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A predisposed motor bias shapes individuality in vocal learning

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The development of individuality during learned behavior is a common trait observed across animal species; however, the underlying biological mechanisms remain understood. Similar to human speech, songbirds develop individually unique songs with species-specific traits through vocal learning. In this study, we investigate the developmental and molecular mechanisms underlying individuality in vocal learning by utilizing F_1 hybrid songbirds (*Taeniopygia guttata* cross with *Taeniopygia bichenovii*), taking an integrating approach combining experimentally controlled systematic song tutoring, unbiased discriminant analysis of song features, and single-cell transcriptomics. When tutoring with songs from both parental species, F_1 hybrid individuals exhibit evident diversity in their acquired songs. Approximately 30% of F_1 hybrids selectively learn either song of the two parental species, while others develop merged songs that combine traits from both species. Vocal acoustic biases during vocal babbling initially appear as individual differences in songs among F_1 juveniles and are maintained through the sensitive period of song vocal learning. These vocal acoustic biases emerge independently of the initial auditory experience of hearing the biological father's and passive tutored songs. We identify individual differences in transcriptional signatures in a subset of cell types, including the glutamatergic neurons projecting from the cortical vocal output nucleus to the hypoglossal nuclei, which are associated with variations of vocal acoustic features. These findings suggest that a genetically predisposed vocal motor bias serves as the initial origin of individual variation in vocal learning, influencing learning constraints and preferences.

individual difference | developmental plasticity | hybrid | learning bias | zebra finch

Significance

The emergence of individuality through learned behavior is common in various animal species. Songbirds develop individually unique songs through vocal learning, influenced by genetic and environmental factors. Using F_1 hybrid songbirds as a model for generating behavioral variability, we investigate the developmental mechanisms underlying individuality in vocal learning. F_1 hybrids exhibit diverse acquired song qualities. Vocal acoustic biases emerge as individual differences among F_1 juveniles, regardless of auditory experience, starting from the vocal babbling stage and persisting throughout the vocal learning process. Individual differences in vocal acoustic variability are associated with specific transcriptional characteristics in glutamatergic projection neurons within the cortical vocal output nucleus. This suggests that a genetically predisposed vocal motor bias initiates individual variation in vocal learning.

Individuality is defined by stable behavioral and physiological differences among individuals of the same species (1, 2). A number of species, from insects to mammals including humans, show individual differences when learning a particular behavior (3–6). This plays a critical role in generating new and unique behavioral phenotypes, such as novel performance skills in primates and birds, subsequently leading to societal variations, such as dialects, during vertical cultural transmission (7–11). Compared with the research on individuality in innate behaviors at the anatomical and molecular levels (12–15), the neurodevelopmental mechanisms underlying individuality in learned behaviors remain elusive.

Birdsong is a learned vocal signal with inter- and intraspecies variations (16–18). Like human speech, birdsong plays a pivotal role in social communication and cultural transmission (7, 19–21). Songbirds learn acoustic elements (syllables) and temporal patterns (sequence) from conspecific adults during the sensitive period of sensory learning for recognition and memorization of the model song. Songbirds use the sensorimotor learning period to match their vocal outputs with the memorized model song (22–24). Song acquisition is considered a gradual development from subsongs, which generate highly variable syllables, to plastic songs characterized by the gradual inclusion of recognizable yet variable syllables, and finally to crystallized songs with an acoustically and sequentially stable set of syllables. In nature, juveniles hear numerous types of sounds during the sensory learning period, yet they display a learning preference biased toward conspecific songs relative to heterospecific songs and unrelated sounds (25, 26). In addition, when prevented from hearing conspecific songs during the sensitive period of song learning, juveniles can still develop individually unique songs with a certain degree of species specificity (27–29). These phenomena imply the existence of genetically predisposed learning biases during sensory and sensorimotor learning, resulting in the development of an idiosyncratic birdsong that characterizes species specificity (26, 30–35).

The advantage of using songbirds as a model system for studying the neural basis of vocal learning and development is that the song circuit, a highly specialized neural circuit for song learning and production, is well defined and conserved among species (*SI Appendix*, Fig. S1) (22, 36, 37). The song circuit comprises various interconnected forebrain song nuclei; it is subdivided into two pathways, the posterior vocal pathway and the anterior forebrain

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pathway (AFP). The posterior vocal pathway connects the premotor vocal nucleus (HVC) and the robust nucleus of the arcopallium (RA) to the brainstem vocal nuclei (38). During song production, HVC and RA regulate the production of syllable sequences and acoustics, respectively (39–41). The AFP, a homolog of the mammalian cortical–basal ganglia–thalamocortical circuit, connects the pallial (cortical) magnocellular nucleus of the anterior nidopallium (MAN) with the basal ganglia nucleus Area X and the dorsolateral nucleus of the medial thalamus (DLM) (42, 43). The AFP is a key neural substrate for vocal-motor learning and plays an integral role in generating vocal exploration and refining vocal performance using auditory feedback (44–48). However, how these conserved neural substrates can generate learned songs with species specificity and individual variability remains unclear.

