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The pallial basal ganglia pathway modulates the behaviorally driven gene expression of the motor pathway

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Abstract

The discrete neural network for songbird vocal communication provides an effective system to study neural mechanisms of learned motor behaviors in vertebrates. This system consists of two pathways – a vocal motor pathway used to produce learned vocalizations and a vocal pallial basal ganglia loop used to learn and modify the vocalizations. However, it is not clear how the loop exerts control over the motor pathway. To study the mechanism, we used expression of the neural activity-induced gene ZENK (or egr-1), which shows singing-regulated expression in a social context-dependent manner: high levels in both pathways when singing undirected and low levels in the lateral part of the loop and in the robust nucleus of the arcopallium (RA) of the motor pathway when singing directed to another animal. Here, we show that there are two parallel interactive parts within the pallial basal ganglia loop, lateral and medial, which modulate singing-driven ZENK expression of the motor pathway nuclei RA and HVC, respectively. Within the loop, the striatal and pallial nuclei appear to have opposing roles; the striatal vocal nucleus lateral AreaX is required for high ZENK expression in its downstream nuclei, particularly during undirected singing, while the pallial vocal lateral magnocellular nucleus of the anterior nidopallium is required for lower expression, particularly during directed singing. These results suggest a dynamic molecular interaction between the basal ganglia pathway and the motor pathway during production of a learned motor behavior.

Introduction

Learned motor behavior is essential for vertebrates to adapt to changing environments and social interactions. To decipher molecular mechanisms associated with production of learned motor behavior, we take advantage of the songbird's discrete vocal communication system. This system consists of two interconnected pathways: the vocal motor pathway (also called the posterior vocal pathway; Fig. 1, blue) responsible for production of learned vocalizations; and the vocal pallial basal ganglia loop (also called the anterior vocal pathway; Fig. 1, red) responsible for sensorimotor vocal learning (Nottebohm *et al*., 1976; Bottjer *et al*., 1984; Sohrabji *et al*., 1990; Scharff & Nottebohm, 1991). The vocal motor pathway, similar to cortical–brainstem motor pathways of mammals (Jarvis, 2004b), sequentially connects vocal nuclei of the pallium [nucleus interface of the nidopallium (NIf) to nucleus HVC to robust nucleus of the arcopallium (RA)] to hindbrain motor neurons [tracheosyringeal part of XII motor nucleus (nXIIts) and nucleus retroambiguus (RAm)] that innervate vocal and respiratory musculature (Fig. 1). The vocal pallial basal ganglia loop, similar to premotor cortical–basal–ganglia–thalamic–cortical loops of mammals (Jarvis, 2004b; Perkel, 2004), connects in its lateral part a nucleus of the anterior pallium [lateral magnocellular nucleus of the anterior nidopallium (LMAN)] to a nucleus in the striatum of the basal ganglia [lateral AreaX (LAreaX); contains both striatal and pallidal neurons; Farries & Perkel, 2002] to a nucleus in the dorsal thalamus [dorsal lateral nucleus of the dorsomedial thalamus (DLM)] back to the pallium (LMAN); a similar loop has been proposed in the medial part (Jarvis *et al*., 1998). The vocal motor pathway sends input to the vocal pallial basal ganglia pathway via a projection from HVC to AreaX; the vocal pallial basal ganglia pathway sends output to the vocal motor pathway via LMAN to RA and medial MAN (MMAN) to HVC (Fig. 1).

LMAN and LAreaX have different functions during the sensorimotor phase of vocal learning in juveniles. LMAN is required for variability in learned song, whereas LAreaX is required for stereotypy (Scharff & Nottebohm, 1991). A balance between the two has been proposed to enable vocal motor learning (Jarvis, 2004b). After vocal learning is complete, lesions to these nuclei have been originally noted to have little or no effect on adult song (Bottjer *et al*., 1984; Scharff & Nottebohm, 1991; Nordeen & Nordeen, 1993). However, subsequent studies have shown that these nuclei are still highly active during the production of adult learned vocalizations and differentially across social contexts (Jarvis & Nottebohm, 1997; Jarvis *et al*., 1998; Hessler & Doupe, 1999a,b). Undirected singing, i.e. singing alone or while not facing another bird, induces high mRNA expression levels of the immediate-early gene (IEG) ZENK throughout MAN and AreaX, as well as in RA and HVC (Jarvis *et al*., 1998). Directed singing, i.e. singing while facing another bird, induces high levels of ZENK mRNA in medial parts of MAN and AreaX (MMAN, MAreaX) and in HVC, but low levels in

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lateral parts of MAN and AreaX (LMAN, LAreaX) and in RA. ZENK, an acronym for zif-268, egr-1, NGFI-A and Krox-24 (Mello *et al.*, 1992), is a transcription factor necessary for hippocampal memory reconsolidation (Lee *et al.*, 2004), and it requires increased electrophysiological activity for its expression (Fields *et al.*, 2001; Dudek & Fields, 2002). Similar to the ZENK expression profiles, the firing rates of LMAN and LAreaX neurons are low during directed singing, and high and more variable during undirected singing (Hessler & Doupe, 1999b). Consistent with this gene expression and neural activity relationship, recent results in adults have revealed that ZENK mRNA levels in LMAN (and in AreaX) positively correlate with variability in song rate (Liu & Nottebohm, 2005), and that LMAN induces or enables small but significant variability in RA activity and song syllables (Kao *et al.*, 2005; Olveczky *et al.*, 2005; Kao & Brainard, 2006).

The above findings and the known connectivity (Fig. 1) led us to hypothesize that after learning is complete the vocal pallial basal ganglia pathway modulates activation of the motor pathway, and differentially in different social contexts, where the lateral part would modulate RA and the medial part would modulate HVC (Jarvis *et al.*, 1998). This would suggest, based upon findings of activity-dependent gene induction in mammals (Lerea, 1997; Berretta *et al.*, 1999; Keefe & Gerfen, 1999), that singing-driven gene expression in a given vocal nucleus requires the input of its afferent vocal nuclei (Jarvis, 2004a). Here we tested this hypothesis. We performed lesions to lateral and medial components of the vocal pallial basal ganglia pathway in adult zebra finches and assessed the effects on singing-regulated ZENK protein expression in the vocal motor pathway nuclei. We found that the motor pathway nuclei have a topographic medial–lateral dependence on the vocal pallial basal ganglia pathway; the expression in RA depends on lateral nuclei while the expression in HVC depends on the medial nuclei. Further, we found that the striatal and pallial nuclei of the vocal pallial basal ganglia pathway can have opposing roles for gene activation in the motor pathway, where LMAN can dampen it and LAreaX (via LMAN) increases it.

Materials and methods

Animals

We used 85 adult (more than 90 days old) male zebra finches (*Taeniopygia guttata*) from our breeding colony. In zebra finches only males learn to sing. Females were used as stimuli for directed singing. Animal treatments and procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Anatomy

Because the boundary between MAreaX and LAreaX had not been determined in the prior study (Jarvis *et al.*, 1998), as their sections were processed in the sagittal plane, we defined it here using directed singing-driven gene expression patterns in frontal sections that spanned medial and lateral AreaX. We found a MAreaX–LAreaX boundary that is diagonally positioned and starts at an angle where AreaX dips ventrally to become larger (Fig. 3A, higher power image in Supplementary material, Fig. S1). Using this functionally defined boundary, we calculated that LAreaX is 19.69 ± 0.48 times larger than MAreaX ($n = 18$ animals singing directed song). Because this boundary is not visible after undirected singing, when we quantified ZENK expression in MAreaX, we did so in an area within MAreaX but more medial to the expected (undirected) or observed (directed) functionally defined boundary.

Surgery

Unilateral ibotenic acid lesions were made to LAreaX, LMAN, LAreaX + LMAN, MAreaX, MMAN and MAreaX + MMAN. We used ibotenic acid, as opposed to electrolytic lesions, to minimize damage to fibers of passage through a vocal nucleus. Ibotenic acid lesions to cell bodies cause degeneration of their axons and terminals to their connected neurons within 48 h (Milner & Veznedaroglu, 1993; Halim & Swerdlow, 2000). Ibotenic acid (Sigma, USA) was dissolved in 1 M NaCl to a concentration of 1% (pH 7.5–8.0). Fresh aliquots were used for each experiment. To lesion LAreaX and LAreaX + LMAN, surgeries were performed with isoflurane anesthesia (1–3%; flow 1 L/min), and ibotenic acid was injected using a single-barrel glass micropipette attached to a Nanoject II injector (Drummond Scientific, USA). The bird was fixed in the stereotaxic apparatus and the location of a vocal nucleus found according to the following coordinates. For LAreaX, two injections of 55.2 nL each were made within 3.9–4.4 mm rostrally, at 1.3 mm laterally and 3.5 mm ventrally, from bregma. For LAreaX + LMAN together, two injections of 55.2 nL were made at the same coordinates and one additional injection of 55.2 nL at 2.2–2.4 mm ventrally. The coordinates were adjusted individually for each bird according to the size and shape of the skull. A different approach was taken to lesion LMAN, MAreaX

TABLE 1. Vocal nuclei lesion sizes (in percentages) in individual birds

LAreaX		LMAN		LAreaX + LMAN		MAreaX	MMAN	MAreaX + MMAN
UD	Dir	UD	Dir	UD	Dir	UD + Dir	UD + Dir	UD + Dir
100	100	100	100	$67^{X} + 100^{M}$	$70^{X} + 100^{M}$	100	100	$100^{X} + 100^{M}$
90	94	100	100	$48^{X} + 91^{M}$	$87^{X} + 60^{M}$	96	100	$100^{X} + 100^{M}$
90	91	100	100	$35^{X} + 26^{M}$	$33^{X} + 47^{M}$	74	100	$84^{X} + 100^{M}$
46	56	99	73	$93^{X} + 8^{M}$		37	100	$83^{X} + 100^{M}$
18	46	87	63			18	88	$81^{X} + 100^{M}$
18		66	45			11	72	$65^{X} + 100^{M}$
11		33					68	$61^{X} + 100^{M}$
							67	$56^{X} + 100^{M}$
							57	$48^{X} + 100^{M}$
							26	$40^{X} + 100^{M}$
								$41^{X} + 90^{M}$
								$41^{X} + 61^{M}$
								$39^{X} + 39^{M}$

Dir, directed singing; UD, undirected singing; ^{X}lesion to lateral or medial AreaX; ^{M}lesion to lateral or medial MAN;

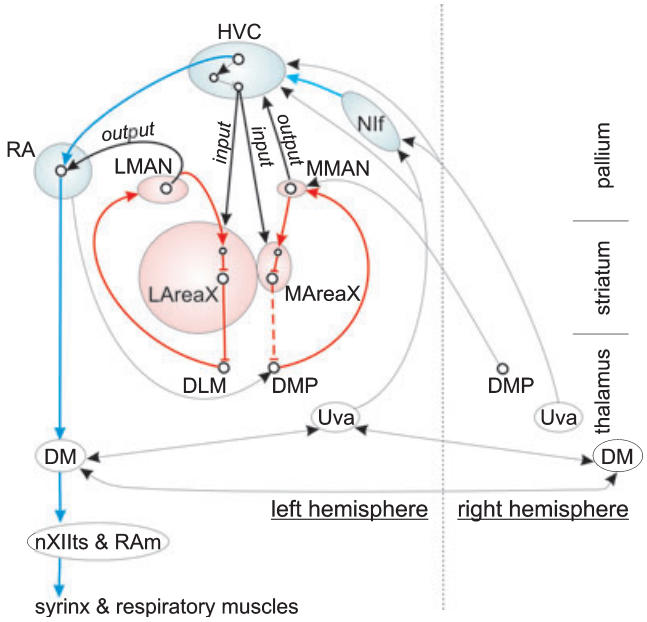

FIG. 1. Connectivity of the vocal motor pathway (blue) and the vocal pallial basal ganglia pathway (red) in songbirds. Thick black lines: inputs and outputs between the two pathways. Thin black lines: thalamic and midbrain (DM) connections between hemispheres. Arrowheads: excitatory connections. Flat-heads: inhibitory connections (Perkel *et al*., 2002). Connections compiled from Nottebohm *et al*. (1976), Nottebohm *et al*. (1982), Bottjer *et al*. (1989), Wild (1993), Johnson *et al*. (1995), Vates & Nottebohm (1995), Foster *et al*. (1997), Vates *et al*. (1997) and Striedter & Vu (1998). The dashed red line from medial AreaX (MAreaX) to the dorsomedial nucleus of the posterior thalamus (DMP) is a predicted connection (Jarvis *et al*., 1998). Abbreviations follow the new avian brain nomenclature (Reiner *et al*., 2004b).

