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EXPLORING LINKS FROM SENSORY PERCEPTION TO MOVEMENT AND BEHAVIORAL MOTIVATION IN THE CAUDAL NIDOPALLIUM OF FEMALE SONGBIRDS

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Abstract

Decision making resides at the interface between sensory perception and movement production. Female songbirds in the context of mate choice are an excellent system to define neural circuits through which sensory perception influences production of courtship behaviors. Previous experiments by our group and others have implicated secondary auditory brain sites, including the caudal nidopallium (NC), in mediating behavioral indicators of mate choice. Here, we used anterograde tracer molecules to define projections that emerge from NC in female songbirds, identifying pathways through which NC influences downstream sites implicated in signal processing and decision making. Our results reveal that NC sends projections into the arcopallium, including the ventral intermediate arcopallium (AIV). Previous work revealed that AIV also receives input from another auditory area implicated in song preference and mate choice (caudal mesopallium, CM), suggesting that convergent input from multiple auditory areas may play important roles in initiating mate choice behaviors. In the present results, NC projects to an area implicated in postural and locomotory control (dorsal arcopallium, Ad), suggesting that NC may play a role in directing those forms of copulatory behavior. NC projections also systematically avoid a vocal motor region of the arcopallium that is innervated by CM (robust nucleus of the arcopallium, RA). These results suggest a model in which both NC and CM project to arcopallial pathways implicated in behavioral motivation. These brain regions may exert different influences on pathways through which auditory information can direct different facets of behavioral responses to information detected in those auditory signals.

Graphical Abstract

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ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: NAB, KL, JFP. Acquisition of data: NAB, KZ, TF, KL, ZZ, JFP. Analysis and interpretation of data: NAB, KZ, KL, TF, EP, HH, ZZ, JFP. Drafting of the manuscript: NAB, JFP. Critical revision of the manuscript for important intellectual content: NAB, JFP. Statistical analysis: NAB. Obtained funding: JFP. Administrative, technical, and material support: NAB, KL, JFP. Study supervision: NAB, JFP.

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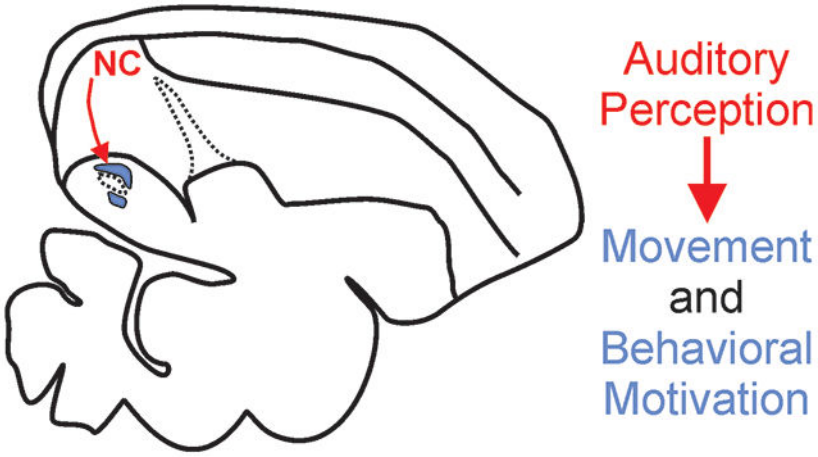
Ethics Approval Statement: All procedures were approved by the University of Wyoming Animal Care and Use Committee, and procedures were in compliance with recommendations from that group and state and federal regulations governing the housing of songbirds.



Keywords

Arcopallium; Nidopallium; Forebrain; Bengalese finch; Anterograde tracer; Courtship signal; Decision making; Mate choice

1. INTRODUCTION

The cognitive process of decision making resides at the interface between sensory perception and the production of movement. This is especially evident in examples of courtship signaling that play important roles in many forms of mate choice. Courtship signals are transmitted by a sender and perceived by a receiver, and the receiver perceives that sensory experience and uses that information to select one behavioral outcome from among many possible alternatives. Female songbirds are an excellent model system to investigate the neural circuits through which sensory perception influences mate choice, as songs performed by males are a primary means through which females evaluate the quality of the associated singers and use that information to choose their mate (Catchpole & Slater, 2008). Song is such a potent influence on mate choice that females will solicit copulation in response to song played through a speaker even if no male is physically present (Catchpole & Slater, 2008). In this study, we harnessed the advantages of this model system to continue to define the neural circuits through which sensory perception can influence activity in downstream areas underlying the production of behavioral responses.

Songbirds possess a network of specific brain structures that underlie auditory processing and express many similarities to corresponding pathways found in mammals (Butler & Hodos, 2005). Auditory information ascends from the ear and is processed through a sequence of brainstem sites followed by a telencephalic primary auditory area and eventually arriving at secondary auditory areas including the caudal nidopallium (NC) and the caudal mesopallium (CM). These NC and CM areas are avian analogs of layers II/III in the secondary auditory cortex in mammals (Karten, 1991; Wang et al., 2010). Experiments by our group and others have revealed that altering activity in either of these secondary auditory areas can induce changes in a female songbird's evaluation of the quality of a song stimulus. Specifically, inactivation of those sites causes females to become less selective in their

production of behavioral indicators of mate preference. Additional experiments by our group have revealed pathways through which CM can influence behavior through projections to motor sites that control behavioral indicators of mate choice such as calls and copulatory posture, and to dopaminergic pathways implicated in reward and behavioral motivation (Dunning et al., 2018). Those results also revealed that NC is a primary recipient of output from CM. Thus, behavioral results reveal an important role for NC in shaping female song evaluation and mate choice, but it remains unknown what collection of neural circuits may enable activity in NC to influence perception and mate choice. One idea is that NC may project back to CM, and it is through that reciprocal connection that NC could indirectly influence behavioral output. Alternatively, NC may project directly to downstream areas implicated in motor activation or behavioral motivation, enabling NC to directly influence behavioral output in parallel with circuits emanating from CM. Interpreting the role of NC in female mate choice requires direct examination of the projections that emanate from NC in female songbirds.

If we are to understand the neural mechanisms through which decision making emerges in the context of mate choice by female songbirds, it is essential that we identify the complete circuit through which auditory information is processed and used to direct the activity of downstream neurons that shape expression of behavioral output. Here we used an anterograde tracer molecule to define the projections that emerge from NC in female songbirds. Our results reveal that NC projects primarily to a telencephalic area that is also the target of projections from CM, revealing convergence of those two pathways onto a dopaminergic pathway implicated in reward and behavioral motivation. NC also projects to another telencephalic area that has been implicated in control of movement and posture. NC does not project to the vocal motor area that receives input from CM and that influences the production of calls (nucleus RA), revealing a distinction in how those auditory areas influence behavioral indicators of mate choice. These data indicate that NC is well positioned to affect motor performance of behavioral indicators of mate choice, and they provide important new insights into the complete circuit through which sensorimotor integration and decision making emerge in this system.

2. METHODS

2.1 Animal Care and Housing

All experiments were performed using adult (age > 120 days post hatch) female Bengalese finches (BF; *Lonchura striata domestica*) obtained from a commercial breeder (Magnolia Bird Farm, CA or Louie's Aviary, RI). Females were identified by the absence of song in continuous recordings of all vocal behavior over a four day period. This was further verified through histological analysis of sexually dimorphic brain structures (e.g., HVC) at the conclusion of each experiment. Birds were housed in same-sex group cages (41 × 31 × 24 cm) that maintained the 15:9 light:dark photoperiod used throughout our colony. To avoid injury or infection, birds were housed individually following surgical procedures. All procedures were approved by the University of Wyoming Animal Care and Use Committee, and procedures were in compliance with recommendations from that group and state and federal regulations governing the housing of songbirds.