To elucidate the neurodevelopmental mechanisms underlying individuality in vocal learning, we take advantage of the species-specific features of birdsongs as well as the individual differences in the capacity to imitate the songs of the respective conspecific adult. Thus, we utilize interspecific F_1 hybrid songbirds as a model to assess song development under the enhanced genetic heterogeneity resulting from the presence of two alleles, one from each parental species (Fig. 1*A*). We select the zebra finch (ZF; *Taeniopygia guttata*) and owl finch (OF; *Taeniopygia bichenovii*) as the parental species for generating an F_1 hybrid offspring because conspecific songs are subject to distinct constraints in these two species (Fig. 1*B* and *SI Appendix*, Fig. S1). In both species, only males possess vocal learning abilities. Vocal acoustic parameters, such as syllable duration, entropy variance, and mean FM, exhibit distinct species-specific traits between the two species (49). ZF males produce songs with a linear sequence comprising 3 to 6 unique syllables, termed motifs, whereas OF songs consist of a repetitive sequence of acoustically similar syllables. Our previous study reveals that F_1 hybrids crossing ZF with OF acquired individually unique songs, even though they are tutored with both ZF and OF songs (49).

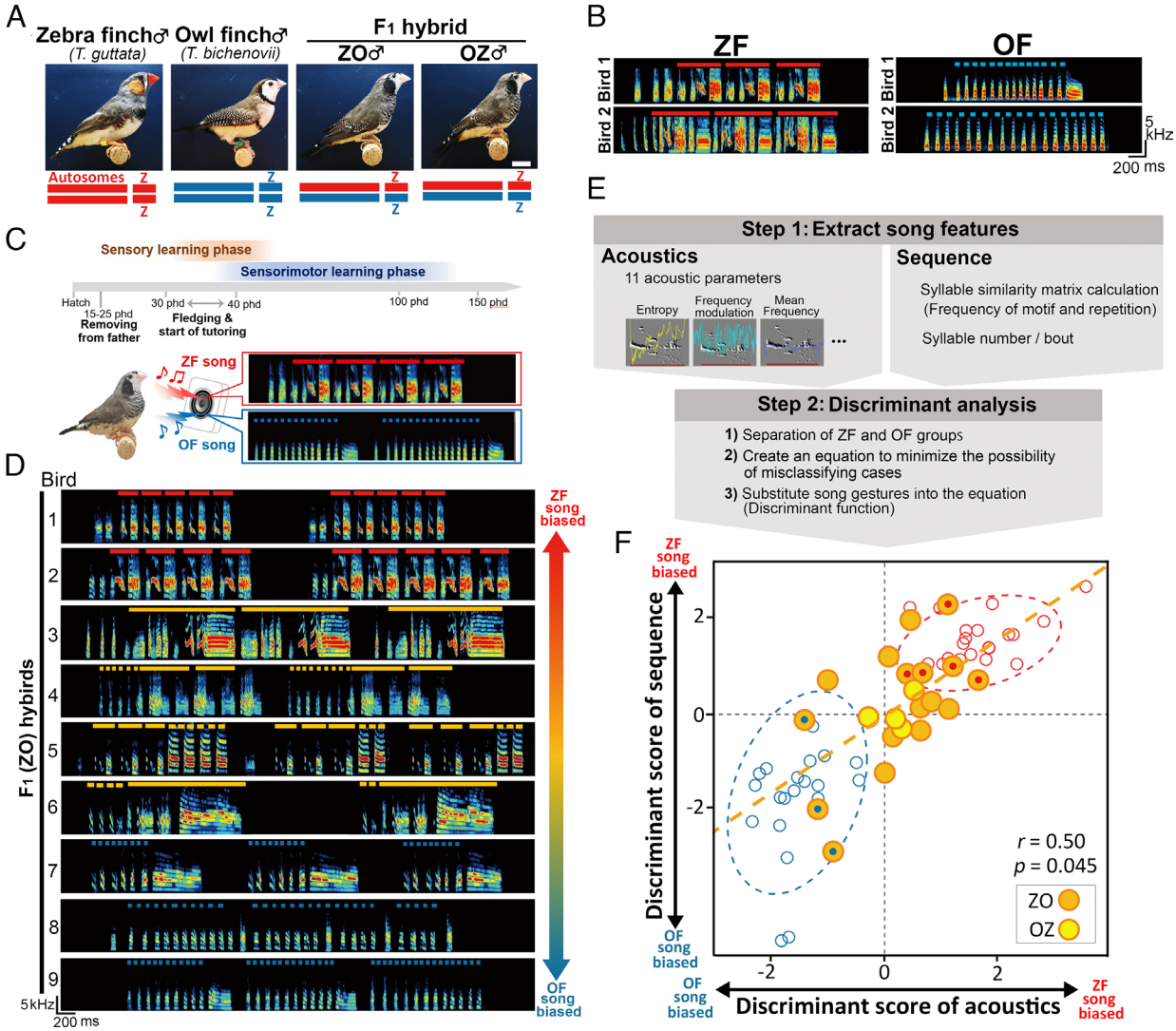

Fig. 1. Song idiosyncrasy of F_1 hybrids tutored using songs of both parental species. (*A*) Zebra finch (ZF), owl finch (OF), and F_1 hybrids (ZO from ZF♀ × OF♂ and OZ from OF♀ × ZF♂). Male ZO and OZ F_1 hybrids share identical autosomal- and sex chromosomes. (Scale bar, 1 cm.) (*B*) Typical examples of songs from ZFs and OFs. Red-solid and blue-dotted lines represent the motif and repetitive song structures, respectively. (*C*) Experimental timeline of song tutoring using two types of songs, conspecific to ZF and OF species, respectively. (*D*) Songs of adult F_1 hybrids (9-ZO bird example) tutored using ZF and OF songs. Motif and repetitive song structures are indicated as solid and dotted lines. Red-solid and blue-dotted lines represent song structures similar to ZF and OF tutor songs, respectively. (*E*) Schematic algorithm of discriminant analysis for assessing bias in song acoustics and sequence features specific to either parental species. (*F*) Discriminant scores of song acoustics and sequence for F_1 hybrids (orange- and yellow-filled circles; $n = 17$ ZO and $n = 4$ OZ hybrids, respectively), ZFs (red circles; $n = 20$), and OFs (blue circles; $n = 20$). Positive and negative values indicate biases for ZF and OF song features, respectively. Ellipses enclosed with red- and blue-dotted lines indicate a 95% probability of typical songs from ZFs and OFs. Orange-filled circles with red or blue dots show ZO hybrids characterized by ZF- or OF-biased songs, respectively.