and MMAN alone, because of their smaller size. Surgeries were performed with ketamine–xylazine anesthesia (15 mg/mL ketamine: 3 mg/mL xylazine; 40 μL/12 mg of bird weight), which enables recording of the spontaneous activity in LMAN. Spontaneous activity of vocal nuclei is different from the surrounding brain areas, allowing us to locate them. To record this activity, one barrel of a double-barrel

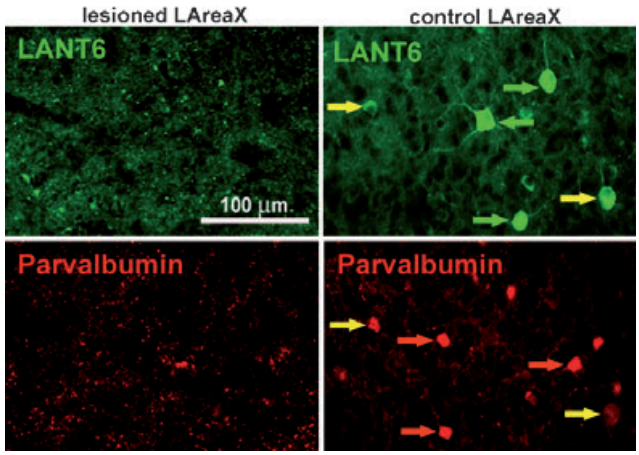

FIG. 2. Immunostaining of lateral AreaX (LAreaX) neurons. Lesioned and contralateral control LAreaX are double-labeled to distinguish DLM-projecting neurons. The projection neurons are LANT6$^+$/parvalbumin$^-$ and are shown by green arrows. Yellow arrows point to LANT6$^+$/parvalbumin$^+$ interneurons. Red arrows point to parvalbumin$^+$ interneurons. None of these neurons was found in LAreaX after ibotenic acid lesion. Neuron types are defined as those identified in Reiner *et al*. (2004a).

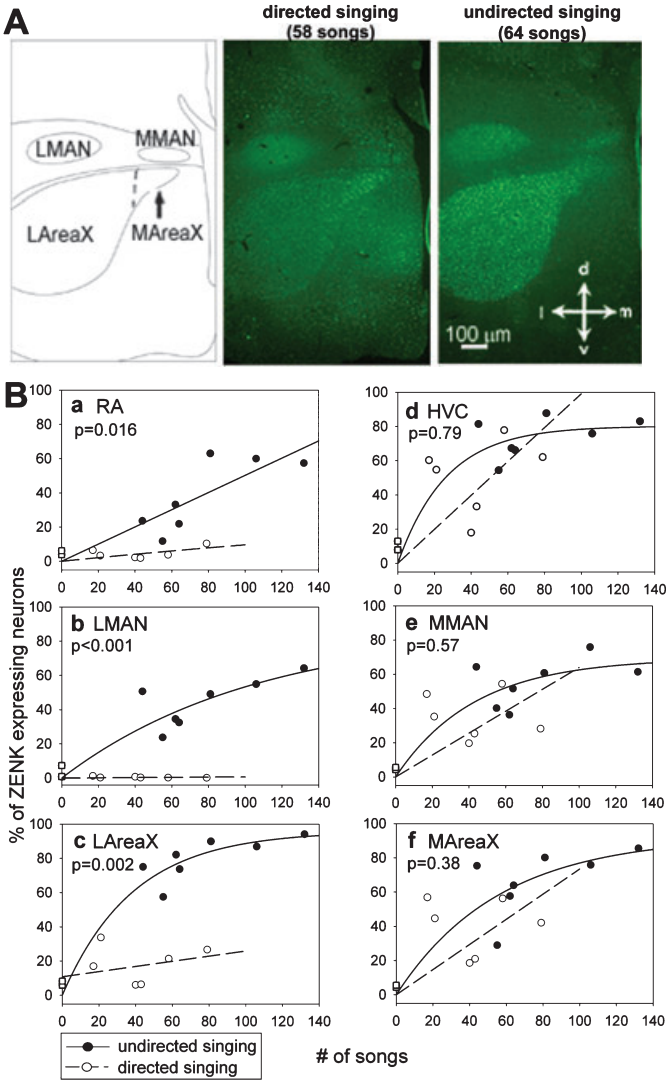

FIG. 3. Singing-driven protein expression. (A) Representative frontal brain sections of ZENK protein expression in birds after female directed or undirected singing. Note the higher expression levels in lateral AreaX (LAreaX) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN; green labeled nuclei) of the undirected singer, but low levels in these nuclei of the directed singer (LMAN has higher neuropil auto-fluorescence relative to the surrounding brain tissue). The dashed line shows the functional boundary between medial AreaX (MAreaX) and LAreaX. (B) The relationship between singing amount and percentage of neurons expressing ZENK protein in vocal nuclei after undirected and directed singing. Squares on the *y*-axis are silent controls. The data were best fitted by an 'exponential rise to maximum, two-parameters' curve, for all graphs (which resulted in curved as well as relatively straight lines); this function gave a better fit than a linear equation. The individual *r*- and *P*-values for each vocal nucleus for undirected singing range from 0.87 to 0.97 and 0.0001 to 0.002, respectively; and for directed singing range from 0.0 to 0.37 and 0.52 to 1.0, respectively. The *P*-values shown in the graphs are for comparisons of each vocal nucleus between undirected and directed singing (multiple regressions). After about 80 undirected songs, the number of ZENK-labeled neurons saturates in LAreaX at ~90%, in MAreaX ~80%, in LMAN ~60%, in the medial magnocellular nucleus of the anterior nidopallium (MMAN) ~60%, in the robust nucleus of the arcopallium (RA) ~60%, in HVC ~80%.

glass micropipette was filled with 1 M NaCl, attached to a silver wire, and used as an electrode. The other barrel was attached to the Nanoject II injector and filled with 1% ibotenic acid. Once found (coordinates: 4.0–4.8 mm rostrally, 1.6–1.8 mm laterally, 2.0–2.6 mm ventrally), LMAN was either lesioned (three injections of 32.2 nL) or the

micropipette was moved medially to MMAN and/or ventrally to MAreaX and these nuclei were lesioned (coordinates: 0.35 mm laterally, 2.0–2.3 mm ventrally for MMAN, 2.7–2.8 mm ventrally for MAreaX; one injection each of 32.2 nL). Left and right sides were alternated. After all surgeries, the bird was kept under a heat lamp overnight and moved the next day to a recording sound isolation box.

Lesion size was determined using Hu^+ immunostaining (a neuronal marker; Barami *et al*., 1995) on brain sections, which unequivocally allowed identification of the lesioned area as opposed to Nissl staining, which is not as unequivocal. In addition, vocal nuclei neuronal morphology and density are different from the surrounding areas allowing identification of their boundaries using Hu staining. Non-lesioned parts of vocal nuclei, when present, were also identified by singing-induced ZENK expression. To determine lesion size, the area of the remaining non-lesioned part of a nucleus (0 when completely lesioned) and the area of the intact nucleus in the control hemisphere was calculated in every fourth section throughout a vocal nucleus using the lasso tool, then histogram, pixel number function in Adobe Photoshop software. The measurements for each hemisphere were summed, and the percentage of the non-lesioned part was calculated and subtracted from 100 to generate lesion size as a percentage. The comparison to the control hemisphere allowed us to control for variations in vocal nuclei sizes between animals. The lesion sizes for each bird and lesion type are shown in Table 1. Because medial vs. lateral divisions of AreaX have not been well studied, specificity of the MAreaX lesions is provided in Supplementary Fig. S2, A. The lesions damaged the whole MAreaX or its medial, dorsal or ventral portion. None of the six birds had LAreaX damaged. Two other birds with lesions that crossed the MAreaX/LAreaX boundary (not shown) were excluded from the analyses for clarity of interpretation.

We used Hu labeling and LANT6 and parvalbumin double-labeling to assess the percentage of neurons and type of neurons lesioned within the lesioned area. $LANT6^+$/$parvalbumin^-$ AreaX neurons project to DLM (Reiner *et al*., 2004a). We found that within the lesioned area, ibotenic acid eliminated more than 96 ± 2.2% and 86 ± 1.6% of the Hu^+ neurons in LAreaX and LMAN, respectively (n = 6 animals each). The small percentages of neurons remaining in the lesioned AreaX were not projection neurons ($LANT6^+$/$parvalbumin^-$, Fig. 2), and thus the lesions removed all output from AreaX. The small percentage of neurons remaining in the lesioned area of LMAN was of neurons small in size (4 ± 0.8 μm diameter vs. 9.2 ± 1.4 μm diameter of large neurons in control LMAN; based on 50 neurons from four birds), and thus are also unlikely projection neurons. Alternatively, the lesions could have reduced the size of residual projection neurons.

Tracer injections

In eight birds we bilaterally targeted MAreaX (n = 16 injections total) with dextran amine Alexa Fluor^R 488 (Molecular Probes, USA),