2.2 Stereotaxic Surgery

Tracer molecules were injected into NC of adult female BFs using either biotinylated dextran amine (BDA, 16 birds) or an adeno-associated virus to induce cells to express green fluorescent protein (GFP, 4 birds). For birds that received BDA injections, each subject was anesthetized with a gaseous solution of 3% isoflurane in oxygen. When deep anesthesia had been achieved (i.e., unresponsive to stimuli such as gentle toe pinch), the bird was moved to a stereotaxic apparatus, and the head was fixed in place using ear bars. With the beak angled 45° below horizontal, bilateral craniotomies were made above NC at specific stereotaxic coordinates in relation to the bifurcation of the mid-sagittal sinus (0.5 mm anterior, 1.6 mm lateral). A syringe (Hamilton, NV) was filled with 10% BDA (10,000 MW, Molecular Probes, OR) in phosphate-buffered saline (PBS, 0.1M) and lowered into NC (1.5 mm ventral), where a total volume of 400 nl was pressure injected into each injection site. To help facilitate focal placement, injections were made in multiple subsets of 100 nl separated by 3-15 minutes, and the syringe was left in place for several minutes following the final injection and prior to removal. After complete injection and withdrawal of the syringe, a silicone elastomer (Kwik-Sil, World Precision Instruments, FL) was used to cover all craniotomies, and a surgical adhesive (Vetbond, 3M, MN) was used to close the scalp. When the closed incision was dried, a local analgesic (2.5% Lidocaine, 2.5% Prilocaine, HI-Tech Pharmacal, NY) was applied to the site. After completion of the surgery, the subject was moved to a recovery cage under a heat lamp and monitored until complete recovery (i.e., upright, eating, drinking) before being placed in an isolated recovery housing cage.

An additional set of four birds received a different treatment to help confirm the identity of projections that were observed in experiments performed using BDA. In those birds, each subject was anesthetized with a gaseous solution of 3% isoflurane in oxygen. When deep anesthesia had been achieved (i.e., unresponsive to stimuli such as gentle toe pinch), the bird was moved to a stereotaxic apparatus, and the head was fixed in place using ear bars. Bilateral craniotomies were made above NC, and NC was targeted using the same stereotaxic coordinates used for birds that received BDA. Instead of receiving an injection of BDA, each of these four birds received an injection of an adeno-associated virus (AAV) into the same location to induce NC neurons to express GFP (serotype 1; product number AAV1.CMV.PI.eGFP.WPRE.bGH from Penn Vector Core, University of Pennsylvania School of Medicine). This means of inducing GFP expression results in vivid labeling of somas at the site of injection with virtually no retrograde transport (fewer than one neuron per brain as documented in Chamberlin et al., 1998; Dunning et al., 2018). Use of this additional method to identify pathways emerging from NC enables us to address and minimize the concern that labeling may have arisen because BDA affected fibers of passage rather than somas that reside in the site of interest (NC). As detailed in the following section, the results obtained using this method served as additional verification that the projections observed are efferents from NC.

2.3 Tissue Processing

Following seven days of post-surgical survival, subjects with BDA injections were deeply anesthetized with an overdose of isoflurane and transcardially perfused through the left ventricle with ice-cold physiological (0.9%) saline followed by 4% paraformaldehyde (PFA)

in PBS. The brain was then carefully extracted, transferred into a vial of 4% PFA, and stored at 4°C for 24 hours before being moved into 30% sucrose PFA and stored at 4°C for another 24 hours to cryoprotect the tissue. Sagittal sections were cut at 40 μm thickness using a cryostat and placed on gelatin-coated slides to dry overnight. The following day, the tissue was processed to visualize the BDA. Sections were first rehydrated in PBS for 20 minutes and then washed for another 20 minutes in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidases. The sections were washed again in three consecutive 15 minute PBS baths before being incubated in a 1% bovine serum albumin in PBS mixture for 1 hour to block non-specific binding of proteins to the tissue. To localize the BDA, an avidin-biotin detection ABC Elite Kit (Vector Laboratories, CA) was applied to the tissue in a humidification chamber for 1 hour. The sections then went through three consecutive 15 minute washes in PBS containing 0.1% Triton X-100 (PBST) to permeabilize the tissue. A DAB substrate (3,3-diaminobenzidine, ImmPACT DAB, Vector Laboratories, CA) was used to visualize the BDA, which was evident as a brown-colored reaction product. The tissue was then washed in two consecutive 15 minute PBS baths to wash out the remaining DAB before being submerged in cresyl violet. Finally, the sections were dehydrated in ascending alcohols, cleared in xylenes, and coverslipped with a Krystalon mounting medium (Fisher Scientific, PA). Tissue was imaged using an Olympus BX51 Fluorescence Microscope with an RT-SE camera. Images of BDA-injected tissue were taken under bright field conditions and analyzed with SPOT software (SPOT Imaging, MI).

Following a survival period of 21 days, subjects that received AAV injections were perfused and the tissue was collected just as was done for BDA-injected subjects. Sagittal sections were cut at 40 μm thickness and placed on gelatin-coated slides and allowed to dry overnight. The next day, the tissue underwent immunohistochemistry for AAV-GFP immunofluorescence. The sections were washed in three consecutive 5 minute PBS baths, followed by one 15 minute PBST bath, before a final 5 minute PBS bath. The sections were then submerged in PBST containing 5% goat serum for 30 minutes before being placed in a humidification chamber where a primary antibody (1:1000 dilution of mouse IgG_{2a} anti-GFP in PBS, Invitrogen, RRID: AB_221568) was applied to the tissue. The humidification chamber was stored overnight at 4°C. The following morning, the tissue was washed in three consecutive 10 minute PBS baths then returned to the humidification chamber where a secondary antibody (1:500 dilution of goat anti-mouse IgG Alexa Fluor 488 in PBS, Invitrogen, RID: AB_263275) was applied for one hour at room temperature. After two final 15 minute PBS baths, the slides were coverslipped using a Fluoromount-G with DAPI mounting medium (Invitrogen, CA). Images of AAV-injected brains were taken using a Zeiss 700 laser scanning confocal microscope and analyzed with SPOT software (SPOT Imaging, MI).

3. RESULTS

3.1 Tracer injections reveal projections from caudal nidopallium neurons

All injection sites were restricted to NC (N = 24 hemispheres, 16 birds, Figure 1a). Specifically, all labeled cells resided posterior of the lamina mesopallialis (LaM) and superior to the dorsal arcopallium lamina (LaD), and in no case did the injection site include

other identifiable regions such as Field L or nucleus interfacialis (Figures 1b–e). The exact location of injection sites varied slightly across subjects, but all were in NC and commonly included the caudal margin of the hemisphere and thus the caudal portion of NC (Figure 1f). Injection sites were recognized by the presence of BDA-labeled cell bodies, dendritic arborizations, and the emergence of axonal projections to downstream locations (e.g., Figure 1e–f), and the affected volume typically extended 250-350 μm in the medial-lateral direction and 200-300 μm in the anterior-posterior direction. There are no clearly defined anatomical boundaries between the medial and lateral portions of NC, therefore we were not able to determine the extent to which cells in those divisions were labeled, and all cells were simply designated as residing in NC (Table 1).

