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Supplementary Information for

Cortico-basal ganglia projecting neurons are required for juvenile vocal learning but not for adult vocal plasticity in songbirds

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Supplementary Text

In situ hybridization

NTS cDNA fragments used for the synthesis of *in situ* hybridization probes were cloned from a whole-brain cDNA mixture of a male zebra finch. Total RNA was transcribed to cDNA using Superscript Reverse Transcriptase (Invitrogen) with oligo dT primers. The cDNAs were amplified by PCR using oligo DNA primers directed to conserved regions of the coding sequence from the NCBI cDNA database (accession # NM_001245684). PCR products were ligated into the pGEM-T Easy plasmid (Promega). The cloned sequences were searched using NCBI BLAST/BLASTX to compare with homologous genes with other species, and genome loci were identified using BLAT of the UCSC Genome Browser. For fluorescence in situ hybridization (FISH), digoxigenin (DIG)-labeled riboprobes were used. A total of 100–200 ng/glass of the DIG-labeled riboprobe was mixed with the hybridization solution [50% formamide, 10% dextran, 1× Denhardt's solution, 1 mM EDTA (pH 8.0), 33 mM Tris-HCl (pH 8.0), 600 mM NaCl, 0.2 mg/mL yeast tRNA, 80 mM dithiothreitol, and 0.1% N-lauroylsarcosine]. Hybridization was performed at 65 °C for 12–14 h. Washing steps were performed as follows: 5× SSC solution at 65 °C for 30 min, formamide-I solution (4× SSC, 50% formamide, and 0.005% Tween20) at 65 °C for 40 min, formamide-II solution (2× SSC, 50% formamide, and 0.005% Tween20) at 65 °C for 40 min, 0.1× SSC at 65 °C for 15 min × 2, 0.1× SSC at RT for 15min, NTE buffer at RT for 20 min, and TNT buffer × 3, and blocking buffer [1% DIG blocking solution (Roche) + 1% normal goat serum/1× TNT buffer] at RT for 30 min. DIG-labeled probes were detected with anti-DIG HRP-conjugated antibody (Jackson Laboratory) and a TSA Plus Cy5 system (Perkin Elmer). Signal images were obtained by fluorescence microscopy (EVOS FL; Thermo Fisher Science; BZ-X700; KEYENCE).

The number of $HVC_{(X)}$ neurons was estimated as the average NTS+ cells/mm^2 in both hemispheres of individuals. On the basis of the value of NTS+ cells/mm^2, the degree of ablation of $HVC_{(X)}$ neurons in individual birds was calculated as a normalized value (%) with the average of NTS+ cells/mm^2 of control birds.

Adeno-associated virus (AAV) construction

All the viral ITR-flanked genomes used in this study were of the self-complementary (sc) AAV vector type (1). The pscAAV-GFP vector containing a CMV promoter was obtained from Addgene (#32396). AAV plasmids containing Cre and DIO (double-floxed inverted open reading frame)/FLEx (Flip excision) inserts were obtained from Dr. Kenta Kobayashi from the National Institute of Physiological Sciences and subsequently cloned into the pscAAV vector plasmids after amplification of the Cre and DIO/FLEx sequences by primers containing the corresponding restriction enzymes in the target plasmid. To cell-specifically ablate the $HVC_{(X)}$ cells, a combination of diphtheria toxin A (dtA) and constitutively active caspase 3 was used (2-4). Diphtheria toxin was cloned from pAAV-mCherry-FLEx-dtA (Addgene, #58536) by primers with specific enzyme sites and inserted into the previously constructed scAAV-DIO/FLEx. Owing to the restricted carrying capacity of the pscAAV vector, it became necessary to generate a constitutively active caspase 3 (2) by insertional mutagenesis of rAAV-flex-taCasp3-Tevp obtained from Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill. This insertion consisted of the substitution of valine with glutamic acid at residue 266 of the protein, with subsequent amplification and cloning into an scAAV-DIO/FLEx vector.