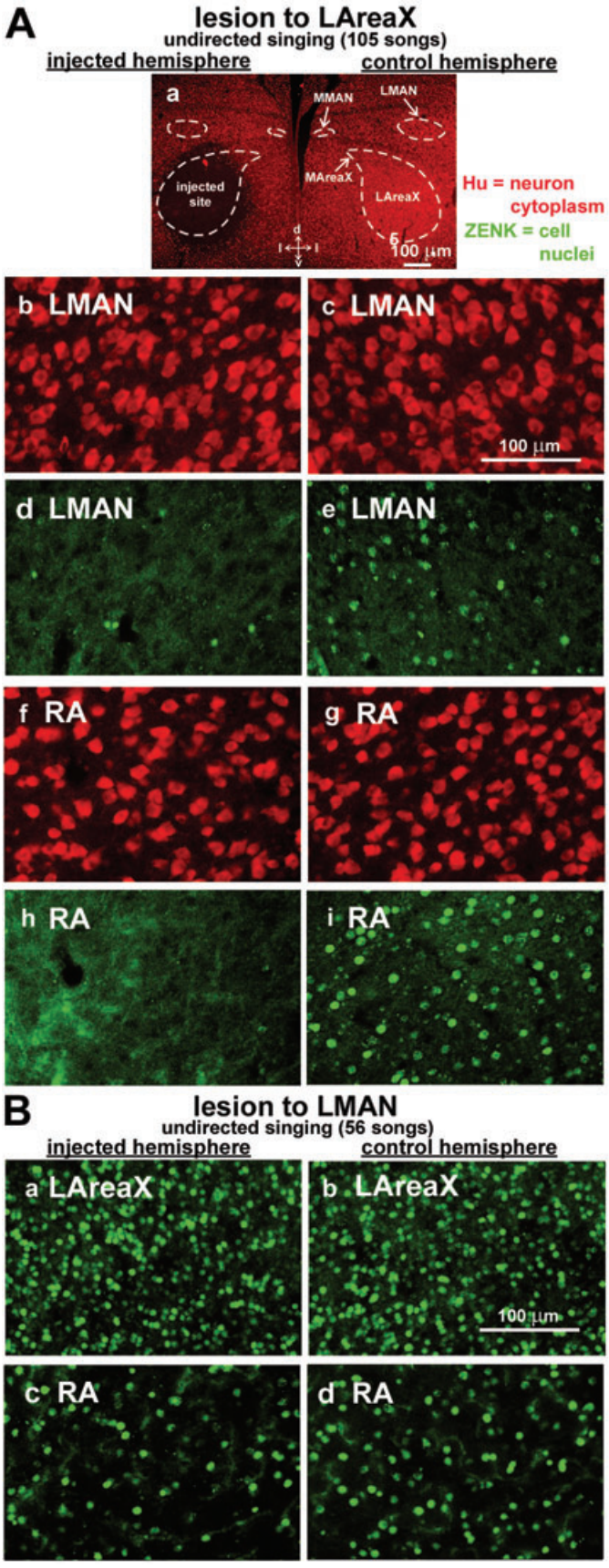

FIG. 4. Singing-driven ZENK protein levels in vocal nuclei after unilateral lesions to lateral portions of the vocal basal ganglia pathway nuclei. (A) Example of unilateral lesion to lateral AreaX (LAreaX; 100%; a) and its effect on singing-driven expression in the ipsilateral (injected hemisphere) and contralateral (control hemisphere) lateral magnocellular nucleus of the anterior nidopallium (LMAN) and robust nucleus of the arcopallium (RA) (b–i). Sections are frontal and double-labeled for Hu (red: b, c, f, g) and ZENK (green: d, e, h, i) proteins. (B) Example of unilateral lesion to LMAN (100%; not shown) and its effect on ZENK expression in ipsilateral and contralateral LAreaX and RA (a–d). MAreaX, medial AreaX; MMAN, medial magnocellular nucleus of the anterior nidopallium.

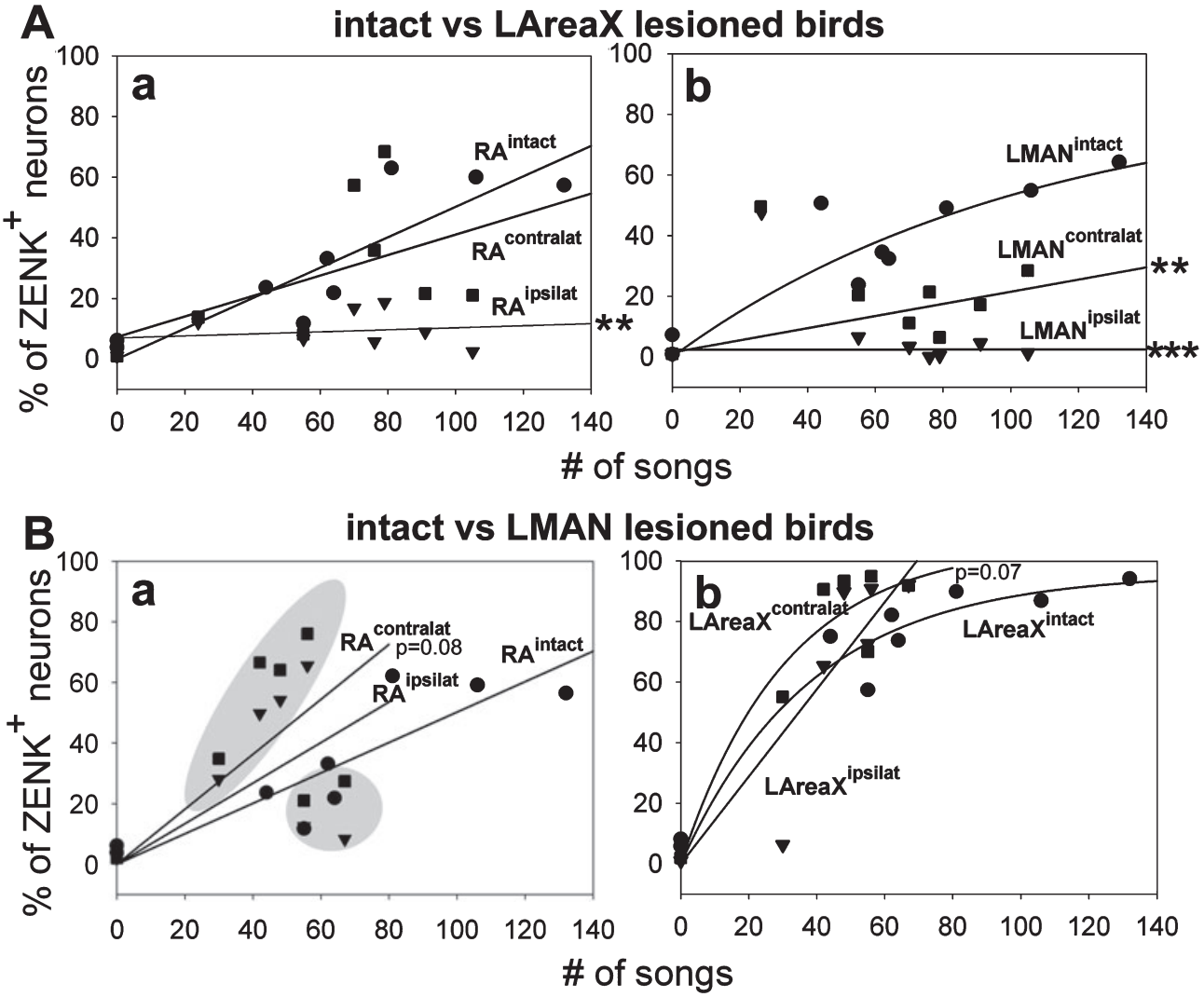

FIG. 5. Regression analyses of the relationship of undirected singing-driven ZENK expression and singing amount in intact relative to (A) lateral AreaX (LAreaX)- or (B) lateral magnocellular nucleus of the anterior nidopallium (LMAN)-lesioned birds. The data were best fitted by 'exponential rise to maximum, two-parameters' curve. For intact birds, the average of both hemispheres (which are not different: $P = 0.83$, ANOVA) is shown. Triangles represent the ipsilateral hemisphere of lesioned birds, squares represent the contralateral hemisphere of lesioned birds, and circles represent intact animals. The LAreaX-lesioned outlier mentioned in the main text with unusually high bilateral LMAN expression is the bird that sang 24 songs (Ab). One bird with an LMAN lesion is omitted as he sang 195 songs, which is well above the amounts sang by intact animals and thus is difficult to compare with intacts; however, like the majority of LMAN-lesioned birds, this bird showed nearly complete saturation of high ZENK expression in LAreaX (99% of neurons in ipsilateral and 97% in contralateral LAreaX) and still high expression in the robust nucleus of the arcopallium (RA; 51% ipsilateral and 59% contralateral). (A) $^{**}P < 0.01$, $^{***}P < 0.001$ lesioned relative to intact animals (multiple regression). The shadowed areas (in Ba) highlight the two groups of LMAN-lesioned birds with either high or intact-like expression in RA.

which is a combined anterograde and retrograde tracer. We used the same coordinates as listed above for lesions and made one 32.2-nL injection of a 5% tracer solution in sterile phosphate-buffered saline (PBS). Five days after surgery, singing behavior was obtained, and the injection location relative to vocal nuclei was identified by singing-driven ZENK protein expression. We found that our exact injection locations varied as follows: predominantly in MAreaX and surrounding striatum ($n = 8$), MAreaX with MMAN along the injection track ($n = 4$), the medial striatum ($n = 2$) and the medial nidopallium caudal to MMAN ($n = 2$).

Singing behavior

Singing behavior was recorded before surgery and the first morning the bird started to sing after surgery. All birds were able to sing after unilateral lesions. The birds were killed on the second−11th day after surgery, but usually on the third–fifth day, after an overnight period of silence in a sound isolation box to prevent examining ZENK induction as a result of unobserved behaviors. The male was either alone in the cage (for undirected singing) or with a female that was placed, while in the dark overnight, in the other half of the cage separated by a cage wall barrier (for directed singing). We found that the barrier encouraged more directed singing. Most birds would start singing within minutes after the lights came on. In the presence of the female, song was scored as directed when the bird was facing the female during singing or undirected when the bird did not face the female during singing. Sometimes an additional female was added midway into the singing session to induce more directed singing. If a bird did not sing 20 or more bouts within a 55-min period, the experiment was repeated on subsequent days until we obtained a sufficient amount of singing. Singing was recorded using Sound Analysis Life 229 (Tchernichovski *et al.*, 2004). The number of song bouts was counted

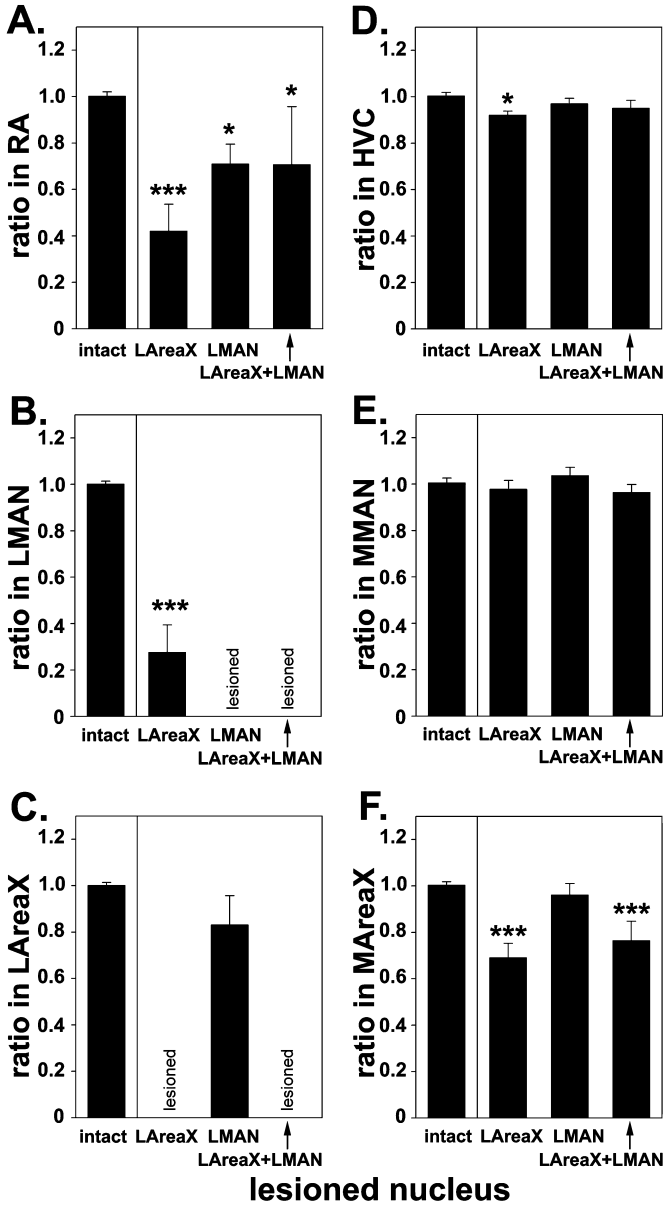

FIG. 6. Ratio analyses of effects of lesions within the lateral part of the vocal pallial basal ganglia pathway and intact birds (*x*-axis) on the expression levels in various vocal nuclei (*y*-axis) after undirected singing. (A–F) Each graph shows lesion effects on one nucleus. Ratios on the *y*-axes represent ZENK-expressing neurons in ipsilateral vs. contralateral hemispheres. $*P < 0.05$; $***P < 0.001$; ANOVA, Fisher's PLSD. LAreaX, lateral AreaX; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MAreaX, medial AreaX; MMAN, medial magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