To identify projections from NC to target locations, we examined tissue to look for the presence of axonal filaments and varicosities that indicate the presence of synaptic connections. In all birds that we studied (N = 24 hemispheres, 16 birds), varicosities were present in NC, indicating local projections to areas of NC both within and beyond the injection site (Figure 2). Such projections from NC neurons to other cells in NC have also been described in male zebra finches (Vates et al., 1996).

NC also projected to more distant targets, and those connections were evident as labeled bundles of fibers (Figure 3a–e). Axonal projections coursed inferiorly from the site of injection and crossed the LaD into the arcopallium (e.g., Figure 1b, 1e). Labeled fibers were clearly evident along that path but were not evident in any other direction emerging from NC. Those labeled fibers terminated as dense fibers and varicosities in the ventral portion of the intermediate arcopallium (AIV, Figure e-f, n = 20 hemispheres, 13 birds). These data are consistent with previous reports of a projection from NC to AIV in male songbirds (Mandelblat-Cerf et al., 2014). Intriguingly, these projections into the arcopallium respected the boundaries of the robust nucleus of the arcopallium (RA; tissue and surrounding areas shown in Figure 4d–e), leaving it clearly devoid of labeled axons and varicosities (n = 16 hemispheres, 11 birds, Figure 4). There was only one hemisphere in which we observed a labeled fiber invading the boundary of RA, but there were no varicosities along the extent of that fiber (Figure 1e). Thus in no case did we detect evidence of a synaptic projection from NC to RA. In addition to the projections observed from NC to AIV, we also observed projections from NC to the dorsal arcopallium (Ad). These projections crossed the LaD and terminated in Ad, located directly superior to RA and inferior to the LaD (Figure 4c). Labeled fibers from NC also projected to another arcopallial area that is most consistent with the caudal part of the anterior arcopallium (AAc; Figure 4g) (Mello et al., 2019).

We analyzed the possible relationships between the precise location of the injection site (e.g., superficial vs. deeper, anterior vs. posterior) and the nature of the injection that we observed in that hemisphere. Across all injections within NC, no consistent relationship was evident between the location of the injection and the nature of the associated projection. Together, those data indicate that neurons residing in the entire range of NC that we sampled project into the arcopallium, and there are no regional distinctions in the patterns of those projections.

In many of the birds we studied, injections were bilateral, making it difficult to determine if the observed projections were ipsilateral, contralateral, or both. To resolve that uncertainty, we placed unilateral injections of the BDA tracer molecule in only one hemisphere of four birds (two placed in the left hemisphere, two placed in the right hemisphere). Results from unilateral injections revealed the types of projections described above in the ipsilateral hemisphere regardless of the side where the injection was placed, and no labeled projections were ever detected in the contralateral hemisphere (n = 4 hemispheres, 4 birds). Thus, all of the projections described here are ipsilateral and express no detectable lateralization (Table 1).

Previous reports have indicated that 10K BDA can occasionally result in retrograde labeling (Veenman et al., 1992). If retrograde labeling occurred here, it could have labeled small numbers of somas in sites that project to NC. In some cases, we detected BDA-labeled somas in Field L. This is consistent with previous reports of projections to secondary auditory areas such as NC from Field L, an area that contains neurons proposed to be similar to layer 4 neurons in the primary auditory cortex of mammals (Vates et al., 1996).

In birds where we investigated these projections using unilateral injections of AAV into NC, we found patterns identical to those detected using 10K BDA (Figure 5, n = 4 hemispheres, 4 birds). Specifically, we observed GFP-labeled somas in NC (Figure 5a), and in no case did we find labeled cells outside of the extent of NC (i.e., there were no labeled somas in Field L or anywhere else apart from NC in these birds). We also observed labeled bundles of axons leaving NC and coursing toward the arcopallium (white arrows in Figures 5a–c), and we detected varicosities in the AIV region of the arcopallium (Figures 5d–f). In no case did we observe any projections in the contralateral hemisphere. Together, these results obtained using AAV to achieve exclusively anterograde labeling confirm and clarify the observations we obtained using BDA, revealing a description of the projections from NC to its downstream targets (Figure 6).

4. DISCUSSION

In the present study, we identified the pathways through which the auditory area NC exerts its influence on downstream brain sites implicated in signal processing and decision making. Pathways were identified primarily using BDA labeling and confirmed using AAV-GFP labeling that enables us to interpret these pathways as emerging from somas residing in NC and minimizes concern that labeling may have arisen from unintended labeling of fibers that pass through NC from elsewhere (Chamberlin et al., 1998; Dunning et al., 2018). In addition to local connections within NC itself, NC neurons send robust projections into a brain region implicated in perception of auditory stimuli (AIV). Work from another group has shown that AIV projects to a brainstem site containing dopaminergic neurons implicated in reward and behavioral motivation (Mandelblat-Cerf et al., 2014). Previous work from our group revealed that another auditory area, CM, also sends dense projections into AIV (Dunning et al., 2018). Injections in those previous experiments could also have encompassed nucleus avalanche, which also resides in that same region (Akutagawa & Konishi, 2010; Feenders et al., 2008; Roberts et al., 2017). Together, those previous data and the present results reveal

that both NC and CM project to AIV, but projections from NC encompass a larger portion of the arcopallium than the area that is spanned by projections from CM.

NC also projects to additional areas of the arcopallium immediately surrounding the vocal motor structure RA, including a region of the dorsal arcopallium implicated in locomotory control (Ad) (Feenders et al., 2008; Mandelblat-Cerf et al., 2014). Notably, NC axons respect the boundaries of RA, sending dense projections to areas surrounding it while not sending synaptic projections into RA itself. This is a key distinction between projections from NC, which innervate AIV and other regions of the arcopallium but systematically avoid RA, and projections from CM, which also innervate AIV and make synaptic connections onto neurons in RA (Dunning et al., 2018). The present data reveal projections from NC into pathways implicated in behavioral motivation and locomotory control. Together with previous results describing projections from CM to its downstream targets, these data reveal pathways through which activity in auditory processing sites can influence perception of sensory input and activation of motor output.

4.1 Functional Contributions of Activity in Auditory Cortical Areas

The auditory areas NC and CM comprise the secondary auditory cortex in the songbird brain and are analogous to layers II/III of auditory cortical regions in mammals (Karten, 1991; Wang et al., 2010). In studies of male songbirds, NC has been implicated in many aspects of auditory processing, including the long-term representation of song memory (Chew et al., 1995), the recognition of auditory patterns (Stripling et al., 2001), and the neural representation of tutor-song memory (Gobes & Bolhuis, 2007). Studies in female songbirds have extended those insights, linking activity in NC to song perception and mate choice (Gentner et al., 2001), recognition of species-specific song (Bailey et al., 2002), and perception of vocalizations to assign identity to specific individuals (Menardy et al., 2012). Activity in CM has also been associated with many of the same song recognition behaviors associated with NC (Bailey et al., 2002; Gentner et al., 2001; Gentner & Margoliash, 2003; Leitner et al., 2005). For example, activity in CM of female birds has been implicated in song perception (Bailey et al., 2002; Gentner & Margoliash, 2003), the perception and discrimination of male quality (Leitner et al., 2005), and the neural representation of the memory of their father’s song (Terpstra et al., 2006). Together, these results indicate that NC and CM play key roles in perception of behaviorally relevant information in auditory signals. An important goal of future studies should be to continue to define the roles of these areas and their respective contributions to auditory processing and behavioral response in both sexes.