In this study, we employ an integrating approach, combining experimentally controlled systematic song tutoring, unbiased discriminant analysis of song features, and single-nucleus RNA sequencing (snRNA-seq) to investigate the developmental learning process and neural substrates associated with individual differences in vocal learning. We find that vocal acoustic biases emerge as initial individual differences in songs among F_1 hybrids during the subsong state and persist throughout the sensitive period of song vocal learning. These biases independently arise regardless of exposure to the biological father's songs and passively tutored songs. SnRNA-seq profiling reveals unique transcriptional signatures in the glutamatergic projection neurons in the cortical vocal output nucleus RA, corresponding to individual differences in acoustic vocal biases.

Results

Individual Differences in Learned Songs in F_1 Hybrid Songbirds. We investigated how interspecies F_1 hybrids obtained from crossing ZF with OF developed their songs under a song tutoring environment based on the playback of recorded songs of both parental species after fledging (mean ± SD = 38.7 ± 5.9 phd) until 150 post-hatching days (phd) (Fig. 1*C*). For this song tutoring, tutor songs were presented passively with a total of 14 daily playback instances. Each song file consisted of both ZF and OF songs separated by a silent interval duration of 1.0 to 2.5 s (*SI Appendix*, Fig. S1 and *Materials and Methods*). In this study, we referred to F_1 hybrids from ZF♀ x OF♂ and from OF♀ x ZF♂ as ZO and OZ, respectively, although males of these F_1 hybrid songbirds share identical sets of auto- and sex chromosomes (Fig. 1*A*). Following fledging and the onset of song tutoring, F_1 hybrid males started producing subsongs, typically within 1 to 9 d. Like their parental species, by 150 phd, F_1 hybrids developed crystallized songs generated as stable song patterns through song renditions. We found that F_1 hybrid offspring developed a vast variety of songs ranging from ZF- to OF-like songs across individuals, including songs that contained a graded mix of acoustic and sequential features drawn from songs of both parental species (Fig. 1*D*).

We performed a linearized discriminant function analysis (LDA) based on the features of song acoustics and sequence using songs of both ZFs and OFs as the supervised model to determine the biases associated with each parental species' traits within F_1 hybrid songs (Fig. 1*E* and *SI Appendix*, Fig. S1). The appropriateness of the LDA was confirmed as significant differences in discriminant scores of songs from normally reared ZFs and OFs (P *** < 1.0×10^{-7} for both acoustics and sequence, Kruskal–Wallis test) (*SI Appendix*, Fig. S2). In addition, we found no difference in the discriminant scores of song acoustics and sequences between the reciprocal F_1 offspring (that is, ZOs versus OZs) (acoustics: $P = 0.53$; sequence: $P = 0.47$, Kruskal–Wallis test). Based on this result and due to the difficulty of breeding to obtain enough OZ hybrids, we pooled all F_1 hybrids for further analyses ($n = 21$, including 17 ZO and 4 OZ hybrids).