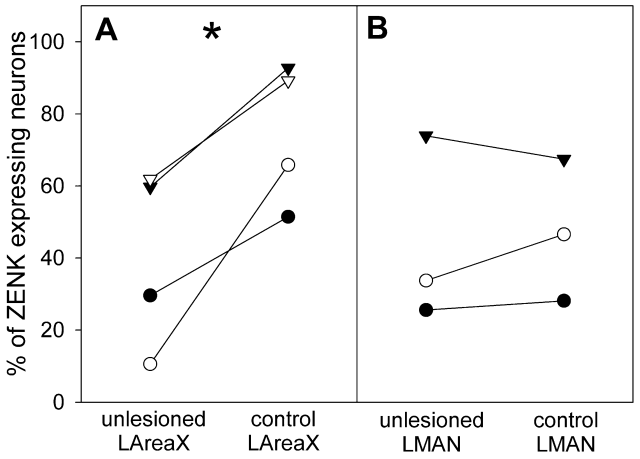

FIG. 7. Comparison of ZENK expression in the unlesioned part of (A) lateral AreaX (LAreaX; for LAreaX lesions) and (B) lateral magnocellular nucleus of the anterior nidopallium (LMAN; for LMAN lesions) with their counterpart in the control hemisphere. $*P < 0.05$, paired *t*-test.

and we refer to them as songs. A song was defined as vocalizations that started usually with several introductory notes, continued with at least one motif, and was surrounded by more than 2 s of silence. We counted song bouts instead of motifs or singing time because in control animals without surgery the number of song bouts showed the highest correlation with the number of neurons that expressed ZENK protein in vocal nuclei ($P < 0.0001$, $r = 0.96$ for number of song bouts; $P = 0.0004$, $r = 0.94$ for number of motifs; $P = 0.0009$, $r = 0.93$ for singing time; correlations for HVC, $n = 7$ birds), similarly as shown for mRNA (Jarvis *et al.*, 1998). After the 55-min singing session, the bird was injected with a lethal dose of equithesin and then within another 5 min the bird was perfused transcardially with PBS and then with 4% paraformaldehyde in PBS. The brain was dissected, postfixed for 5 h, and cryoprotected in 20% and then 30% sucrose in PBS at 4 °C. The frozen brain was stored at −80 °C until used for immunocytochemistry. Brains of intact singers were taken under the same behavioral paradigm, and those of silent intact controls were taken after an overnight period followed by at least 1 h of lights on with no singing.

Immunocytochemistry

The brains were cut on a cryostat in 30-μm coronal sections, and the sections were collected in PBS. Every fourth section was used for Hu and ZENK double-labeling. Up to eight animals from different groups were processed together for each immunocytochemistry experiment. Adjacent sections from several brains were used for LANT6 (pallidal neuron and striatal interneuron marker) and parvalbumin (striatal interneuron marker) double-labeling (Reiner *et al.*, 2004a). We used the mouse monoclonal anti-Hu (Molecular Probes), diluted 1 : 500, the rabbit polyclonal anti-egr-1 (ZENK; Santa Cruz Biotech, Santa Cruz, CA, USA), diluted 1 : 200, the rabbit polyclonal anti-LANT6 (provided generously by Dr R. Carraway, University of Massachusetts, Worchester, USA), diluted 1 : 1000, and the mouse monoclonal anti-parvalbumin (Sigma), diluted 1 : 1000. The specificity of these antibodies for birds has been described in previous studies (Reiner & Carraway, 1987; Barami *et al.*, 1995; Mello & Ribeiro, 1998; Reiner *et al.*, 2004a). The free-floating sections were washed 3 × in PBS, and non-specific binding was blocked in 0.1% bovine serum albumin with the addition of 0.3% Triton X-100 in PBS for 1 h. The sections were then incubated overnight (Hu and ZENK) or over 2 nights (LANT6 and parvalbumin) in the mixture of primary antiseras in blocking solution at 4 °C. After three washes in PBS, the sections were incubated for 2 h with Cy3-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, USA); their concentrations were 1 : 200 and 1 : 50, respectively. For the tracer-injected animals, we used Cy3-conjugated donkey anti-rabbit IgG against the egr-1 antibody. After three washes in PBS, the sections were mounted on silanated slides, washed in deionized water and coverslipped with DAPI to stain cell nuclei (Vectashield, USA).

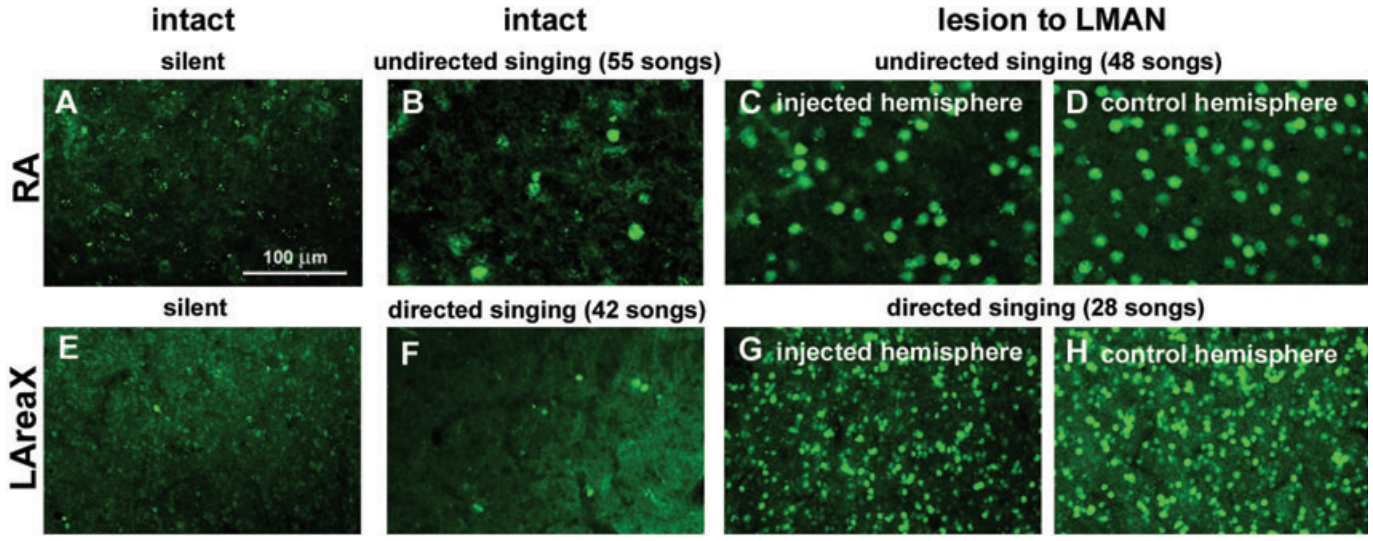

FIG. 8. Examples of ZENK expression increases in target vocal nuclei of lateral magnocellular nucleus of the anterior nidopallium (LMAN)-lesioned birds. (A) Robust nucleus of the arcopallium (RA) of a silent bird. (B) RA of a bird that sang undirected song. (C and D) RA in both hemispheres of an unilateral LMAN-lesioned bird that sang a similar amount of undirected song (C, LMAN-lesioned hemisphere; D, control hemisphere). (E) Lateral AreaX (LAreaX) of a silent bird. (F) LAreaX of a bird that sang directed song. (G and H) LAreaX in both hemispheres of an unilateral LMAN-lesioned bird that sang a similar amount of directed songs (G, LMAN-lesioned hemisphere; H, control hemisphere). The fluorescent images show ZENK (green) protein located in cell nuclei. The size of cells in LAreaX is smaller than those in RA.

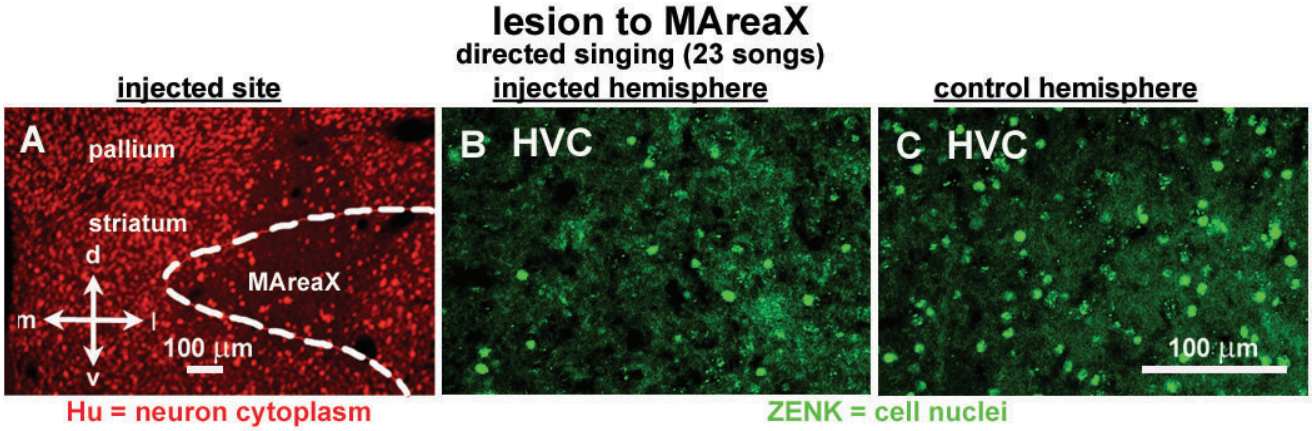

FIG. 9. Singing-driven ZENK protein levels in vocal nuclei after lesions to the medial part of the vocal basal ganglia pathway. (A) Example of unilateral lesion to medial AreaX (MAreaX; 96%). (B and C) The lesion effect on singing-driven expression in the ipsilateral (injected hemisphere) and contralateral (control hemisphere) HVC (a bird with one of the largest differences seen in ipsilateral vs. contralateral HVC). Sections are frontal and double-labeled for Hu (red) and ZENK (green).

Gene expression quantification

We measured ZENK protein levels because protein is the functional molecule and we could perform double-label immunohistochemistry to determine the portion of neurons that co-expressed ZENK. Images of immunolabeled vocal nuclei from injected and control hemispheres were taken with a Spot III CCD camera attached to a Leica DMRXA2 microscope under 2.5 × and 10 × objectives using Spot 3.2.4 software (Diagnostic Instruments). The images taken with the 10 × objective were used for counting. The total number of Hu-labeled and ZENK-labeled cells was manually counted in representative 0.06-mm^2 areas for LAreaX, LMAN and HVC, in 0.02-mm^2 areas for MAreaX and MMAN, and in the whole nucleus in sections with RA. To count double- and single-labeled neurons, we created a layer in Adobe Photoshop of the ZENK image, then marked the 0.02-mm^2 or 0.06-mm^2 area, and counted all the labeled nuclei using the brush tool to mark counted cells; we considered the cell as ZENK$^+$ if we could recognize the circle-shaped cell nucleus. Then we copied this layer and superimposed it to the Hu image; all ZENK$^+$ cells were Hu$^+$. Then we counted the rest of the Hu-labeled neurons in the area. The counts were taken from the center of the vocal nucleus in a given section. Because the cell densities near the rostral and caudal ends of a vocal nucleus can vary, the sections were taken from the middle of a vocal nucleus. For HVC, there has been reported a trend of less directed singing-driven mRNA levels in lateral part in comparison to the medial part (Jarvis *et al.*, 1998). We did not find such a trend in the protein levels in control animals ($r = 0.64$ vs. 0.74, $P = 0.89$; multiple regression, $n = 6$), and thus for all other animals we measured the protein expression from the center of HVC, in frontal sections. For all nuclei, we counted at least two sections containing the

