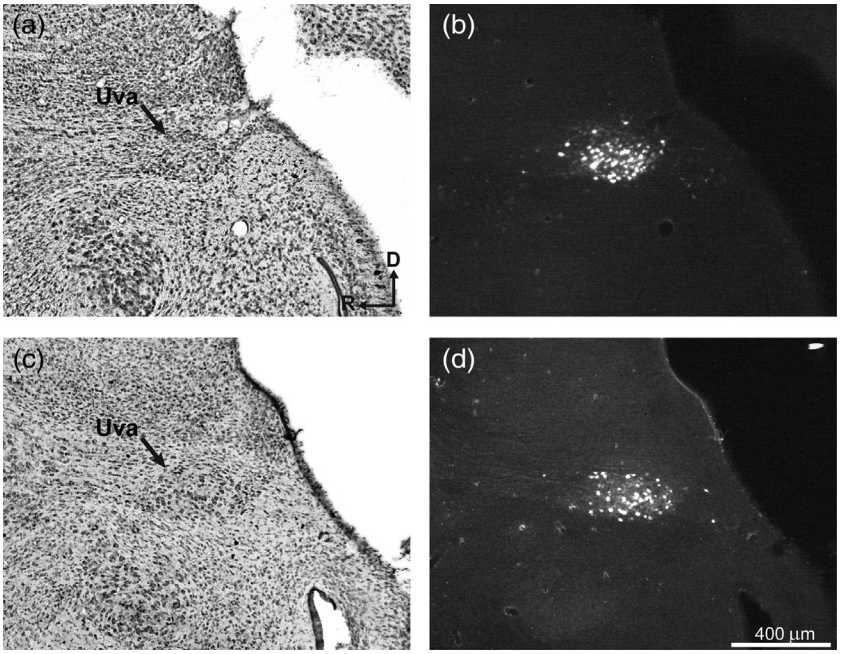

FIGURE 8 Sagittal views compare Nissl staining with hodologically defined Uva in female (a, b) and male (c, d) zebra finches. The same Nissl-stained sections in (a, c) are shown in (b, d) under epifluorescence to reveal the retrograde labeling produced by injection of a fluorescent tracer into HVC. The borders of Uva are somewhat ambiguous in Nissl-stained tissue from both sexes (a, c) but particularly so in females. However, under epifluorescence Uva is visible as a distinct cluster of labeled neurons in the dorsolateral portion of the posterior thalamus in females (b) and males (d). See Figure 3e for volumetric comparison of female and male Uva

appears in 4 or 5 sagittal sections using the same section thickness and measurement of alternate sections (Elliott et al., 2017). Thus, it seems likely that zebra finch MMAN, along with HVC, RA, Area X, and NIf shows a M > F sex difference in size.

3.7 | Retrograde labeling of female Uva and the magnitude of sex difference across measured song regions

HVC receives ascending thalamic input from a single source—Uva. Note that in males, Uva ascending input to HVC arrives via a direct pathway (Uva-HVC) as well as via an indirect pathway through NIf (Uva-NIf-HVC, Akutagawa & Konishi, 2010). Named for its small grape-like appearance in Nissl-stained tissue, Uva is discernable in males and females alike (Nottebohm et al., 1982; Wild, 1994). Although the size of Uva does not appear to be sexually dimorphic upon visual inspection (Wild, 1994, Figure 8a,c), the absence of a sex difference in the size of Uva has never been quantified. Retrograde labeling following a fluorescent tracer injection into HVC reveals a border that appears to roughly match the Nissl border of Uva in males (Akutagawa & Konishi, 2010, present results) and in females (present results). Moreover, the overall pattern of fluorescent Uva labeling appears identical in females and males—in location, shape, and size (compare Figure 8b,d).

We measured the volume of male and female Uva by tracing the border of fluorescent-backfilled neurons that populated the region, finding that female Uva (mean ± *SEM* = 0.011 ± 0.002 mm^3; n = 6) and male Uva (mean ± *SEM* = 0.011 ± 0.001 mm^3; n = 6) were not significantly different in volume (t_{10} = 0.04837, p = 0.9624, see Figure 3e). Uva was the only measured region in this study that did not show a sex difference in volume.

Figure 3f summarizes the magnitude of the sex difference in the volume of the five song regions measured in this study (expressed as percent male mean for each region). Viewed together, the volumes of HVC and two regions targeted by HVC efferents (RA, Area X) show the largest sex differences (25%, 15%, 7%, respectively). In contrast, one afferent input to HVC shows a substantially smaller sex difference (NIf, 48%) while another input (Uva) is not sexually dimorphic at all. This lessening of the morphological sex difference in afferent input to HVC suggests that HVC in both sexes may be driven by similar patterns of ascending input (particularly from Uva), with a larger sex difference emerging as HVC and its efferent targets transform afferent input into a motor-related output.