This LDA revealed that 61.9% of F_1 hybrids (n =13, including 9 ZOs and 4 OZs of the 21 birds) produced songs with merged traits from the parental species, plotted outside the 95% probability ranges of the two parental species' songs (Fig. 1*F*). The remaining 38.1% of F_1 individuals learned songs that were strongly biased toward song traits specific to one of the parental species (ZF-biased song learners: $n = 5$ ZOs and OF-biased song learners: $n = 3$ ZOs, shown as orange-filled circles with a red or blue dot in Fig. 1*F*). The presence of ZF-biased song learners among ZOs indicates that early exposure to their genetic father's songs (i.e., OF songs) before receiving song tutoring was not the primary factor influencing their individual biases during song learning (Fig. 1*F* and *SI Appendix*, Fig. S2). In addition, we found that the biases in the acoustic features were correlated with biases in the sequence features ($r = 0.50$, $P = 0.045$, Spearman's rank correlation using only F_1 hybrids' songs), indicating a consistent acquisition of these two song traits from tutored model songs.

Development of Individual Characteristics in F_1 Hybrid Bird Songs. To characterize the developmental timing of the individual variable traits observed in F_1 hybrid songs, we traced the ontogeny of parental species bias in their acoustic and sequence features. We observed that F_1 hybrids already showed individual differences in the vocal acoustic features of their subsongs, which are generated at the early stage of vocal learning (Fig. 2). For instance, F_1 hybrids that eventually acquired either ZF- or OF-like songs at the adult stage already produced syllables mainly consistent with ZF- or OF-like acoustics, respectively, during their subsong stage (3-ZO bird example shown in Fig. 2*A*). Furthermore, the individual differences in vocal acoustic biases persisted throughout the sensitive period of vocal learning (Fig. 2*B*). We observed the preservation of unique vocal acoustic biases over song development in the entire F_1 population, as captured by the significant correlation between juvenile subsong and adult crystallized song stages ($r = 0.80$, $P = 1.51 \times 10^{-5}$, Spearman's rank correlation) (Fig. 2*C*). In contrast to the acoustic features, apparent parental species biases in sequence features were not observed at the subsong stage. During the subsequent process of sensorimotor learning, although there was a trend indicating that sequence biases gradually became clearer, the degree of developmental changes in sequential biases varied among F_1 individuals (lower panel in Fig. 2*B*). Indeed, unlike the acoustic features, an analysis of sequence features did not reveal as strong an association between juvenile subsong and adult crystallized song stages ($r = 0.35$, $P = 0.12$, Spearman's rank correlation; Fig. 2*C*).

Predisposed Vocal Acoustics Bias. Next, we examined the extent to which the auditory experience of hearing tutored songs influences the individual characteristics of vocal acoustics biases in F_1 hybrid songs. To this end, we tutored F_1 hybrid juveniles by playing back only ZF or OF songs during the sensitive period of song learning, to test whether some F_1 hybrid individuals could still generate songs biased toward the acoustic characteristics of the nontutored species. Through this approach, we found that F_1 hybrid juveniles exclusively listening to the song of only a single-parental species still developed a wide variety of individually unique songs, including nontutored species traits, by the adult stage (Fig. 3 *A* and *B*). Through the analysis of the discriminant scores of song features, we found a learning effect on F_1 hybrids' songs of tutored song types, identified as significant differences in both acoustic and sequential features at the adult stage between the birds tutored using ZF or OF songs (**$P < 0.01$, Wilcoxon rank-sum exact test) (Fig. 3*C*). However, approximately half of the birds developed songs with unexpected biases based on the species specificity of the tutored songs (Fig. 3*B*, birds indicated by arrowheads). As prominent examples, some F_1 hybrids acquired songs with consistent biases in acoustics and sequence patterns contradictory to the species traits contained in both playback-tutored and biological father's songs (birds #3 and #6 in Fig. 3*A*). In addition, as observed during the two-parental species' songs tutoring (Fig. 2), F_1 hybrids tutored with songs conspecific to either of the two parental species developed individually unique vocal acoustic biases that emerged during the initiation of subsong production. These vocal characteristics were maintained throughout the vocal learning period (Fig. 3 *D*–*F* and *SI Appendix*, Fig. S3). In addition, no significant differences were