AAVs were produced in-house using AAVpro 293T (Takara) cells transfected with a polyethyleneimine (PEI)-condensed recombinant DNA mixture, based on a protocol kindly provided to us by the Gradinaru Lab at Caltech. AAVpro 293T cells were amplified in 10 cm sterile culture dishes under standard cell culture medium [D-MEM, 10% fetal bovine serum (FBS), 1% penicillin, 1% GlutaMAX] until at least 1.5×10^8 cells could be collected. Cells were then plated onto 15 plates of 15 cm culture dishes at 1.0×10^7 cells per plate in standard cell culture medium. The confluence of the 15cm plates was checked visually, and the medium was changed again to standard 10% FBS medium when the confluence reached 80%, before proceeding to the transfection step. The transfection mix contained a triple plasmid system (pPack2/9 for serotype 2 Rep and serotype 9 Cap genes, pHelper for the adenoviral helper genes and the ITR-flanked viral genome containing plasmid) mixed with 40 kDa PEI in a 1: 3.5 DNA: PEI weight ratio, all dissolved in warm PBS. The cells were maintained in this transfection mix for 24 h, and then the medium was changed to a low serum one (D-MEM, 5% FBS, 1% penicillin, 1% GlutaMAX) to promote protein synthesis instead of cell division. Cells were scraped and collected in buffer (150 mM NaCl, 100 mM Tris-HCl pH 8.0) 3 days after transfection and maintained in a –80 °C freezer until viral purification. The purification procedure was performed as follows. Cells were freeze-thawed between a 37 °C water bath and

a –80 ℃ cooled ethanol bath for at least four cycles and then incubated for 30 min in Benzonase (Merck-Millipore) nuclease after adding of 60 µL of 1 M $MgCl_2$ to the cell solution. At the end of this incubation, the cell solution was centrifuged at 4℃ and 7,000 rpm for 1 h at 4°C, and then its supernatant was added as the top layer of a polycarbonate centrifuge column filled with an iodixanol gradient (15%, 25%, 40%, and 54% iodixanol layers). After ultracentrifugation for 6 h at 28,000 rpm, the 40% layer was extracted with a syringe and concentrated in four VivaSpin (Sartorius) cycles. Samples were finally aliquoted and stored in PBSF at –80 ℃.

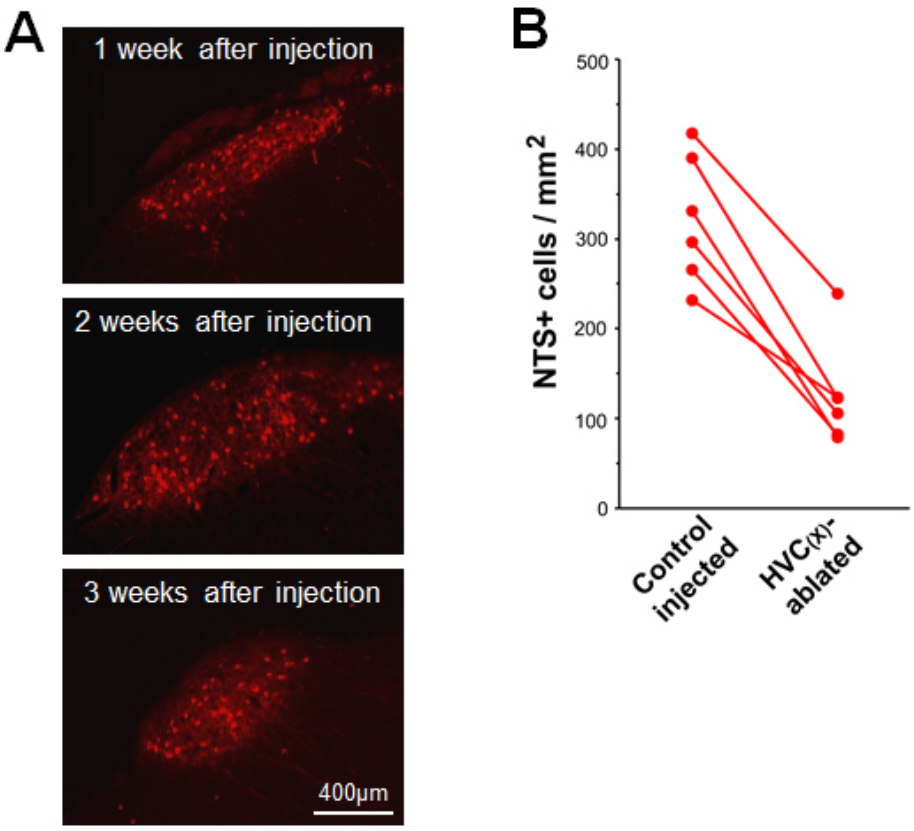

Fig. S1. Retrograde transported Cre-dependent FLEx inversion timing after scAAV9 injection and $HVC_{(X)}$ neuron ablation