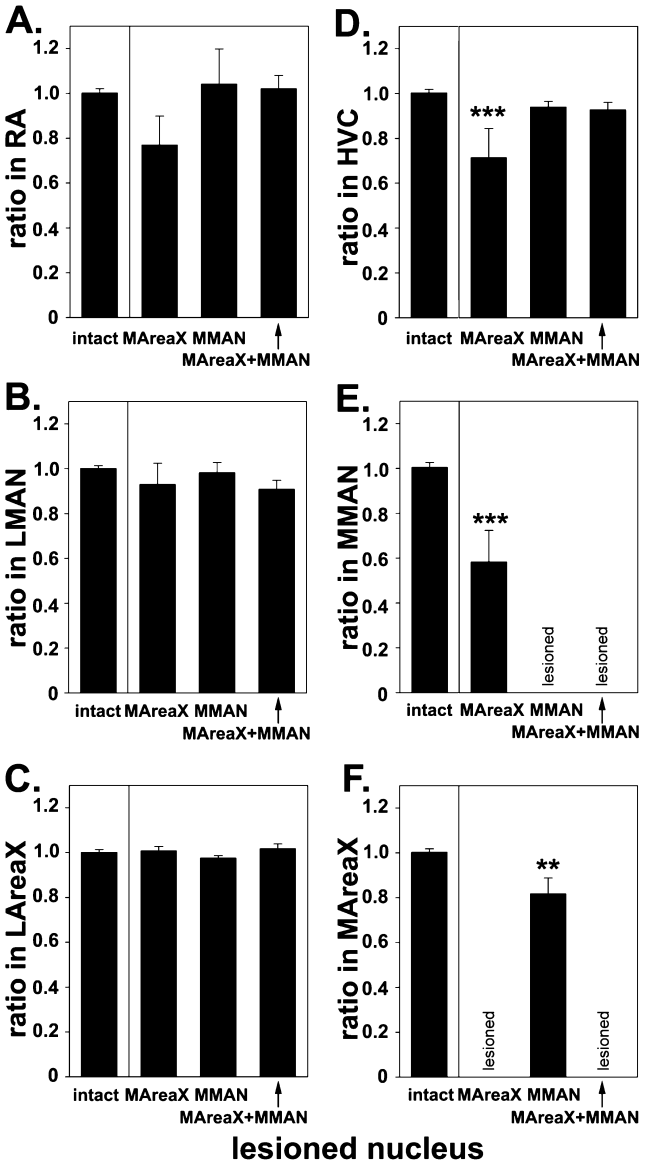

FIG. 10. Ratio analyses of effects of lesions within the medial part of the vocal pallial basal ganglia pathway and intact birds (*x*-axis) on the expression levels in various vocal nuclei (*y*-axis). (A–F) Each graph shows lesion effects on one nucleus. Ratios on the *y*-axes represent the ratios of ZENK-expressing neurons in ipsilateral to contralateral hemispheres. The data are combined from birds singing undirected and directed song. $^{**}P < 0.01$; $^{***}P < 0.001$; ANOVA, Fisher's PLSD. LAreaX, lateral AreaX; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MAreaX, medial AreaX; MMAN, medial magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

vocal nucleus in each hemisphere. The variability in counts between adjacent sections was low; the standard deviations of the mean in HVC and LAreaX in intact birds were 3.5% and 4.4%, and in LMAN-lesioned birds they were 3.5% and 2.8%. The number of ZENK-labeled neurons was divided by the number of Hu-labeled neurons to obtain the percentage of ZENK-expressing neurons (% of ZENK expressing neurons = 100 × # of $ZENK^+$ neurons/# of Hu^+ neurons). Counting was done blind to group.

Song analysis

Song motif duration was measured in 20 undirected motifs before surgery and 20 undirected motifs from the last day of observation, and also at two different comparable time points for intact birds ($n = 7$ intact birds, 5 birds with LAreaX lesion, 5 birds with LMAN lesion, and 3 birds with LAreaX + LMAN lesion).

Statistics

We present the data of intact and lesioned animals in two complementary analyses: first as regression analysis of gene expression in each vocal nucleus with singing amount; and second as ratio analysis between the same nuclei of each brain hemisphere. The regressions allow for comparisons of each hemisphere relative to control animals but are sensitive to only relatively large differences between groups because they do not control for within-animal variability. The ratios control for within-animal variability, and as such are sensitive to subtle but significant changes in the expression levels between hemispheres of individual animals because each animal serves as its own control. The ratio analysis also normalizes for singing amount. The information lost in the ratio analysis is the direction (increase or decrease) of a change that occurs relative to intact controls.

For the regression analysis, we calculated *P*- and *r*-values from simple regressions when comparing expression of a single vocal nucleus and singing amount. We used multiple regressions when comparing undirected and directed singing and when comparing the effect of vocal nuclei lesions on gene expression in other vocal nuclei between lesioned and intact animals. For the ratio analysis, the number of ZENK-expressing neurons in a vocal nucleus of the lesioned side was divided by the number on the intact side, and statistical differences across groups were assessed by ANOVA followed by the Fisher's protected least significant difference (Fisher's PLSD) *post hoc* test. The ANOVA analyses were done separately for each nucleus and social context. The independent variable was lesion type and the dependent variable was percentage of ZENK-expressing neurons in the ipsilateral : contralateral vocal nucleus.

Singing-driven ZENK protein levels and social context

The social context-dependent differences in LAreaX, LMAN and RA are known for ZENK mRNA levels per cell (Jarvis *et al.*, 1998), but not for ZENK protein (but see Castelino & Ball, 2005). Before performing experiments in this study, we first established whether the social context mRNA differences persist at the protein level in control animals without lesions. Consistent with the mRNA regulation (Jarvis *et al.*, 1998), the number of neurons expressing ZENK protein in LAreaX, MAreaX, LMAN, MMAN, RA and HVC increased with the number of undirected songs produced (Fig. 3B, filled circles and solid lines). However, unlike the amount of mRNA/cell, which continues to increase linearly even after 120 songs in 45 min (Jarvis *et al.*, 1998), the number of ZENK protein-expressing neurons began to show saturation with about 80 songs. The saturation was not at 100% of the neurons, but less, with the level of saturation depending on vocal nucleus (Fig. 3B). When a bird directed its singing to a female, the number of neurons expressing ZENK protein in MAreaX, MMAN and HVC also increased, while in LAreaX, LMAN and RA the numbers were low (Fig. 3B, open circles and dashed lines), which is consistent with the amount of mRNA/cell (Jarvis *et al.*, 1998). We could not ascertain for directed singing whether saturation occurs in MAreaX, MMAN and HVC, as it was difficult to obtain birds that would sing more than 80 songs to a female in the time period (55 min) tested. The variations between the mRNA and protein results could be due to post-translational regulation of ZENK (Whitney *et al.*, 2000; Whitney & Johnson, 2005) or differences in the relationships of singing amount

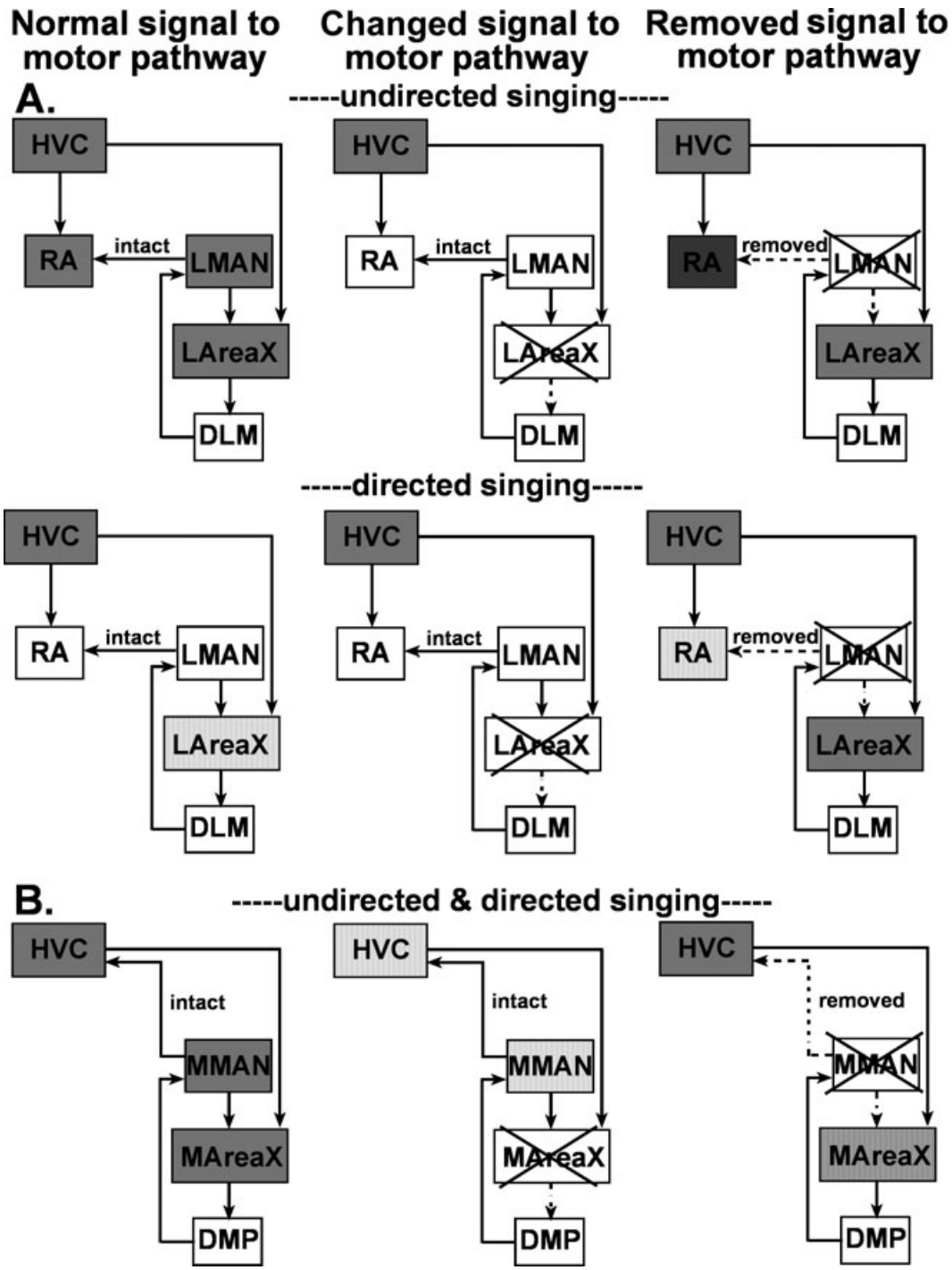

FIG. 11. Schematic diagram summarizing the results of this study. (A) The lateral part of the vocal pallial basal ganglia pathway and modulation of the robust nucleus of the arcopallium (RA). (B) The medial part of the vocal pallial basal ganglia pathway and modulation of HVC. When the lateral magnocellular nucleus of the anterior nidopallium (LMAN) or medial magnocellular nucleus of the anterior nidopallium (MMAN) is lesioned, singing-driven ZENK expression in RA or HVC is increased or not affected. However, when they are intact and the upstream nucleus lateral AreaX (LAreaX) or medial AreaX (MAreaX) is lesioned, then singing-driven ZENK expression in RA and HVC is decreased. The difference in ipsilateral HVC is smaller in magnitude than it is for RA, and is suggested from ratio analysis (ipsilateral expression < contralateral). Shading: relative ZENK expression levels after singing. ZENK is not induced in the dorsal lateral nucleus of the dorsomedial thalamus (DLM), and so it is not shaded. DMP, dorsomedial nucleus of the posterior thalamus.

and 'expression/cell' vs. 'percentage of expressing neurons'; the latter by definition has to saturate below or at 100% of the neurons.