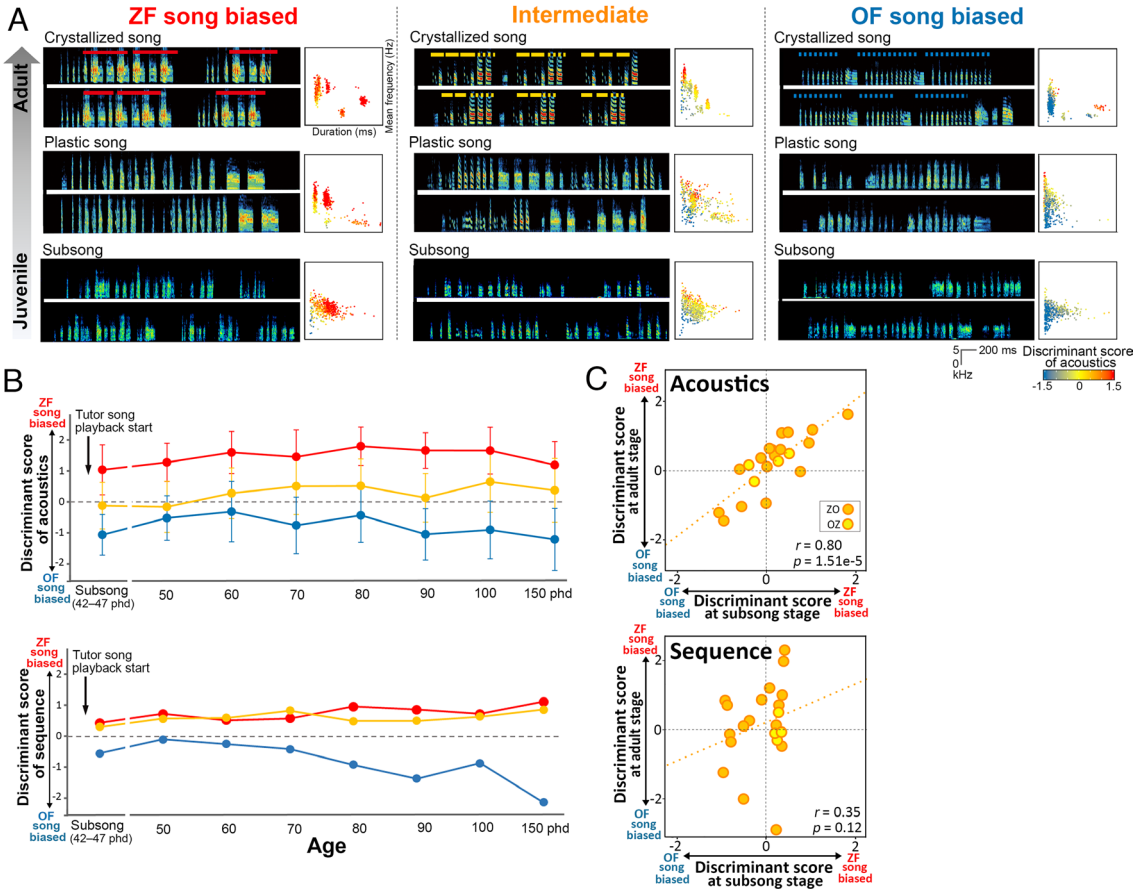

Fig. 2. Song development of F_1 hybrids tutored using songs of both parental species. (*A*) Examples of song development in three ZO hybrids tutored using songs of both parental species. Song spectrograms at the subsong, plastic song, and crystallized song stages of development (2 songs at each stage). Syllable scatter plots to the right of the spectrograms are based on 500 syllables (each depicted by a separate symbol), with symbol colors indicating the discriminant score of acoustic features (red and blue hues corresponding to ZF and OF biases, respectively). Motif and repetitive song structures are indicated as solid and dotted lines, respectively. (*B*) Developmental trajectory of the biases in song acoustics (*Upper* panel) and sequence (*Lower* panel) in the three ZO F_1 hybrids shown in panel *A*. Red, orange, and blue dots indicate ZO F_1 hybrids biased toward ZF, intermediate, and OF songs, respectively. Data plotted represent median ± SD of 500 syllables for discriminant scores of acoustics. Song playback tutoring started 3 to 6 d prior to the onset of subsong production. (*C*) Comparisons of song acoustics and sequences between early development (at the subsong stage) and the adult stage in F_1 hybrids tutored using songs of both parental species [orange- and yellow-filled circles; ZOs (n = 17) and OZs (n = 4), respectively].

observed in subsong acoustic biases between the two tutor groups (P = 0.0947, Wilcoxon rank sum exact test) (*SI Appendix*, Fig. S3). There was a developmental trend indicating that the content of the tutored songs reinforced the initial subsong acoustic bias throughout the sensorimotor learning phase, especially when the tutor songs shared the same species bias directions as the subsong of F_1 juveniles. Together, these results indicate that the emergence of individual differences in vocal acoustics biases was associated with the later development of idiosyncratic songs among F_1 hybrids, which was not predominantly determined by the auditory experience of hearing song contents.