CM is the source of pathways connecting the auditory system to downstream targets implicated in vocal, locomotory, and hormonal control, as well as context-dependent release of dopamine (Dunning et al., 2018). Interconnections between CM and NC and their convergence onto AIV reveal pathways through which activity in each of these areas may influence multiple types of behavioral output. Behavioral experiments by our group have shown that female BFs indicate their mate preference through the production of copulation solicitation displays and calls in response to strongly preferred song stimuli (Dunning et al., 2014). Each of these forms of behavioral output is clearly related to processing of auditory

signals, as they are produced in response to male songs even if they are played through a speaker and no male is physically present (Dunning et al., 2014). Additional work from our group has revealed that inactivation of either NC or CM results in decreased selectivity of song preference, and optogenetic activation of neurons in CM results in strongly increased production of behavioral indicators of preference (calls) in response to songs that were previously unattractive (Elie et al., 2019). Together, these data strengthen the link between activity in NC and CM of female songbirds and social decision making in the context of mate choice, with activity in NC associated with intensity of preference, and activity in CM associated with both intensity of preference and the production of behavioral indicators of mate preference (Brenowitz, 1991; MacDougall-Shackleton et al., 1998). This suggests that NC and CM may work together in processing auditory signals. Other authors have found that patterns of immediate early gene expression can differ between neurons in CM and NC due to hearing song or performing movements (Feenders et al., 2008), suggesting that the pattern of coordinated activity may be complicated and perhaps variable from one context to the next. These results highlight the importance of additional investigations in which electrophysiological recording and optogenetic manipulation are employed to define the functional contributions of these auditory brain sites (e.g., Elie et al., 2019).

4.2 Projections from NC to Areas Implicated in Behavioral Motivation and Activation

Projections from NC to AIV in female BFs are consistent with what has been reported previously in male zebra finches (Mandelblat-Cerf et al., 2014). Previous studies have demonstrated that neurons in AIV are responsive to auditory stimuli, and inactivation of those cells results in deficits of vocal imitation (Mandelblat-Cerf et al., 2014). Some authors have speculated that deficits in imitation could emerge because of reduced contributions to detection and modification of vocal errors (Mandelblat-Cerf et al., 2014), however, those deficits could also emerge from altered behavioral salience of a song stimulus and decreased motivation to achieve imitative accuracy. That link to motivation is made plausible by the finding that neurons in AIV project to dopaminergic neurons in the ventral tegmental area (VTA) in the brainstem (Mandelblat-Cerf et al., 2014). Neurons that reside in AIV and project to VTA are sensitive to changes in auditory feedback (Mandelblat-Cerf et al., 2014), providing a link between auditory experience and activity in VTA. VTA neurons also project to regions implicated in behavioral control (Mandelblat-Cerf et al., 2014), providing an additional link between activity in VTA and influence on behavioral output. Together, these findings reveal a circuit that can be tested as a possible mechanism through which activity in areas implicated in perception and evaluation of song quality may influence activity in areas associated with control of the associated courtship behaviors.

In the same study that revealed projections from auditory areas to AIV in male songbirds, those authors also noted distinctions between the portion of AIV that is innervated by NC and the portion that is innervated by CM (Mandelblat-Cerf et al., 2014). Specifically, they proposed distinctions between the anterior and posterior portions of AIV. Neurons that reside in the anterior portion of AIV receive greater input from CM, whereas neurons that reside in the posterior portion receive greater amounts of input from NC (Mandelblat-Cerf et al., 2014). In the present study and previous results from our group (Dunning et al., 2018), we extend those findings by showing that CM and NC both project to AIV in female

songbirds. Comparison of the locations within the female AIV that receive input from NC and CM suggest that at least part of AIV receives input from both areas. Investigating this further and determining the degree to which any single neuron in AIV may receive different amounts of input from each area will be an important goal of future studies. If individual neurons in AIV receive input from both NC and CM, those cells could serve as a station where input from both sites is required to activate downstream pathways. This idea is consistent with other examples where coincident input plays a key role in the perception of auditory input (Koppl et al., 2000).

A connection between NC, CM and AIV could contribute to sensory based decision making through an arrangement in which neurons in NC and CM may contribute different facets of auditory processing and may be integrated through their convergence onto AIV. In preliminary studies, we have identified populations of CM neurons that respond in ways that could support this type of processing (JF Prather, unpublished observations). In recordings of CM neurons in awake and freely behaving birds as they are engaged in behavioral tests of song preference, we have detected cells with auditory responses that are related to different facets of the song stimulus. The majority of cells that we detect have tonic, step-like responses to songs. The magnitude of those responses is closely related to the bird's preference for that song, with more preferred songs evoking greater amounts of activity. Other cells in CM have responses to song stimuli that are very phasic, consisting of brief bursts of activity that are tied to specific features within the song. A recent study from another group has also revealed different classes of neurons in CM with different physiological and morphological characteristics (Chen & Meliza, 2018). Those data are also consistent with the idea that different classes of auditory neurons may encode auditory information on different timescales. An important goal of future experiments will be to further characterize the auditory response properties of such neurons within and across CM and NC. If subjective evaluation and song identity were represented in different streams comprising different groups of auditory neurons, those facets of perception could be processed independently then converge onto AIV to enable the brain to bind preference to the identity of the song and the associated singer. Integrated output from AIV could be passed on to dopamine-recipient areas to affect motor output and thus influence subsequent behaviors. This possible model can be investigated using behavioral, electrophysiological, and optogenetic approaches. An important future goal will be to test the possibility that such a circuit may be a key mechanism through which salient features of sensory stimuli are extracted and used to selectively activate reward systems in service of decision making.

In addition to robust projections to AIV, we also observed extensive projections from NC to Ad. These results confirm and extend previous reports of projections from NC to Ad that were found in male zebra finches (Bottjer et al., 2000), indicating that these circuits are a general feature of functionality in both sexes. Previous studies have offered contrasting insights into the possible function of activity in Ad. One study reported that lesions in Ad result in impaired vocal learning (Bottjer & Altenau, 2010). Another study, however, demonstrated that immediate early genes are activated in Ad and elsewhere during locomotor activities, such as hopping, but are not activated during singing (Feenders et al., 2008). Subsequent studies of the functional contributions of specific areas of the arcopallium revealed that lesions in AIV are associated with impaired vocal imitation, but lesions in

Ad result in severe akinesia and immobility (Mandelblat-Cerf et al., 2014). These results are most parsimoniously explained by the possibility that Bottjer and Altenau may have unintentionally affected small regions of AIV as well as neurons in Ad. Together, these and other insights have given rise to the idea that Ad and RA may form the output of a general cortical motor circuit, with RA specialized for vocal production and Ad serving a more general role in locomotory behaviors (Feenders et al., 2008).