(A) Restricted expression of FLEx-inverted mRuby2 fluorescent protein in $HVC_{(X)}$ cell populations at 1, 2, and 3 weeks after virus injection.

(B) Comparison of $HVC_{(X)}$ neuron density between control and lesioned HVC. The control hemisphere was injected with scAAV9-Cre in Area X and with scAAV9-FLEx-mRuby2 in HVC. The lesioned hemisphere was injected with scAAV9-Cre in Area X and with a mixture of scAAV9-FLEx-dtA and scAAV9-FLEx-caCasp in HVC.

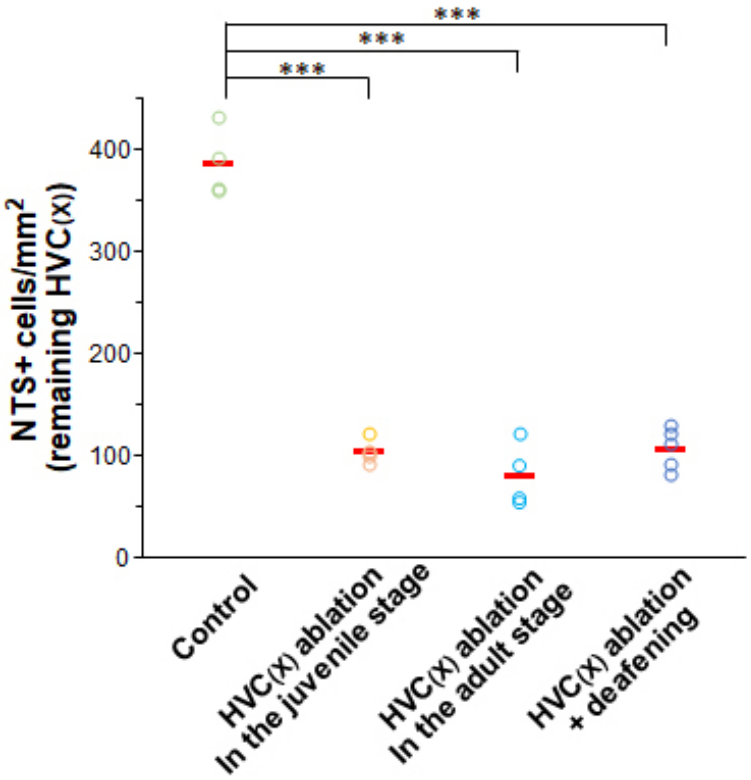

Fig. S2. Comparison of remaining $HVC_{(X)}$ neurons among control, ablation in the juvenile stage, adult stage, and following deafening conditions

Each dot corresponds to the average density of NTS+ cells ($HVC_{(X)}$ neurons) in one bird. Red horizontal bars represent the mean values for each group (Tukey HSD, ***p <1e−07).