A Neural Substrate of Vocal Acoustic Bias. We subsequently investigated structural, neural circuits, and genetic factors that could account for the vocal acoustic biases in F_1 hybrids. First, we conducted a structural analysis of the beak and the syrinx, the periphery vocal organs, to assess their relationship with individual variability in F_1 hybrid songs. This is because the structure of these periphery vocal organs could influence song properties (50–52). For each bird, we obtained three measures of beak morphology (height, depth, and width) and two of the syrinx (width and thickness of the syringeal muscle). All of these parameters were significantly smaller in the OF than in the ZF (*SI Appendix*, Fig. S4). However, we found no significant relationship between the structure of vocal organs and the parental species bias of the acoustic features of F_1 hybrid songs (*SI Appendix*, Fig. S4). This suggests that the observed variations in parental species bias observed within F_1 hybrid songs may not be linked to structural disparities in peripherical vocal organs.

Second, to elucidate the neural circuits presumed to generate vocal acoustic biases during development, we examined the potential contribution of the AFP to this process. The AFP is the primary neural circuit for subsong production with varying syllable duration in the ZF (53). However, HVC projection neurons also generate singing-related firing during the subsong stage (54). Thus, to test whether the AFP alone generates the biased tendency of acoustic features of syllables in subsong, we lesioned the bilateral HVC at the subsong stage of F_1 hybrid development (n = 5 ZOs) to rule out inputs from HVC to RA and Area X (Fig. 4*A*). We quantified the percentage of lesions in HVC per hemisphere to be 94%–100%, based on the decrease in immunohistochemical labeling using a neuronal marker NeuN antibody (Fig. 4*B*). We then found that the vocal acoustic bias was consistently maintained between pre- and post-HVC lesions ($^{n.s.}$ $P > 0.05$, Wilcoxon signed-rank test) (Fig. 4 *C* and *D*), indicating that the AFP without inputs from HVC was sufficient for generating the individual characteristics of the vocal acoustic bias at the subsong stage.