In the present study, we very commonly observed projections from NC to arcopallial areas immediately surrounding RA (Ad, AIV). We also detected projections from NC to another area that is most consistent with it being the caudal part of the anterior arcopallium (Mello et al., 2019). Notably, we never observed projections from NC to RA itself. In light of speculation regarding possible functional contributions of activity in Ad and RA, projections from NC to Ad but not to RA suggest that NC may exert a greater influence on gross motor behaviors than vocal output such as calls or songs. In contrast to the pattern we observe for NC projections, CM projects to RA as well as AIV (Dunning et al., 2018). These results suggest a possible model in which both NC and CM project to arcopallial pathways implicated in behavioral motivation, but these brain regions exert different influences on pathways through which auditory information can be used to direct different facets of the behavioral response to information detected in those auditory signals.

A previous study by our group revealed a robust projection from CM to NC. Those data also revealed small numbers of labeled somas in NC, indicating a sparse reciprocal connection from NC to CM (Dunning et al., 2018). Other authors have also reported bidirectional connectivity between NC and CM, and those authors also reported that the connection from NC to CM was sparse and sometimes difficult to detect (Vates et al., 1996). Data in the present study did not reveal a connection from NC to CM, with none of the cells that we labeled in NC projecting exclusively or collaterally into CM. This result is also consistent with a possible connection from NC to CM that is sparse and difficult to detect. Taken together, these findings suggest that NC and CM are interconnected, but the connection from CM to NC is much more robust than the connection from NC to CM.

We previously demonstrated the existence of projections from the auditory area CM to pathways implicated in perception of auditory experience (NC), behavioral motivation and reward (AIV), motor control of the vocal behaviors that female BFs use to indicate their mate preference (RA), motor control of copulatory behaviors such as copulation solicitation displays (hypothalamic sites and spinal motor areas downstream of RA), and to areas implicated in initiation and integration of motor behaviors (caudal striatum) (Dunning et al., 2018). Here, we describe another important element in this emerging animal model of decision making. The present data reveal projections from the auditory area NC into the same pathway implicated in behavioral motivation and reward (AIV), as well as projections to other pathways implicated in control of behavioral output (Ad). These results collectively reveal a circuit through which subjective evaluation of sensory signals may be linked to selective initiation of behavioral action. Such a mechanism could enable an organism to take advantage of information detected in sensory experiences. Our preliminary results in other experiments also reveal that manipulating activity in sites within this pathway induces changes in a female BF’s evaluation of the attractiveness of a song stimulus. Together

with continuing emergence of a comprehensive circuit-level understanding of the system that underlies song evaluation and mate choice, the development of optogenetic tools opens the door to detailed studies of the causal role that each of these pathways plays in those decisions. The experimental tractability of this system makes it very attractive as a context for studies seeking to understand the neural basis of sensory perception and initiation of consequent behavioral responses in service of decision making.

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Data Availability Statement:

Data will be made openly available in a public repository that issues datasets with DOIs

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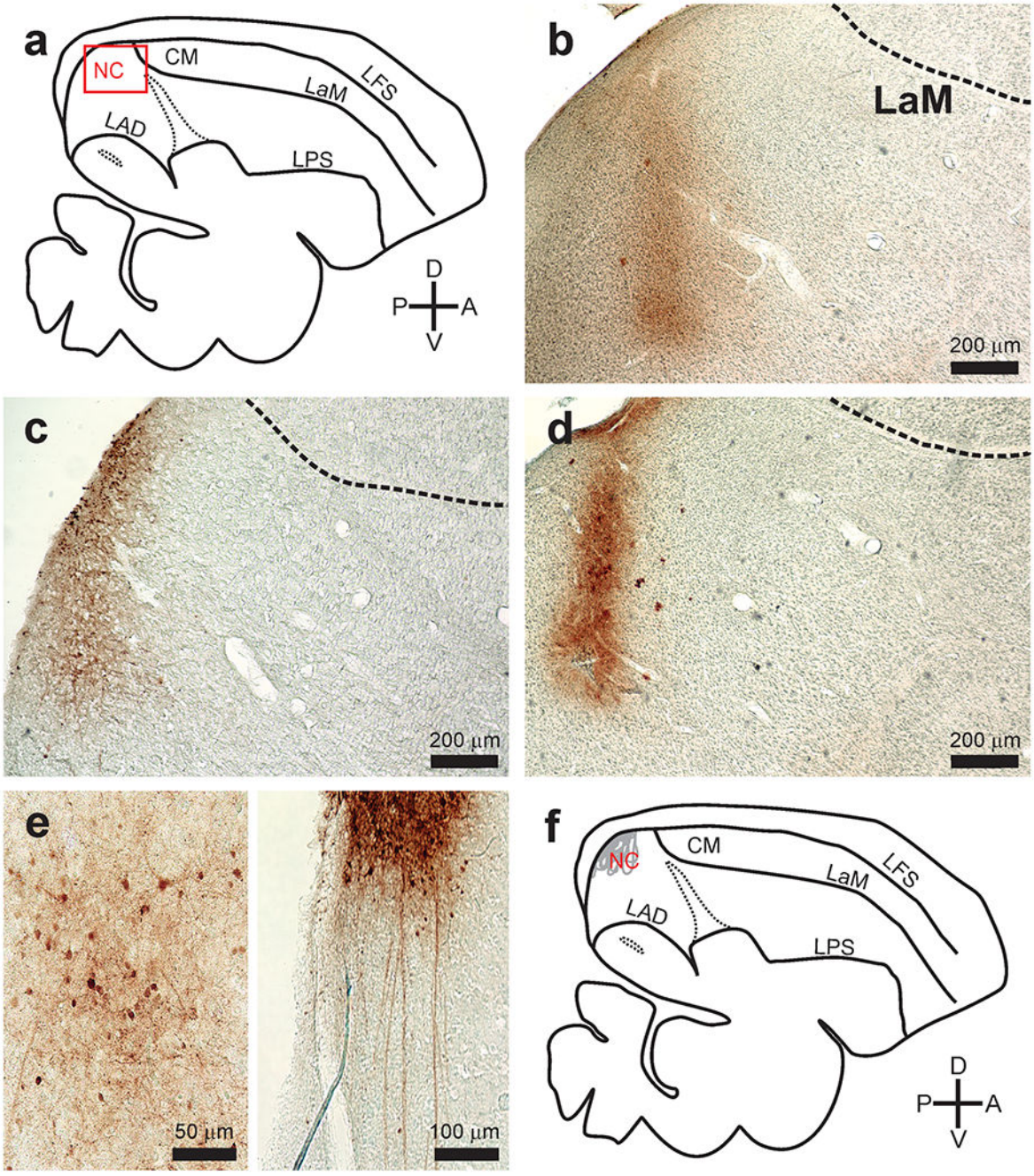

Figure 1. BDA injections remained within NC.

(a) schematic of a parasagittal section of the female Bengalese finch brain. The red box indicates the approximate location of the photographs in the panels below. (b-d) BDA injections were restricted to NC with BDA-labeled cells located posterior to LaM and superior to LaD. NC injection sites did not include other identifiable regions. (e) NC injection sites were identified by individually labeled cell somas in NC. (f) Schematic of all NC injection sites that vary in exact location but remain within the bounds of NC (injection sites are indicated by gray lines). LaM = lamina mesopallialis; LaD = lamina arcopallius

dorsalis; LFS = lamina frontalis superior; LPS = lamina pallio-subpalliallis. Dashed lines signify lamina location.