4 | DISCUSSION

Thought previously to be incomplete or vestigial (see reviews by Wade, 2001, Wade & Arnold, 2004, Condro & White, 2014), we revisited the extrinsic connectivity of HVC in nonsinging female zebra finches with a visually guided surgical approach to target injections of bidirectional tracers. Visual guidance, which substantially increased the accuracy of HVC tracer injections, was made possible by backfilling HVC_X neurons with fluorescent tracer prior to making HVC tracer injections. The present findings demonstrate a pattern of extrinsic HVC connectivity in female zebra finches that follows the same general pattern observed in male zebra finches. As in males, female NIf, MMAN, and Uva project to HVC, and female RA and Area X both receive dense terminal fields from HVC projection neurons. We were,

however, unable to identify reciprocal connectivity from RA to HVC or bidirectional HVC-Av connectivity in females. Interestingly, while the downstream targets of female HVC (RA and Area X) are substantially reduced in volume compared to males, the sex difference of ascending inputs to HVC is either smaller (NIf) or absent (Uva). These categorical (input vs. output) differences in the magnitude of the sex dimorphism suggest that male and female HVC may be driven by similar patterns of upstream activity, and that the behavioral sex difference in zebra finches is a result of a divergence in the function of HVC and its downstream targets.

4.1 | Relationship to prior findings

4.1.1 | Efferent HVC-RA connectivity

Konishi and Akutagawa (1985) and Holloway and Clayton (2001) both reported near-binary developmental sex differences in the extent to which RA is innervated by axons projecting from HVC. In these reports, axon terminals (which originated in HVC) robustly filled RA in 25- to 35-day-old males yet appeared to cluster densely along the dorsal border of RA in age-matched females, with few axons immigrating into RA proper. In contrast, our study of adult female zebra finches finds that while there is indeed a large sex difference in the volumes of HVC and RA, female RA is fully and robustly innervated by axons projecting from HVC. Moreover, we find no evidence of HVC axon terminals clustering along the dorsal border of adult female RA. The most parsimonious explanation for the discrepancy between our findings and those of Konishi and Akutagawa (1985) and Holloway and Clayton (2001) is that female HVC axons grow into RA over a slower developmental time scale.

Our HVC-RA findings in adult female zebra finches agree with those of Gurney (1981), who reported scattered retrograde labeling in HVC following tracer injections in and around RA of adult females, as well as those of Wang et al. (1999), who used an in vitro slice preparation to show physiological evidence of a functional HVC-RA connectivity in adult females. Wang et al. (1999) also reported that application of a GABA antagonist to female slices dramatically increased the excitatory activity recorded in HVC and RA, yet the same manipulation had little effect on activity in HVC and RA in male slices. Because we show a pattern of HVC-RA connectivity in females that could potentially support singing (Figure 4b), the sex difference in response to pharmacological suppression of GABAergic inhibition could reflect a means to suppress singing-related neural activity in female RA, or a unique female-typical RA functionality—perhaps both.

Recent data suggest that RA of adult female zebra finches may be specialized for a female-typical behavioral response (Benichov et al., 2016). That is, compared to adult males, adult female zebra finches show a greater capacity for predictive call timing than males. RA appears to be necessary for the expression of this sex difference. Consistent with a role for female RA in the control of vocalizations, retrograde labeling experiments in female zebra finches demonstrate descending connectivity from RA to the vocal hindbrain (Johnson & Sellix, 2000). Interestingly, experiments in a related species (canary) suggest the descending connectivity of RA may also influence female copulatory solicitation displays (CSDs) via disynaptic control of spinal motoneuron pools (Wild & Botelho, 2015). These data lead to the view that HVC and RA may be a part of a more general network that controls courtship and reproductive behavior (including learned song and CSDs) in both sexes (reviewed by Perkes et al., 2017).

In adult males, HVC and RA are reciprocally connected—a small population of neurons in dorsal RA send and receive connectivity with HVC (Basista et al., 2014; Elliott et al., 2017; Roberts et al., 2008). In adult females, we did not observe retrogradely labeled cells in dorsal RA following HVC tracer injections. However, we do not rule out the possibility that the small size of female RA coupled with the intensity of anterograde terminal labeling from HVC-RA neurons may have obscured a small population of RA-HVC projecting neurons.

4.1.2 | Efferent HVC-Area X connectivity

Building on the prior work of Gahr (2007), we used anterograde labeling of HVC axon terminals to provide the first delineation of the size and position of Area X in female zebra finches (Figure 5). Because it cannot be identified in Nissl-stained tissue, Area X has been considered absent in female zebra finches (Bottjer et al., 1985; , Condro & White, 2014; Nixdorf-Bergweiler, 1996; Nottebohm & Arnold, 1976; Wade, 2001; Wade & Arnold, 2004). However, we know from work in male zebra finches that Nissl identification of brain regions is not a necessary precondition for functional importance—neither MMAN nor Av can be identified as distinct regions in Nissl-stained tissue, yet both regions influence the developmental learning and adult production of song (Foster & Bottjer, 2001; Roberts et al., 2017). Indeed, like female Area X, the anatomical position of Av in male zebra finches can only be identified using tract-tracing techniques (Akutagawa & Konishi, 2010).