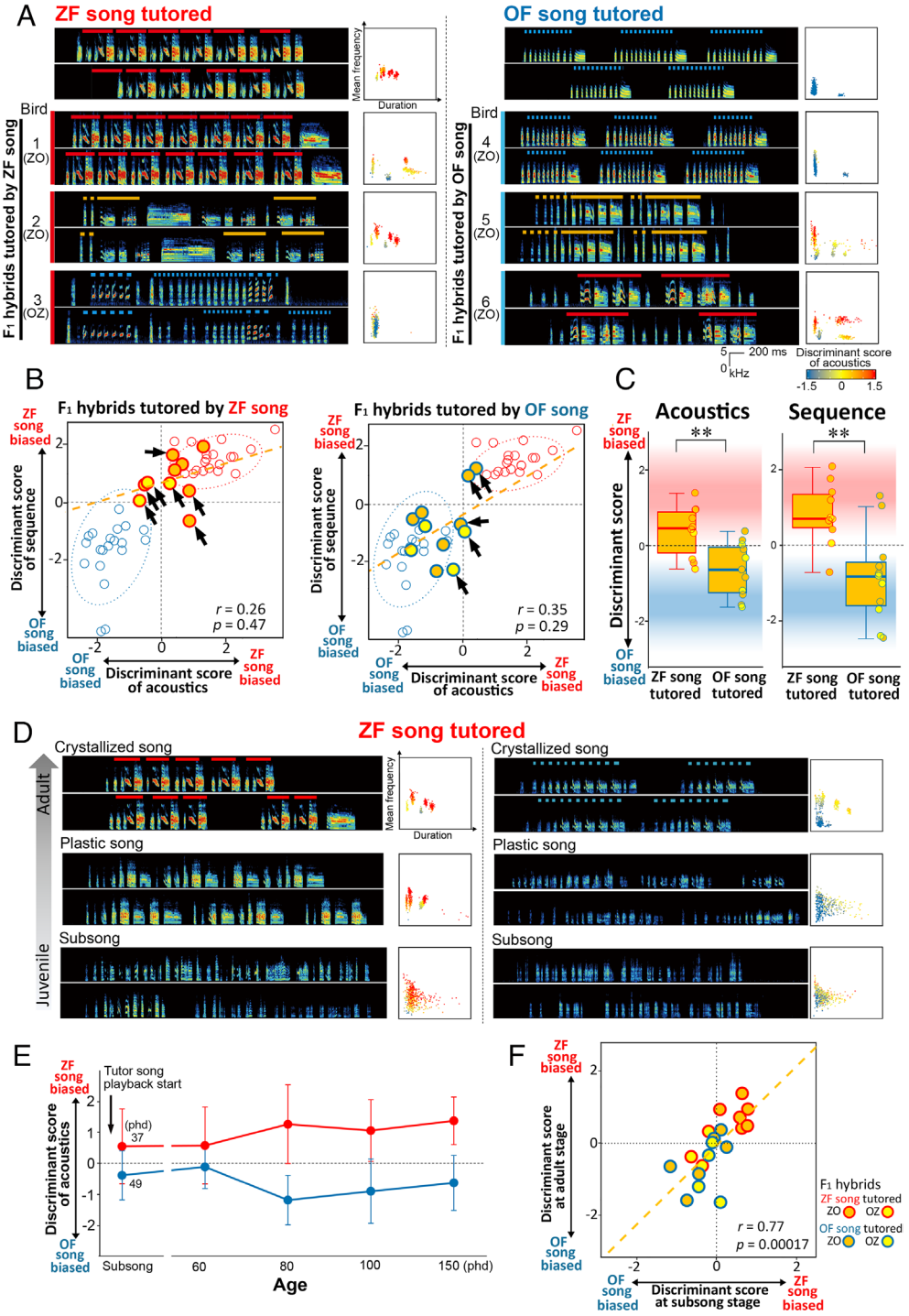

Fig. 3. Song features and development of F_1 hybrids tutored using the song conspecific to a single-parental species. (*A*) Examples of individual differences in the adult songs of F_1 hybrids tutored using ZF or OF songs (three birds each). Motif and repetitive song structures are shown as red-solid and blue-dotted lines above each spectrogram. Each scatterplot displays 500 syllables with a symbol color determined by the discriminant score of acoustic features (red and blue color according to ZF and OF biases, respectively). (*B*) Discriminant scores of song acoustics and sequence determined for F_1 hybrids tutored using (*Left*) ZF songs (n = 10 including 7 ZOs and 3 OZs, r = 0.26, P = 0.47) or (*Right*) OF songs (n = 11 including 7 ZOs and 4 OZs, r = 0.35, P = 0.29, Pearson correlation coefficient). All songs were recorded at the adult stage (>150 phd). Ellipses in red and blue broken lines indicate the 95% probability region of the song features conspecific to ZFs and OFs, respectively. Arrowheads indicate individual F_1 hybrids that developed a song deviating from (outside the 95% probability region of) the tutored song traits. Orange- and yellow-filled circles represent ZO and OZ hybrids, respectively. (*C*) Comparison of the discriminant scores of song acoustics and sequences between F_1 hybrids tutored using either ZF or OF songs (**P < 0.01, Wilcoxon rank-sum exact test). Shaded red and blue colored areas represent typical ranges (the 95% probability regions) for ZF and OF birds, respectively. (*D*) Two examples of song development by F_1 hybrids tutored using ZF songs alone, one (ZO pupil: *Left* panels) developing ZF-like biases and another (OZ pupil: *Right* panels) developing OF-like biases. (*E*) Developmental trajectory of the discriminant scores of song acoustics of the two F_1 individuals shown in panel *D*. Data plotted are median ± SD. Song playback tutoring started 1 to 3 d prior to the onset of subsong production. (*F*) Developmental correlations of song acoustic features between the subsong and adult stages in F_1 hybrids tutored with the song conspecific to a single-parental species (ZF song tutored: r = 0.91, P = 0.001; OF song tutored: r = 0.83, P = 0.003. Spearman's rank correlation).