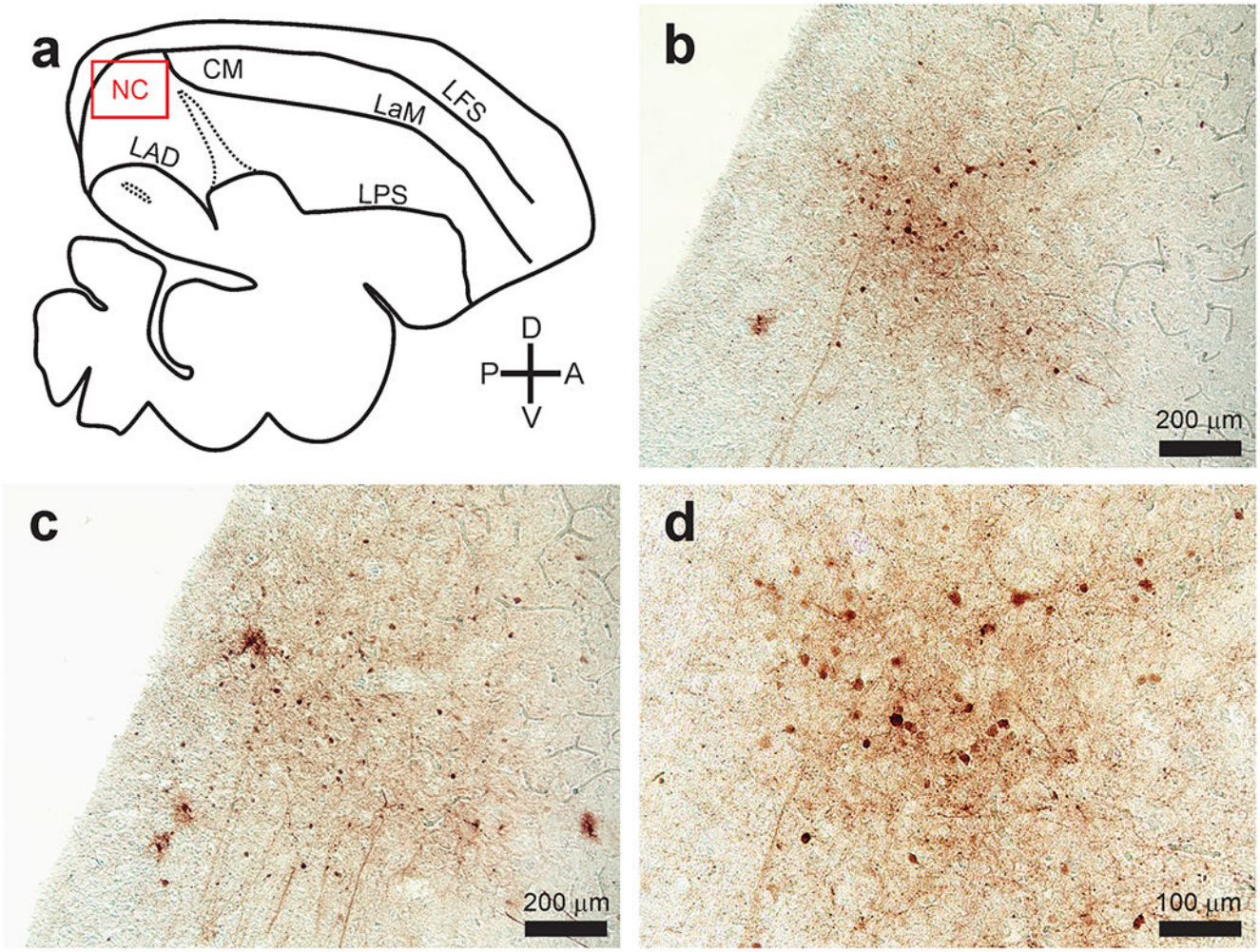

Figure 2. NC projects locally within NC.

(a) Schematic of a parasagittal section of the female Bengalese finch brain. The red box indicates the approximate location of the photographs in the panels below. (b-d) BDA injections into NC resulted in dense projections to other areas within NC, evident as axons and varicosities. These local projections reveal extensive local connectivity of NC with other neurons in NC.

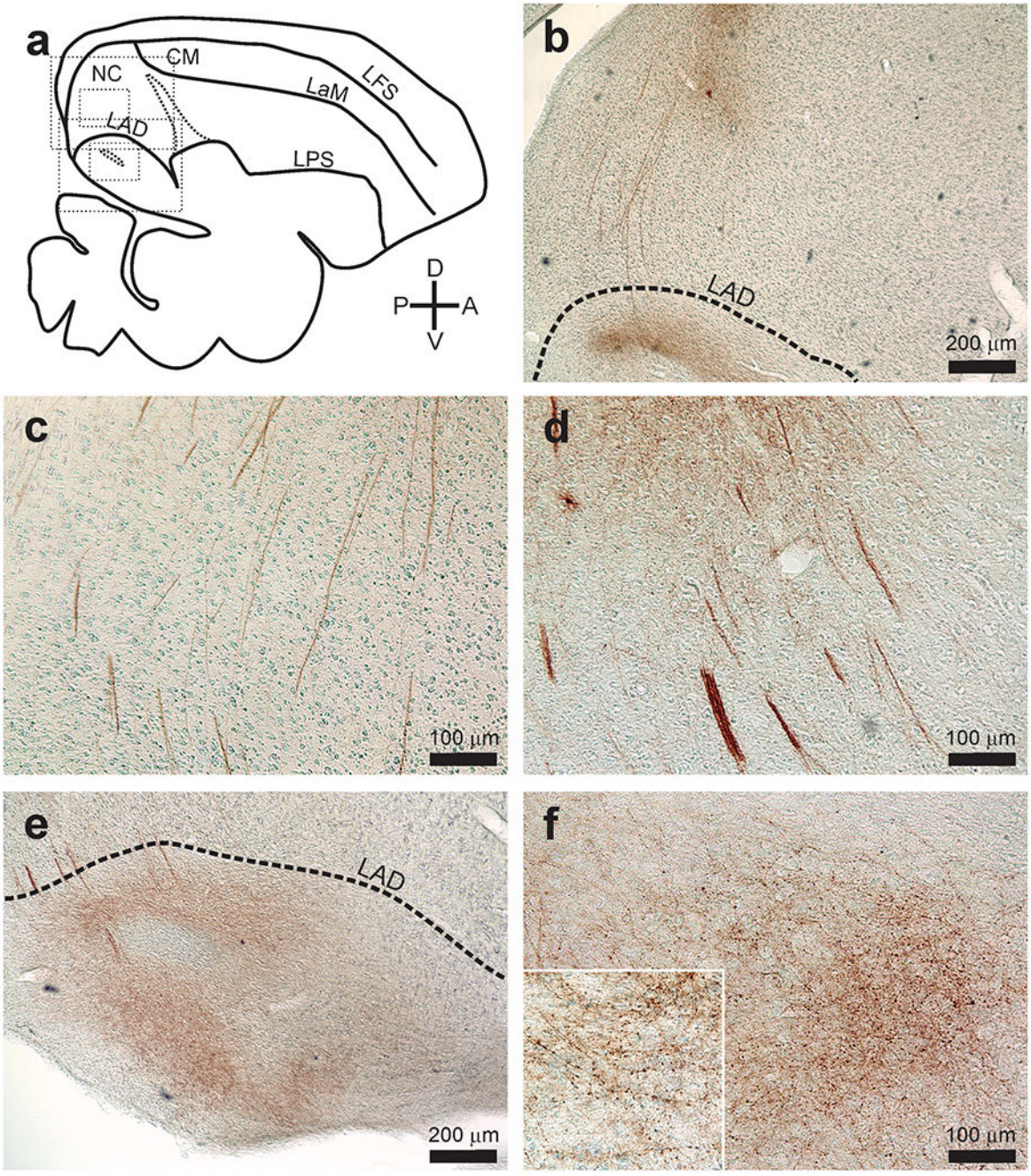

Figure 3. NC projects to the arcopallium.