Furthermore, after undirected singing the ZENK protein expression levels in LAreaX, LMAN and RA were strongly correlated (graphs not shown; $r = 0.85$–0.94; $P = 0.0004$–0.007; simple regressions); after directed singing, no significant correlations were found with any of these three nuclei among each other or with other nuclei, due to little or no increases ($r = 0.09$–0.74, $P = 0.06$–0.83; simple regressions). The expression levels in MAreaX, MMAN and HVC were strongly correlated with each other in both singing contexts ($r = 0.91$–0.95,

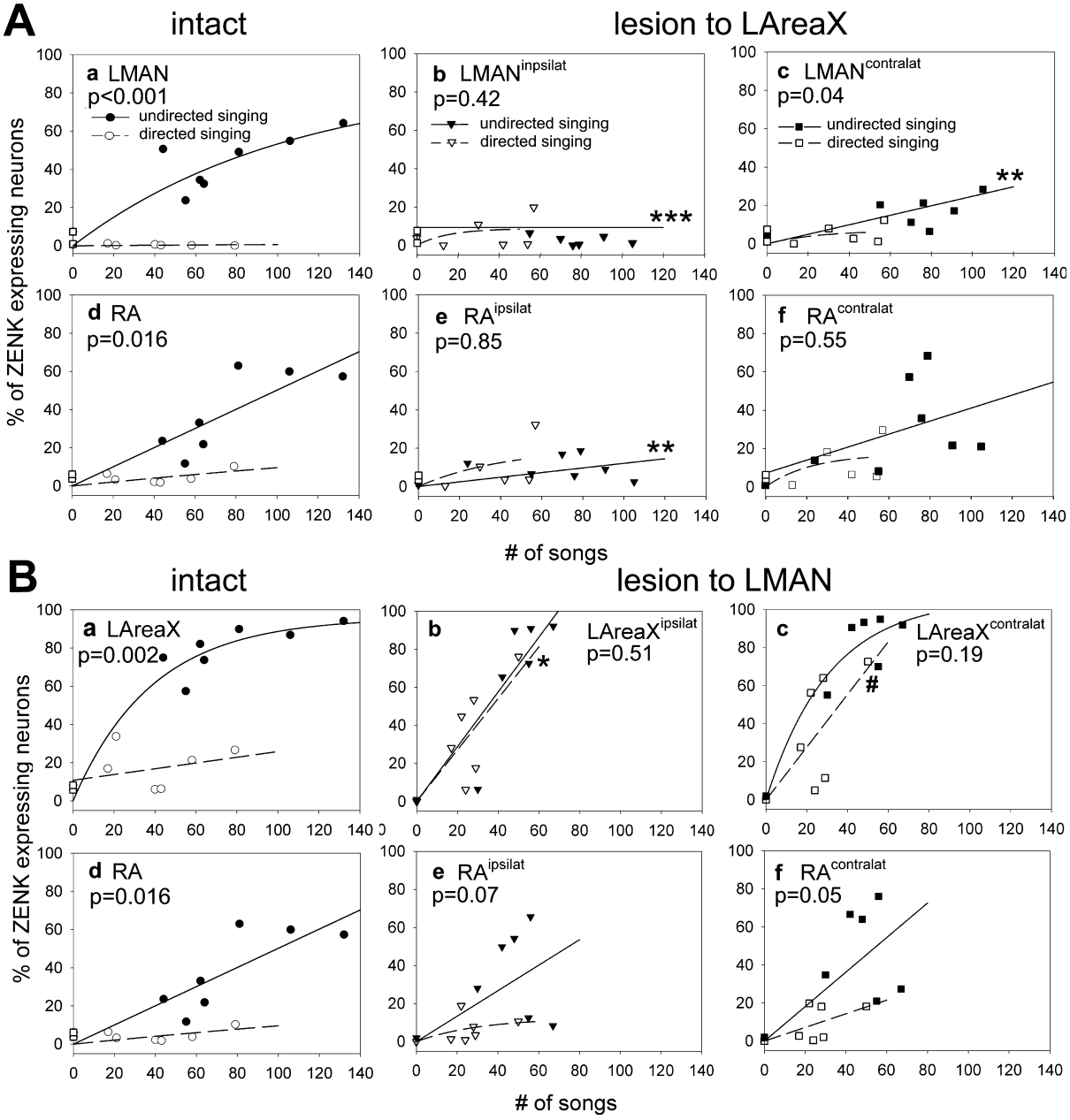

FIG. 12. Singing amount and ZENK protein levels in vocal nuclei of (A) lateral AreaX (LAreaX)- and (B) lateral magnocellular nucleus of the anterior nidopallium (LMAN)-lesioned birds for undirected and directed singing together with intact controls from Fig. 3B to make the comparisons clear. The values for the undirected singing lesioned groups are the same as those in Fig. 5. The data were best fitted by 'exponential rise to maximum, two-parameters' curves. The effects of LAreaX and LMAN lesions on the social context-dependent gene expression were assessed by two statistical comparisons: (1) comparison between undirected and directed singing (*P*-values shown in the graphs, multiple regressions, solid vs. dashed lines); and (2) comparison between undirected or directed singing of lesioned birds relative to intact birds (symbols in graphs, #$P = 0.056$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, multiple regressions). A lesion was considered to completely eliminate the social context difference if the *P*-value for comparison #1 was not significant and for comparison #2 was significant for at least one social context. It was considered reduced if only one of these criteria were met. RA, robust nucleus of the arcopallium.

$P = 0.0007$–0.0014 after undirected singing; $r = 0.95$–0.98, $P = 0.0001$–0.0007 after directed singing; simple regressions), and with LAreaX, LMAN and RA only during undirected singing ($r = 0.88$–0.93, $P = 0.0001$–0.005; simple regressions). Thus, the social context-dependent differences persist at the protein level, where the lateral part of the vocal pallial basal ganglia pathway (LAreaX and LMAN) together with RA function in a coordinated manner, whereas the medial part (MAreaX and MMAN) together with HVC may function in a coordinated manner.

Results

We found that lesions to lateral and medial portions of the vocal pallial basal ganglia pathway nuclei affect singing-regulated gene expression predominantly in RA and HVC, respectively. Such lesions also affected the singing-driven gene expression within connected vocal nuclei of the pallial basal ganglia pathway. We first describe the findings for the effects that lateral and medial lesions have on undirected singing-driven gene expression, and then the effects on the

social context-dependent differences between undirected and directed singing. Within each lesion description, we first present the findings of regression analysis, which is sensitive to relatively large differences between groups and shows the direction of change between intact and lesioned animals, and second present the findings of ratio analysis, which enable us to detect subtle but significant changes in the expression levels between hemispheres.

Lateral part of pallial basal ganglia pathway

LAreaX lesions and undirected singing-driven gene expression

Unilateral ibotenic acid lesions to LAreaX (Fig. 4A, a) had a dramatic effect of preventing high levels of undirected singing-driven ZENK expression in ipsilateral RA and LMAN (example images in Fig. 4A, d and e, h and i). Regression analyses showed that the decrease in the ipsilateral nuclei relative to intact controls was significant, with very few ZENK-labeled neurons in ipsilateral RA and LMAN regardless of singing amount (Fig. 5A, a and b; triangles relative to circles; except one outlier in LMAN, which was the only bird that had some leftover neurons inside the LAreaX lesion site that expressed ZENK). This was not the result of neuron loss due to removal of presynaptic LAreaX input, as the number of neurons in ipsilateral RA and LMAN were comparable to the numbers in contralateral RA and LMAN (Fig. 4A, b and c, f and g; $P = 0.99$ for LMAN, $P = 0.98$ for RA; ANOVA). Unilateral LAreaX lesions also prevented the full undirected singing-driven ZENK expression in the contralateral LMAN, although not in the contralateral RA (Fig. 5A, a and b; squares relative to circles). Further, we found a decreased expression in ipsilateral MAreaX relative to intact controls (graphs not shown; comparison between lesioned and intact animals: $P = 0.03$ for ipsilateral MAreaX, $P = 0.80$ for contralateral MAreaX; multiple regression).

Ratio analyses confirmed the large effect that unilateral LAreaX lesions had on ipsilateral relative to contralateral RA (Fig. 6A) and LMAN (Fig. 6B), as well as the effect on MAreaX (Fig. 6F; seen even when the lesions spared some of LAreaX adjacent to MAreaX in two out of seven birds). Further, the ratio analysis revealed a more subtle effect on HVC where there was less singing-driven ZENK expression in ipsilateral relative to the contralateral nucleus (Fig. 6D). We did not find a relationship between these effects and unilateral LAreaX lesion size ($r = 0.14$–0.56, $P = 0.19$–0.76; simple regressions). A possible explanation is that we noted that lesion to one part (even a small part) of LAreaX reduced the ZENK expression levels in the remaining non-lesioned part of ipsilateral LAreaX to 16–66% of that of contralateral LAreaX (Fig. 7A; $P = 0.02$, paired *t*-test).

LMAN lesions and undirected singing-driven gene expression

Regression analyses showed that unlike LAreaX lesions, unilateral ibotenic acid lesions to LMAN did not cause a consistent change in the expression in ipsilateral or contralateral RA and LAreaX relative to intact animals, when including all LMAN lesion animals as a group (Fig. 5B, a and b: triangles or squares relative to circles). However, within the group of six LMAN-lesioned birds, two had intact-like singing-driven ZENK expression levels in RA while the other four had increased expression to 200–300% in both hemispheres relative to intact animals that sang similar amounts of song, and these increases were higher in the contralateral RA (Fig. 5B, a, the shadowed areas encompass the two and four birds). These increases in RA were also visually apparent (Fig. 8B vs. C and D; also see Fig. 4A, i, RA of an LAreaX-lesioned bird vs. Fig. 4B, c and d, RA of a LMAN-lesioned bird that only sang half the amount of song). To examine this finding more objectively, we performed a distribution analysis on the LMAN-lesioned and intact animals together with the null hypothesis that these two groups do not differ. To control for singing amount, we normalized the number of ZENK-expressing neurons to the amount of singing for each bird. This analysis resulted in a bimodal distribution that separated the LMAN-lesioned animals into two subgroups: those with intact controls and those with increased RA expression relative to intact controls (Supplementary Fig. S3). When regression analysis was performed on the later subgroup of four birds relative to intact birds, they had significantly increased levels of ZENK-expressing neurons in both hemispheres ($P = 0.01$ for ipsilateral hemisphere, $P = 0.005$ for contralateral hemisphere; multiple regression). At present, we do not know the source of this bimodal effect. Because individual LMAN neurons send axon collaterals to RA and LAreaX (Fig. 1) we would expect the increase also in LAreaX. However, the number of undirected singing-driven ZENK-labeled neurons in LAreaX of our intact animals is already close to 100% saturation, whereas in RA it is not (Fig. 5B, a and b), making it difficult to determine whether an enhancement occurs in LAreaX following LMAN lesions (but see directed singing condition below). To further assess confidence that LMAN and LAreaX lesions have different consequences on RA ZENK gene expression, we performed additional analysis and found that the effect of LMAN lesions on RA was significantly different from the effect of LAreaX lesions on RA ($P = 0.02$ for ipsilateral RA; $P = 0.11$ for contralateral RA; including all LMAN-lesioned birds; $P = 0.03$ and $P = 0.003$ for comparison of four birds with high expression after LMAN lesion with seven LAreaX-lesioned birds; multiple regressions).