In males, Area X links the functions of the avian basal ganglia into processes of song learning and production, contributing to a cortico-basal ganglia-thalamo-cortical loop hypothesized to regulate the motor variability necessary to adjust the vocal output of a juvenile male toward a specific acoustic target (the song of an adult tutor, Fee & Goldberg, 2011, Woolley & Kao, 2015). We have documented a portion of this loop in females (HVC-Area X, the cortico-basal ganglia component), but connectivity from Area X to the medial portion of the dorsolateral thalamus (DLM), from DLM to the lateral magnocellular nucleus of the anterior nidopallium (LMAN), and from LMAN to Area X remain to be investigated. Because females do not learn to produce song, it remains to be seen what if any behavioral functions might be influenced by a cortico-basal ganglia-thalamo-cortical loop in female zebra finches.

4.1.3 | Ascending thalamic input to HVC

In males, the thalamic nucleus Uva contains two distinct neural populations, one ascending directly to HVC and the other projecting to the nidopallial cortical nucleus NIf, which in turn also projects to HVC (Akutagawa & Konishi, 2010). The present findings show that female HVC, like male HVC, receives input from NIf and Uva (Figures 6 and 8). In adult males, ablation, pharmacological, and recording experiments demonstrate that Uva and NIf provide ascending afferent drive on HVC premotor activity, resulting in production of the learned syllable sequence (Coleman & Vu, 2005; Danish, Aronov, & Fee, 2017; Lewandowski, Vyssotski, Hahnloser, & Schmidt, 2013; Naie &

Hahnloser, 2011; Otchy et al., 2015; Vyssotski, Stepien, Keller, & Hahnloser, 2016; Williams & Vicario, 1993). In juvenile males, NIf also makes premotor as well as auditory contributions to song learning (Piristine, Choetso, & Gobes, 2016; Roberts, Gobes, Murugan, Ölveczky, & Mooney, 2012).

Because females do not sing, it may seem surprising that Uva and NIf—given their necessary premotor role in male song production—show only a modest volumetric sex difference (NIf, Figure 3d) or none at all (Uva, Figure 3e). One possibility is that Uva and NIf play a similar premotor role in both sexes, conveying a generic ascending afferent drive that serves to elicit HVC activity in both sexes. If so, the binary sex difference in song production would be the result of a transformation of Uva and NIf afferent signaling within HVC and/or the associated downstream pathways from HVC to RA or HVC to Area X. It should be emphasized, however, that the female functions of any of the ascending projections from Uva and NIf to HVC are yet untested.

4.1.4 | Other cortical connectivity with HVC

In male zebra finches, HVC receives input from another nidopallial cortical region (MMAN) and has bidirectional connectivity with a mesopallial cortical region (Av). MMAN and Av cannot be identified in Nissl-stained tissue in male or female zebra finches—both were initially revealed through retrograde labeling in tract-tracing experiments (Bottjer et al., 1989; Nottebohm et al., 1982). The lack of a distinctive nuclear organization in Nissl-stained tissue belies the importance of these regions in male singing as MMAN (Foster & Bottjer, 2001) and Av (Roberts et al., 2017) influence both juvenile learning and adult production of song.

Using a tract-tracing approach, which was employed to detect MMAN and Av connectivity with HVC in males (Elliott et al., 2017), MMAN was identified as a source of afferent input to HVC in females (Figure 7). However, we were unable to identify a pattern of retrograde or anterograde labeling that would correspond to female Av. Despite robust retrograde labeling within nearby NIf, no labeling was observed within the portion of the caudal mesopallial region that corresponds to male Av. One possibility is that Av input to HVC conveys activity that is needed only for male-typical functions of HVC.

5 | CONCLUSION

Although future investigation will be needed to confirm the apparent absence of RA-HVC and Av-HVC connectivity in females, the present findings are sufficient to conclude that the overall network connectivity of male and female HVC is remarkably similar. Sex differences (male > female) in the volume of HVC and its two primary efferent targets (RA and Area X) highlight the role of these three regions in male-typical song learning and production. However, the persistence of these regions and pathways in females points to the possibility of female-typical functions such as predictive call timing (Benichov et al., 2016) or CSDs (Perkes et al., 2018; Wild & Botelho, 2015). Diminished sex differences in the morphology Uva and NIf—regions that convey ascending thalamic input to HVC—further suggest that similar types of sensory information may drive the appropriate sex-typical output from male and female HVC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

DWS and FJ conducted all experimental work and collection of anatomical data, assisted by Laurel Moore, Lexi Perrini and Matt Branch. The authors thank Kevin C. Elliott for assistance in developing the surgical approach used for tracer injections. DWS, RLH, RB, WW, and FJ all contributed to data analysis and manuscript preparation.

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