(a) Schematic of a parasagittal section of the female Bengalese finch brain. The orientation of tissue in this schematic is the same as in the panels b-e below. Dotted boxes indicate locations where tissue in those panels was imaged. Top large box = panel b. Top small box = panels c and d. Bottom large box = panel e. Bottom small box = panel f. (b-e) NC projections to more distant targets are evident as labeled bundles of fibers. Those fibers course inferiorly from the injection site and cross the LaD into the arcopallium. (e, f) Labeled NC projections terminated as dense fibers and varicosities within AIV. Dashed lines

indicate lamina location. Inset in Figure 3f shows labeling of terminal fields at 2x higher magnification than in the surrounding panel.

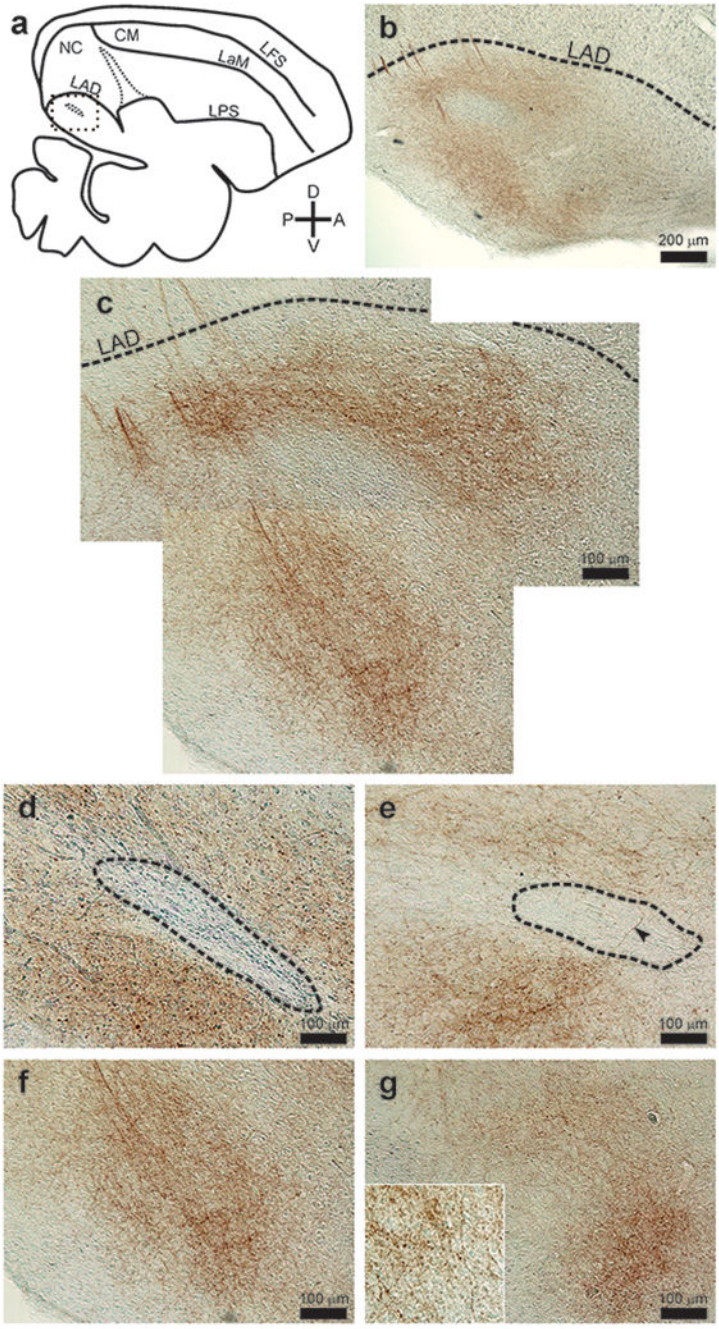

Figure 4. NC projects to AIV and respects the boundary of RA.

(a) Schematic of a parasagittal section of the female Bengalese finch brain. The orientation of tissue in this schematic is the same as in the panels below. The dotted box indicates the approximate location of the photographs in each of the panels b-g below. (b, c) NC projections cross the LaD into the arcopallium, terminating as dense fibers and varicosities within Ad (superior to RA) and AIV (inferior to RA). (d) Projections from NC to AIV and Ad surround but do not invade RA. The unlabeled tissues in panels d and e could also have included other arcopallial tissue immediately surrounding RA. (e) In one hemisphere, the

boundary of RA was invaded by a labeled fiber (indicated by arrow). This is the only case in which we observed invasion of RA by fibers from NC. This fiber lacks varicosities, revealing that even in this case NC did not innervate RA. (f) In contrast, axons and varicosities were prevalent within AIV, indicating the presence of functional connections from NC to AIV. (g) Labeled fibers from NC also projected to the anterior arcopallium (anterior/inferior to AIV). The dashed lines in panels b and c indicate lamina locations; the dashed outlines in panels d and f represent the boundary of RA. Each of panels f and g are taken from the region indicated by the dotted box in panel a. The inset in Figure 4g shows labeling of terminal fields at 2x higher magnification than in the surrounding panel.