Ratio analyses confirmed the finding that unilateral LMAN lesions result in higher expression in the contralateral relative to ipsilateral RA (Figs 4B, c and d, and 6A). There were no detectable large or subtle changes on any of the other vocal nuclei (Figs 4B, a and b, and 6C–F). The effect of LMAN lesions on RA ratios was significantly different from the effect of LAreaX lesions on RA ratios ($P < 0.001$, ANOVA, Fisher's PLSD). Similarly to LAreaX lesions, with LMAN lesions we did not find a relationship with lesion size and the effect on RA ratios ($r = 0.46$, $P = 0.55$; simple regressions), although there may not be enough statistical power, as most LMAN-lesioned birds had lesions sizes above 87% (Table 1). Unlike LAreaX lesions, we noted that for the birds where LMAN was not completely lesioned, ZENK expression levels in the remaining non-lesioned part of ipsilateral LMAN were similar to that of contralateral LMAN (Fig. 7B).

LAreaX + LMAN lesions and undirected singing-driven gene expression

The shortest known path for LAreaX to influence ZENK expression in RA is through ipsilateral LMAN (Fig. 1). If this is the case, then combined lesions to LAreaX and LMAN should result in similar findings as lesions only to LMAN. Regression analysis showed that three of four birds had increased expression that was 150–300% of the ZENK-labeled neurons in RA relative to intact animals with a larger difference on the contralateral side ($P = 0.04$ for the contralateral hemisphere vs. intact birds; but $P = 0.57$ for the ipsilateral hemisphere with one bird not showing high expression; multiple regression), similar to what happens in most birds with lesions only to LMAN (Fig. 5B). The bird that did not show higher expression in RA with combined lesions was the only one with a small lesion (8%) in LMAN (Table 1). Ratio analysis showed that the difference of singing-driven gene expression in RA between hemispheres is significant (Fig. 6A). The combined lesions to LAreaX + LMAN had an effect on

MAreaX ratios (Fig. 6F), similar to that seen with lesions only to LAreaX (Fig. 6F).

Summarizing, the results above show that: (1) LAreaX is required for the high undirected singing-driven ZENK expression in RA and LMAN (Fig. 11A, top middle panel); (2) in turn LMAN is required for lowering levels of undirected singing-driven ZENK expression in RA of some birds (Fig. 11A, top right panel); and (3) LMAN input to RA is required for the regulation that LAreaX has on RA (i.e. LAreaX + LMAN lesion result is the same as Fig. 11A, top right panel). LAreaX has a smaller modulatory role on HVC that is perhaps mediated by MAreaX, as MAreaX is affected after LAreaX lesions and may be connected more directly in a pathway with HVC (Fig. 1).

Medial part of pallial basal ganglia pathway

MAreaX lesions and singing-driven gene expression

Regression analyses showed that unilateral ibotenic acid lesions to MAreaX (Fig. 9A, Supplementary Fig. S2, A) did not result in any large differences in ZENK expression in ipsilateral or contralateral vocal nuclei relative to intact animals ($P > 0.05$; multiple regressions; not shown). However, the more sensitive ratio analyses revealed significant differences between hemispheres with less singing-driven ZENK protein expression in ipsilateral relative to contralateral HVC and MMAN (Figs 9B and C, and 10D and E; for the effect of medial pathway lesions on MAreaX, MMAN and HVC we combined results from birds singing undirected and directed song as no differences between contexts were seen). There were no effects detected on LAreaX, LMAN or RA ratios (Fig. 10A–D).

MMAN lesions and singing-driven gene expression

Regression analyses showed that unilateral ibotenic acid lesions to MMAN did not result in any large differences in ZENK expression in ipsilateral or contralateral vocal nuclei relative to intact animals ($P > 0.05$; multiple regressions; not shown). However, the ratio analysis revealed a significant difference in ipsilateral relative to contralateral MAreaX (Fig. 10F). In contrast to MAreaX lesions, MMAN lesions did not have an effect on HVC ratios (Fig. 10D). The absence of an effect of MMAN lesions on HVC was significantly different from the effect of MAreaX lesions on HVC ($P = 0.003$, ANOVA). There was no effect on RA, LMAN or LAreaX ratios (Fig. 10A–C).

MAreaX + MMAN lesions and singing-driven gene expression

To determine whether the effect of MAreaX lesions on HVC was through a possible loop with MMAN (Fig. 1), we removed both MAreaX and MMAN together. If this is the case, then combined lesions to MAreaX and MMAN should result in similar findings as lesions only to MMAN. Regression and ratio analyses revealed that combined unilateral lesions to MAreaX + MMAN (Supplementary Fig. S2, B) were similar to MMAN lesions alone and prevented the effect that MAreaX lesions had on singing-driven gene expression in HVC ($P > 0.05$, multiple regression and Fig. 10D). There were no effects on the RA, LMAN or LAreaX ratios (Fig. 10A–C). We also performed preliminary tracer experiments, using directed singing-driven gene expression for identifying MAreaX, to test the predicted, but not proven, projection of MAreaX to the dorsomedial nucleus of the posterior thalamus (DMP; Fig. 1). These preliminary experiments revealed that in parallel to LAreaX and surrounding striatum projections to DLM of the lateral thalamus (Iyengar *et al.*, 1999; Luo *et al.*, 2001), injections of a combined retrograde/anterograde tracer targeted to MAreaX and surrounding striatum relative to the overlying nidopallium labeled axons that specifically projected to DMP of the medial thalamus (DMP as defined, Foster *et al.*, 1997; Supplementary Fig. S4). Thus, although this is not a detailed anatomical study, the findings do support the hypothesis that the medial part of the vocal pallial basal ganglia pathway may form a medial loop, separate and in parallel with a lateral loop, and are consistent with the finding that MAreaX lesions specifically affect HVC and not RA.

Summarizing, the results above show that: (1) MAreaX is required for the normal levels of singing-driven ZENK expression in HVC and MMAN (Fig. 11B, middle panel); (2) MMAN is not required for singing-driven ZENK expression in HVC (Fig. 11B, right panel); but (3) MMAN input to HVC is required for the regulation that MAreaX has on HVC (i.e. MAreaX + MMAN lesions result is similar to Fig. 11B, right panel).

Pallial basal ganglia pathway and social contexts

Given that the lateral part of the pallial basal ganglia pathway modulates singing-driven ZENK expression in RA and given that the lateral part is differentially active during undirected and directed singing, this leads to the prediction that the lateral part may modulate RA expression differentially in different social contexts. To test this idea, we lesioned the lateral basal ganglia pathway nuclei and assessed whether social context-dependent differences remained in RA and other nuclei using multiple regression analyses. We found that such lesions either eliminated or reduced the social context-dependent differences.

LAreaX lesions and directed vs. undirected singing-driven gene expression

Unilateral lesions to LAreaX eliminated the social context-dependent ZENK expression differences in RA and LMAN in the ipsilateral hemisphere and reduced it in the contralateral hemisphere (Fig. 12A; compare *P*-values for undirected vs. directed singing in the graphs for intact and lesioned birds; statistical definitions of elimination vs. reduction are in the figure legend). The social context differences were lost (or reduced) because relative to intact animals, LAreaX lesions resulted in lower ZENK expression levels in RA and LMAN after undirected singing and had no effect on the already low levels in LMAN and RA after directed singing (Fig. 12A; *P*-values as symbols ** and *** next to the lines for the comparison with intact birds).

LMAN lesions and directed vs. undirected singing-driven gene expression

Unilateral lesions to LMAN also eliminated or reduced the social context-dependent differences in LAreaX and RA, respectively, but in an opposite fashion (Fig. 12B; *P*-values in the graphs). The difference in LAreaX was lost because relative to intact animals, LMAN lesions resulted in a dramatic increase in expression levels during directed singing in ipsilateral as well as contralateral LAreaX and had very little effect on the already high levels in LAreaX after undirected singing (Fig. 12B, a–c; *P*-value as symbols * and # next to the lines for comparison with intact birds). The dramatic increase in LAreaX after directed singing was visually apparent, i.e. such birds had visibly many more labeled neurons in LAreaX relative to intact controls that produced even double the amount of directed songs (Fig. 8E–H). The difference between directed and undirected singing in RA was reduced because relative to intact birds some LMAN-lesioned birds showed an increase in both contexts (Fig. 12B, d–f).

LAreaX + LMAN lesions and directed vs. undirected singing-driven gene expression

Combined lesions to ipsilateral LAreaX and LMAN also eliminated the social context difference in RA of both ipsilateral and contralateral hemispheres ($P = 0.32$ and 0.11, respectively; multiple regressions comparison within lesion groups; not shown). The difference between directed and undirected singing in RA was lost because relative to intact birds some LAreaX + LMAN-lesioned birds showed an increase in both contexts, similar to that seen with lesions only to LMAN.

Summarizing, the results above show that LAreaX and LMAN are required for social context-dependent gene expression in opposite directions for different contexts. LAreaX is required for 'high' expression in LMAN and RA when producing 'undirected song' (Fig. 11A, top middle panel). In a reciprocal manner, LMAN is required for 'low' expression in LAreaX when producing 'directed song' (Fig. 11A, bottom right panel). Because RA can still have some low expression during directed song after a LMAN lesion or combined LAreaX + LMAN lesion, an additional input must be responsible for the full low expression in RA during directed singing. We have not yet investigated the source of this additional input.

Behavior

There was no statistical difference in singing amount between unilateral lesion and intact birds either for undirected singing ($P = 0.52$, ANOVA) or for directed singing ($P = 0.37$, ANOVA). Although we performed unilateral and not bilateral lesions, we carried out a measurement of song tempo that has been previously shown to change after bilateral lesions to LMAN (Williams & Mehta, 1999). Our analysis showed that there are only subtle changes towards slower singing in unilateral LAreaX-lesioned birds and no changes for unilateral LMAN or LAreaX + LMAN-lesioned birds (ANOVA, $P = 0.06$; Fisher's PLSD is $P = 0.01$ for LAreaX lesion, $P = 0.16$ for LMAN lesion, and $P = 0.12$ for LAreaX + LMAN lesion; mean percentage change ± SEM was 2.14 ± 0.7% for LAreaX lesion, 0.9 ± 0.9% for LMAN lesion and 1.3 ± 0.5% for LAreaX + LMAN lesion).

Discussion

Our results suggest: (1) that the songbird vocal pallial basal ganglia pathway modulates the behaviorally driven gene expression of the vocal motor pathway; (2) that this modulation occurs in a topographic manner, where a functionally defined larger lateral part modulates expression mainly in RA, and a smaller medial part modulates expression, albeit to a smaller degree, in HVC; and (3) that the pallial or cortical-like MAN nucleus and the striatal AreaX nucleus can modulate motor pathway gene activation in opposite directions, including for social context-dependent gene expression. Previous studies on cortical–basal–ganglia–thalamic–cortical loops have found that functional manipulation of mammalian striatum affects cortical IEG activation (Steiner & Kitai, 2000; Blandini *et al*., 2003), and likewise manipulation of the cortex affects striatal gene activation (Parthasarathy & Graybiel, 1997; Sgambato *et al*., 1999). In those studies, manipulation of excitatory or inhibitory striatal dopamine receptors up- or downregulated IEG expression in multiple cortical regions, respectively, and electrical stimulation of cortex upregulated topographically defined patches of IEG expression in the striatum. In this study, we were able to address relationships between striatal and pallial (i.e. cortical-like) components of similar pathways in a more defined circuit that controls learning and production of vocalizations. Below we propose potential mechanisms of this modulation and possible functional consequences.

Medial and lateral subdivisions of vocal basal ganglia pathway

Traditionally, the role of the vocal pallial basal ganglia pathway is said to process information from HVC and then send output to RA (Fig. 11A; Williams, 1989; Nottebohm, 1993). The instructive signal has been proposed to change RA activity to correct the song produced (Brainard & Doupe, 2000). We argue that this is only part of the story, the lateral part, which has been the inadvertent focus of most studies on this pathway in songbirds. Our results suggest that information from HVC is also processed in the medial part of the vocal pallial basal ganglia pathway, which then sends feedback to HVC (Fig. 11B). In this view, the main role of the vocal pallial basal ganglia pathway may be to process motor pathway input from HVC and use it to modulate activity of both major nuclei, HVC and RA, of the motor pathway. For RA, this modulation could be for learning or maintaining syllable acoustic structure, and for HVC, learning or maintaining syllable sequences (Hahnloser *et al*., 2002).