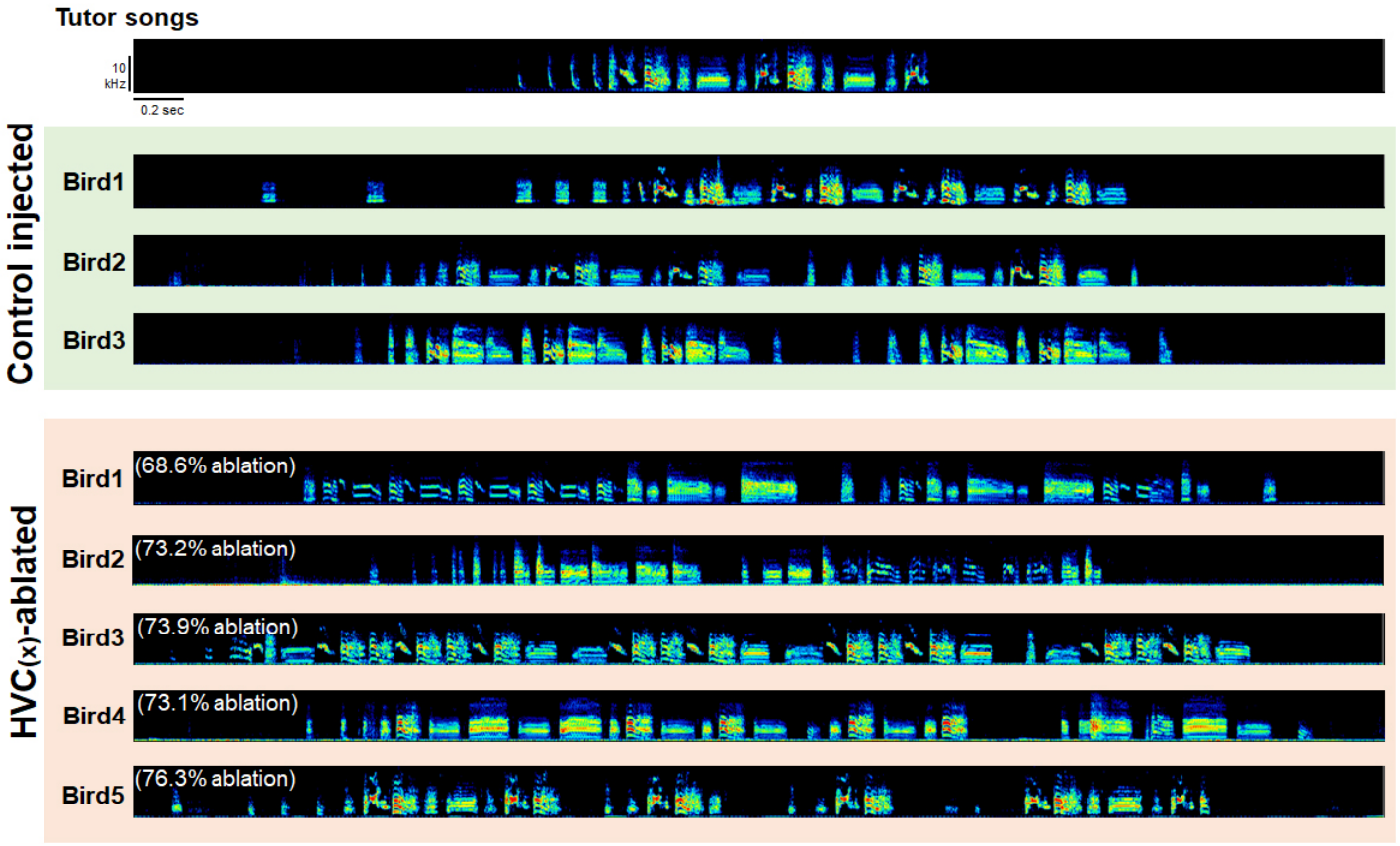

Fig. S3. Examples of acquired songs at phd 180 from control and HVC(X)-ablated birds in the juvenile stage

Bird numbers are consistent in Figs 2–4.