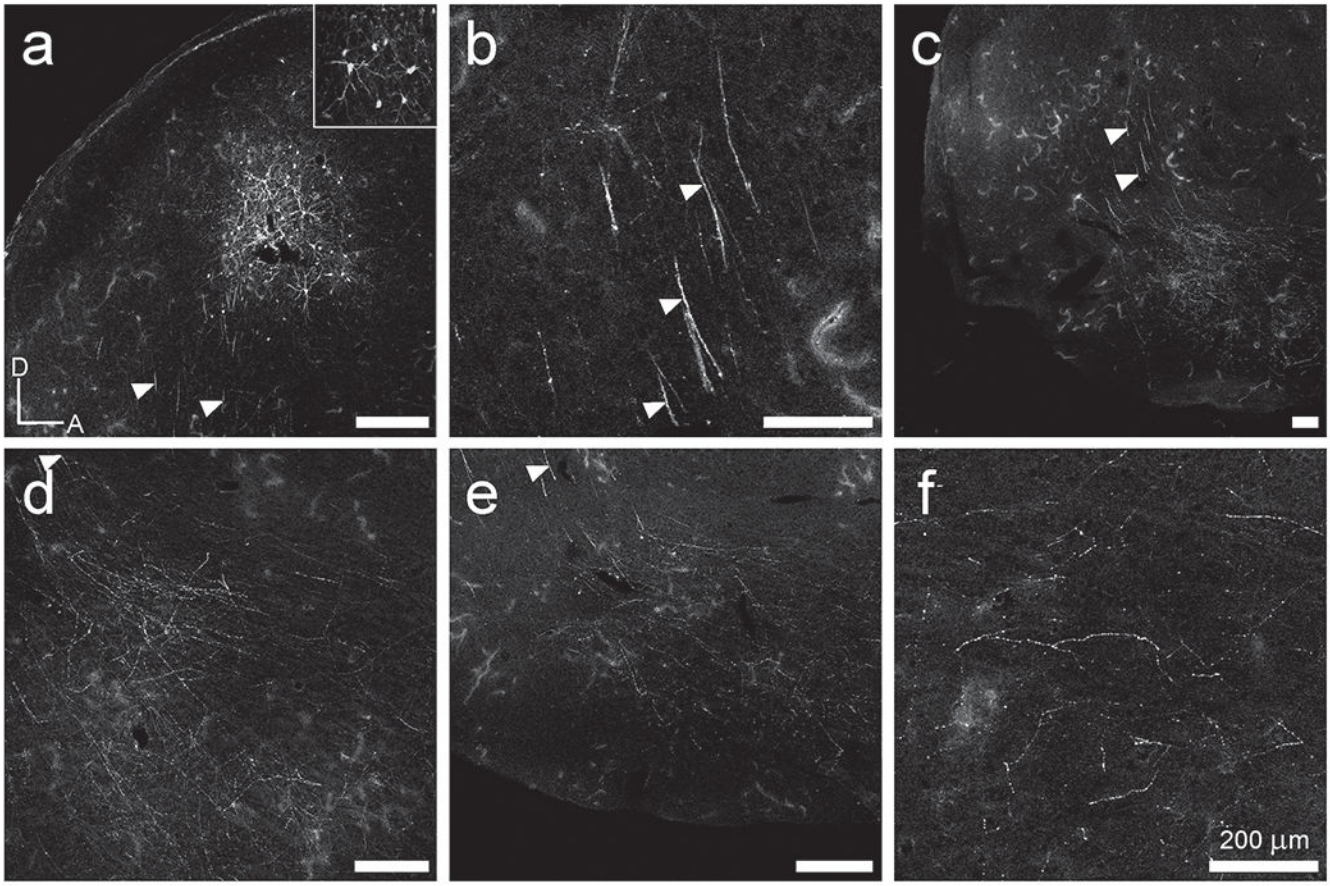

Figure 5. Projections detected using BDA were confirmed using adeno-associated viral infection of NC neurons.

(a) Injections of AAV into NC resulted in GFP-labeled somas within NC. The orientation of tissue in this schematic is the same in all panels. (a-c) Labeled bundles (indicated by arrows) leave NC and course inferiorly toward the arcopallium. (d) Projections from NC crossed the LaD. Labeled axons and varicosities revealed synaptic connections from NC neurons on cells in the Ad (e) and AIV (f) regions of the arcopallium. All scale bars are 200 µm as shown in panel f. Inset in Figure 5a shows labeling of somas in NC at 2x higher magnification than in the surrounding panel.

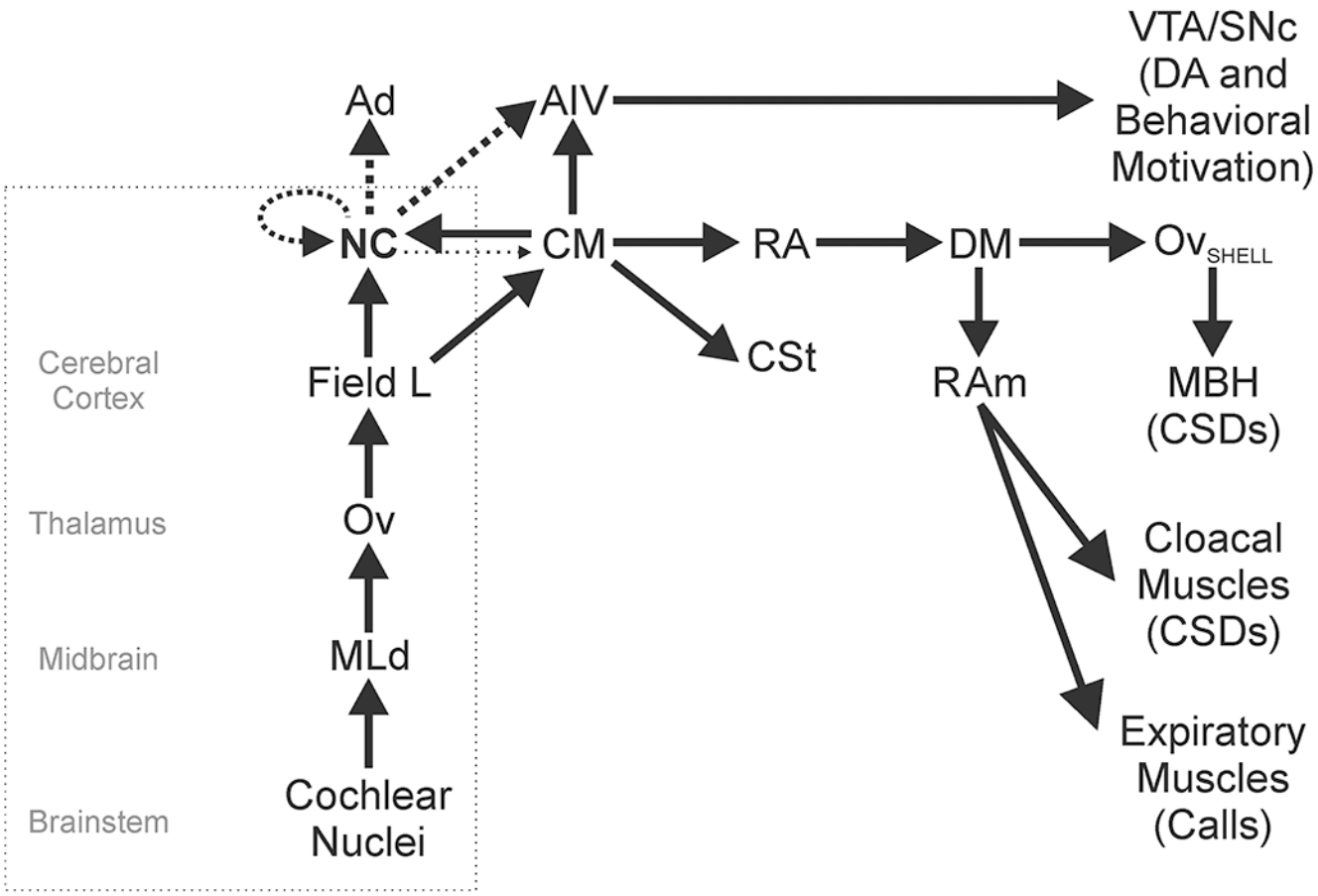

Figure 6. Schematic of the circuit in the female songbird brain through which auditory activity in NC may influence moor performance of courtship behaviors.

Auditory stimuli are processed by the ascending auditory pathway, beginning with cochlear nuclei in the brainstem, then projecting to the dorsal lateral nucleus of the mesencephalon (MLd), which then projects to the thalamic site, Ovoidalis (Ov). Field L, the primary thalamorecipient of projections from Ov, innervates both NC and CM. The connections reported in this study (dotted line arrows emerging from NC) reveal that the female NC projects to NC (n = 24), AIV (n = 20), and Ad (n = 16). These circuits and the pathways into which they project provide a mechanism through which subjective evaluation of sensory signals is linked to selective initiation of behavioral action.

Table 1

Projections from NC to Downstream Targets

			Injection Site	Projections from NC		
	Birds	**Hemispheres**	**Localized to NC**	**to NC**	**To AIV**	**to Ad**
Number	16	24	24	24	20	16
Percent			*100%*	*100%*	*83%*	*67%*