Our lesion results indicate that the lateral part of the vocal pallial basal ganglia pathway has stronger unilateral control over RA than the medial part does over HVC. This could be because of either a larger lateral pathway volume or unilateral vs. bilateral connectivity. The lateral part of the pallial basal ganglia pathway and RA has mainly unilateral connections (Fig. 1). In contrast, the medial part and HVC have bilateral feedback connections: DMP to MMAN, and nucleus uvaeformis (Uva) to NIf and HVC (Fig. 1). Thus, when MAreaX is lesioned, ipsilateral MMAN and HVC could still be activated and compensated by input from the contralateral side.

Our results also suggest that there is an interaction between the medial and lateral components. Birds with LAreaX lesions had an effect on the singing-driven gene expression in HVC. This might be due to two reasons. First, the downstream series of connections from LAreaX to DLM to LMAN to RA to dorsal medial nucleus of the midbrain (DM) to Uva to NIf to HVC or from RA to DMP to MMAN to HVC (Fig. 1) could influence HVC (Akutagawa & Konishi, 2005). A second possibility is that LAreaX and MAreaX are interconnected. The lesion to LAreaX influenced the expression in MAreaX, which then via a loop with MMAN could have affected the expression in HVC. This interconnection is supported by the fact that numerous striatal spiny neurons terminate on other striatal neurons in AreaX (Reiner *et al*., 2004a), and lesions in one part of LAreaX affected ZENK expression throughout other parts of AreaX. However, because MAreaX lesions did not affect ZENK expression in LAreaX, the interconnections may not be reciprocal (and we did not note projections from MAreaX to LAreaX in our tracer injections). Alternatively, LAreaX is much larger than MAreaX and thus LAreaX may have enough redundancy to withstand damage of input from MAreaX.

Opposing roles for striatal vs. pallial nuclei

Our data show that relative to intact animals, LAreaX lesions lead to a lower ZENK expression in downstream nuclei and LMAN lesions lead to the same or higher ZENK expression in downstream nuclei. Given the later bimodal effect, we were still concerned whether we can conclude that LMAN lesions can have an effect of increased ZENK expression in RA. Our concern, though, is assuaged by the facts that first there are clearly much higher levels of visible ZENK expression in

RA of the majority of LMAN-lesioned birds at levels we have never seen before in intact birds; second there is a similar result with combined LAreaX + LMAN lesions; third there is a significant difference in the RA expression in LMAN-lesioned vs. LAreaX-lesioned groups; and fourth a large increase in RA ZENK expression has been previously found following LMAN lesions in juvenile birds (Whitney *et al.*, 2000). A parsimonious explanation of these results is that LAreaX enhances while LMAN permits or dampens (possibly by suppression) the expression in their targets, the two working in concert to balance each other. The enhancing effect that LAreaX has on RA expression during undirected singing appears to go through LMAN (and thus also through DLM), where LAreaX influences the effect that LMAN has on RA. That is, in intact animals activation of LAreaX may induce (via DLM) the high activation levels in LMAN, which in turn permits HVC (or other upstream nuclei) to induce high levels in RA up to a certain amount (Fig. 11A, top left panel); a role for HVC activation of RA is supported by electrophysiology studies of Hahnloser *et al.* (2002) and preliminary gene expression findings of ours (where unilateral HVC lesions prevented singing-driven ZENK activation in ipsilateral RA). When LAreaX is removed and LMAN connections to RA are intact, LMAN may no longer be activated or is altered in a manner that does not permit HVC to induce ZENK expression in RA (Fig. 11A, top middle panel). But, when LMAN is removed, HVC can then induce high levels in RA (and in LAreaX) without the need for permissive signals from LMAN (Fig. 11A, top right panel). A similar activation scenario can be made for directed singing (Fig. 11A, bottom panels), with the exception that other as yet to be determined input to RA still does not permit HVC to fully activate ZENK expression in RA when LMAN is lesioned. This other input to RA might be dopaminergic or noradrenergic innervation from the midbrain (Appeltants *et al.*, 2002). A recent test of noradrenergic involvement for LAreaX showed that lesions to noradrenergic neurons result in abnormally high levels of directed singing-driven ZENK expression in LAreaX, but still relatively low levels in RA and LMAN (Castelino & Ball, 2005), similar to what we find in RA and in LAreaX following directed singing in LMAN-lesioned birds. Thus, like LMAN input, noradrenergic input to LAreaX is required for the low expression levels during directed singing. Consistent with the idea of opposing effects that LMAN and LAreaX (via LMAN) have on downstream nuclei is the prior behavior findings that LAreaX is required for song stereotypy and LMAN is required for song variability (Scharff & Nottebohm, 1991; Kao *et al.*, 2005; Olveczky *et al.*, 2005).

As for the role the medial nuclei MAreaX and MMAN have on the activation of each other and on HVC, the changes following lesions were too small for regression analyses to reveal the direction of change, enhancement or suppression. However, the results of the ratio analyses of MAreaX-lesioned birds with lower ipsilateral than contralateral expression in MMAN and HVC suggest similar effects as its lateral counterpart LAreaX has on LMAN and RA. Further, in parallel with the lateral counterpart, the change in HVC requires MMAN input, MMAN lesions alone do not cause a lowering of expression in HVC, and there is a significant difference in the HVC expression in MMAN-lesioned vs. MAreaX-lesioned groups. To reveal the full effect of medial pathway lesions on HVC expression may require bilateral lesions in comparison with intact controls to prevent possible contralateral compensation.

Proposed synaptic mechanisms

It has been hypothesized that presynaptic neurons regulate postsynaptic IEG expression, where neurotransmitters released by presynaptic neural activity onto postsynaptic receptors induce the motor-driven expression of postsynaptic IEGs during behavior (Lerea, 1997; Sgambato *et al.*, 1999; Jarvis, 2004a). Our results are consistent with this hypothesis, as lesions to the presynaptic nuclei disrupt normal postsynaptic IEG induction. As for RA, there are two main inputs: HVC (Fig. 11A), which releases glutamate onto AMPA and *N*-methyl-D-aspartate (NMDA) receptors of RA neurons; and LMAN (Fig. 11A), which releases glutamate mainly onto NMDA receptors of the same RA neurons (Mooney & Konishi, 1991; Stark & Perkel, 1999). When bound to glutamate, NMDA receptors activate ZENK transcription (Lerea, 1997). But if LMAN input to RA (and presumably also to LAreaX) is glutamatergic, i.e. considered excitatory (Perkel, 2004), how could LMAN suppress ZENK expression in the connected nuclei during singing? One possible explanation is that LMAN activates in RA and in LAreaX not only the excitatory NMDA receptors but also some inhibitory glutamate receptors. Potential candidates are metabotropic glutamate group II receptors, such as mGluR4, which is rich in both RA and AreaX (Wada *et al.*, 2004). Differential activation of NMDA and mGluR4 receptor types in different social context could then possibly allow high and low ZENK expression during undirected and directed singing, respectively. A second possibility is that LMAN preferentially activates RA inhibitory neurons and these inhibit ZENK activation in RA's tonically active projection neurons. This feedforward inhibition of neural firing from LMAN to RA inhibitory neurons that synapse onto excitatory neurons has been shown in electrophysiological studies (Spiro *et al.*, 1999). A similar argument can be made for LMAN input to AreaX and the ZENK activation in its striatal neurons. Detailed cellular analysis will be required to find out which hypothesis is correct.

As for the contralateral changes that we found, perhaps some compensation occurs in the contralateral hemisphere, but we do not suppose that the expression changes are simply due to compensation, because we would expect to see an opposite pattern of expression in the contralateral hemisphere. For example, compensation for the high expression in LAreaX after directed singing and unilateral LMAN lesions would be a decreased expression in the contralateral LAreaX and vice versa for the LAreaX-lesioned birds. Instead, in LAreaX-lesioned birds we see bilateral decreases in downstream nuclei, and in LMAN-lesioned birds we can see bilateral increases in downstream nuclei. These contralateral effects presumably occur via communication through the anterior commissure and/or bilateral thalamic and midbrain vocal nuclei connections (Fig. 1). Birds do not have a corpus callosum (fibers connecting the two hemispheres), as mammals. In mammals, although social context and behavior were not evaluated, unilateral blocking of dopamine D1 receptors in the striatum causes bilateral reduction of basal egr-1 (ZENK) levels in the cortex (Steiner & Kitai, 2000), with less reduction on the contralateral side, similar to our findings with striatal lesions in songbirds. Thus, even without a corpus callosum, bilateral control of pallial/cortical gene activation by the basal ganglia pathway could be a general feature of vertebrate brain function.

Possible behavioral consequences

The modulation of the vocal motor pathway by the vocal pallial basal ganglia pathway could potentially lead to immediate and long-term changes in vocal behavior output. The absence of any effect on adult singing following LMAN and LAreaX lesions (Bottjer *et al.*, 1984; Sohrabji *et al.*, 1990; Scharff & Nottebohm, 1991) is being

revised in the literature. An immediate effect on adult vocal output was shown in bilateral lesion studies where MMAN is necessary for consistent stereotyped sequencing of syllables (Foster & Bottjer, 2001), LMAN is necessary for generation of variability in syllable structure (Kao *et al*., 2005; Kao & Brainard, 2006), and LAreaX is necessary for producing repeated syllables without stuttering (Kobayashi *et al*., 2001). However, the immediate behavioral changes are presumably not the result of ZENK activation, as ZENK is induced after the behavior is produced (Jarvis & Nottebohm, 1997). ZENK can have long-term effects; gene manipulation studies in the mammalian hippocampus have shown a functional role of ZENK on memory reconsolidation, but not on acquisition (Lee *et al*., 2004). If a similar functional role exists for other neural circuits, then high activation of ZENK throughout the vocal pathways during undirected singing may be useful for reconsolidation of motor networks for the songs produced. In this context, perhaps for RA and HVC, AreaX is required for higher ZENK expression and higher reconsolidation, while MAN is required for lower ZENK expression and thus more plasticity. During directed singing, however, the lateral part of the basal ganglia pathway may be utilized less or inhibited and thus LMAN may not have such control of RA gene expression, but reconsolidation is still needed for HVC. In this context, the behavioral performance to the listening animal may be more crucial than memory reconsolidation.

Supplementary material

The following supplementary material may be found on www.blackwell-synergy.com

Fig. S1. Detailed camera lucida drawing and photomicrograph of the medial AreaX/lateral AreaX boundary in the directed singing bird shown in Fig. 3.

Fig. S2. Lesions to the medial part of the pallial basal ganglia loop.

Fig. S3. ZENK induction in the vocal motor nucleus RA in intact and lateral magnocellular nucleus of the anterior nidopallium-lesioned birds.

Fig. S4. A possible medial anterior vocal pathway loop.

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Abbreviations

DLM, dorsal lateral nucleus of the dorsomedial thalamus; DM, dorsal medial nucleus of the midbrain; DMP, dorsomedial nucleus of the posterior thalamus; HVC, nucleus HVC; IEG, immediate-early gene; LAreaX, lateral AreaX; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MAreaX, medial AreaX; MMAN, medial magnocellular nucleus of the anterior nidopallium; NMDA, *N*-methyl-D-aspartate; NIf, nucleus interface of the nidopallium; nXIIts, tracheosyringeal part of XII motor nucleus; PBS, phosphate-buffered saline; RA, robust nucleus of the arcopallium; Ram, nucleus retroambiguus; Uva, nucleus uvaeformis.

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