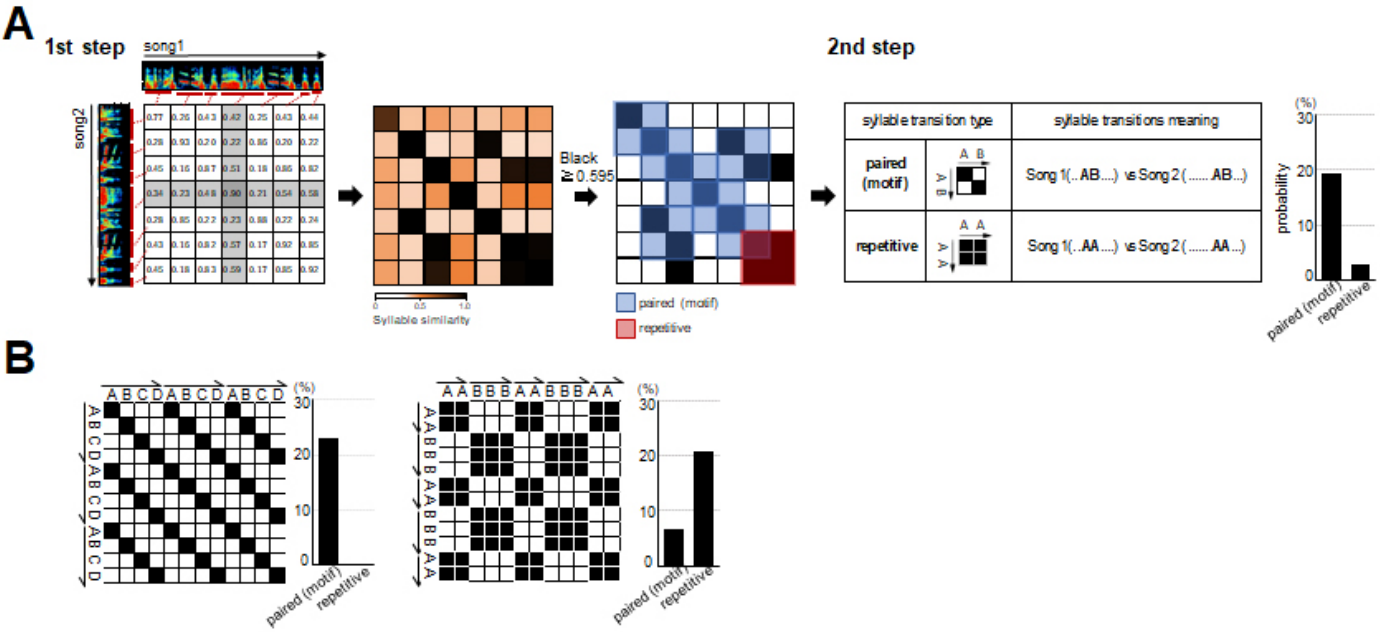

Fig. S4. Syllable similarity matrix (SSM) method for the detection of syllable transition patterns

(A) The SSM method consists of two steps. In the first step, a correlation matrix including syllable similarity scores was prepared by the round-robin comparison of all the syllable comparisons in two songs, maintaining the sequential order of the syllables in the songs. These similarity scores in the matrix were binarized at a threshold at 0.595. In the second step, the occurrence rate of two patterns of binarized "2 row × 2 column" cells in the SSM was calculated as a percentage of the paired (motif) and repetitive-syllable transition types (see Materials and methods).

(B) Test examples of the SSM method using artificial song models mimicking the songs with motif and repetitive sequences.

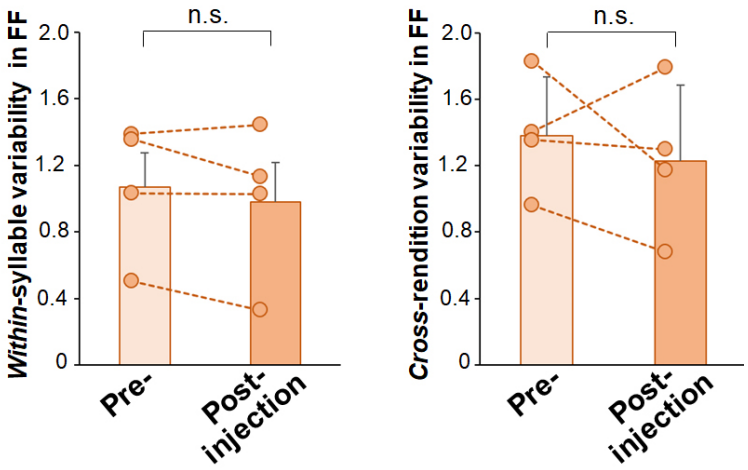

Fig. S5. Within- and cross-rendition syllable variability in fundamental frequency (FF) between pre- and post-2 weeks injection in HVC$_{(X)}$-ablated birds (n = 4; paired *t* test: n.s., $p > 0.05$)

References

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