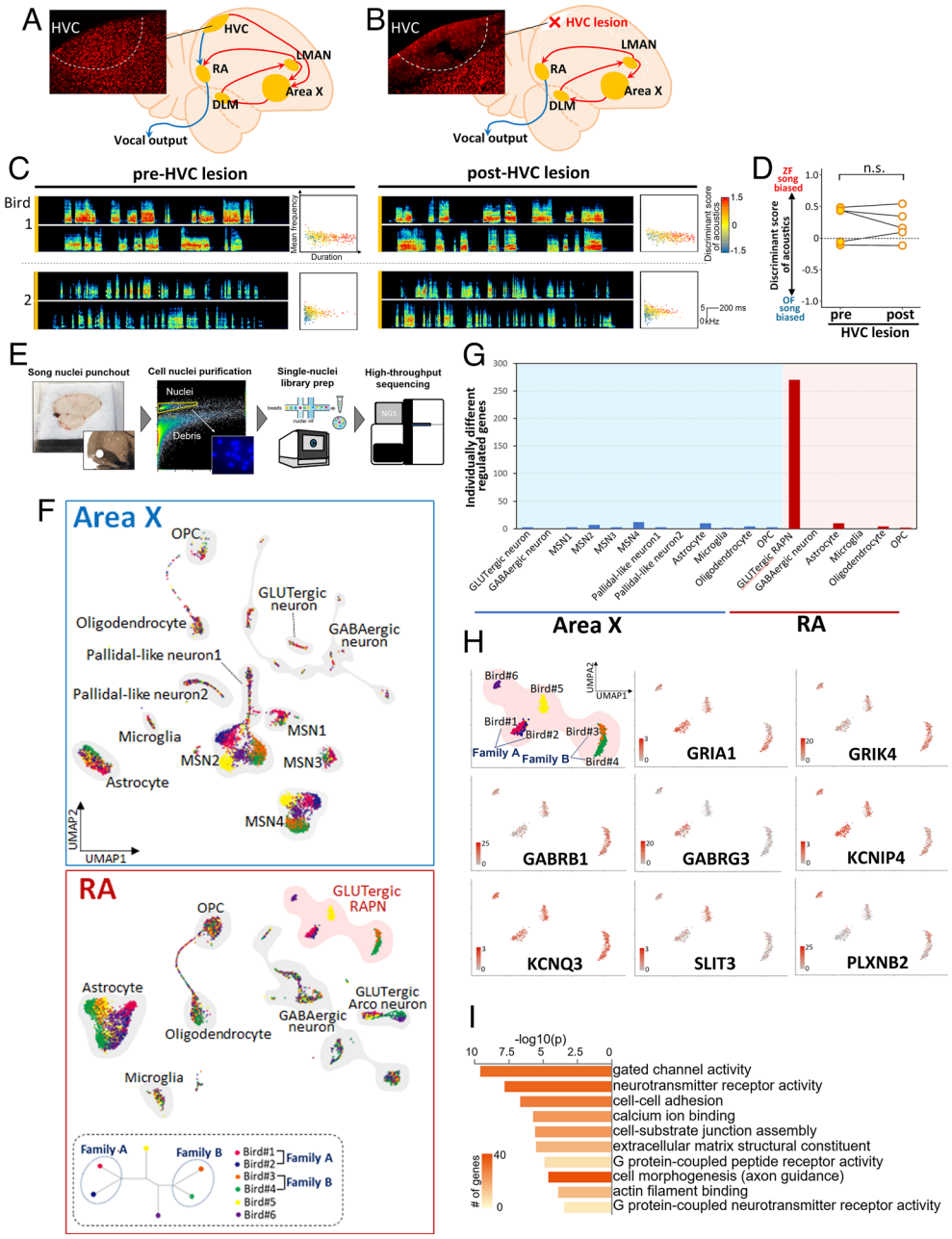

Fig. 4. Individual transcriptional signature differences at the initiation of vocal learning. (*A*) (*Right*) Schematics identifying the song nuclei, which play a role in vocal learning and production, and their connections in the songbird brain. Area X, Area X of the basal ganglia; DLM, dorsal lateral nucleus of the medial thalamus; HVC, used as a proper name, a vocal premotor nucleus; LMAN, the lateral magnocellular nucleus of the anterior nidopallium; RA, the robust nucleus of the arcopallium. The posterior vocal and anterior forebrain pathways (AFP) are shown as blue and red lines, respectively. (*Left*) An intact HVC stained with a neural marker NeuN antibody (red). (*B*) (*Right*) Remaining neural pathway connections after HVC lesion. (*Left*) Typical example of a chemical lesion in HVC stained with NeuN antibody staining (red). (*C*) Examples of subsongs and syllable scatter plots for two ZO F_1 hybrids before and after HVC lesions. Songs were recorded 1 d before and 2 to 3 d after lesions, indicated as pre- and postlesioned, respectively. (*D*) No change in song acoustics bias was observed after HVC lesion (n = 5 ZO hybrids; n.s., $P > 0.05$, Wilcoxon signed-rank test). (*E*) Experimental procedure for single-nucleus RNA sequencing (snRNA-seq). (*F*) Uniform manifold approximation and projection (UMAP) mapping of Area X and RA of F_1 hybrid juveniles at the initiation stage of song sensorimotor learning. Each color represents an F_1 hybrid individual (n = 6 ZO hybrids). RA glutamatergic (GLUTergic) projection neurons (red shading) indicate clear individual differences in their transcriptional signatures, but arcopallium (Arco) GLUTergic and other cell types did not. *Right* box: Genetic distance based on SNP information sharing rate. Birds #1 and #2 and birds #3 and #4 were siblings from two different families (families A and B, respectively). (*G*) Number of genes with individually different expressions in each cell type in Area X and RA. (*H*) Examples of the expression of individually different genes (GRIA1, GRIK4, GABRB1, GABRG3, KCNIP4, KCNQ3, SLIT3, and PLXNB2) in the glutamatergic projection neurons in RA. The analyzed cell number of RAPNs for each F_1 hybrid ranged from 73 to 217 cells/bird (mean ± SD = 151.8 ± 53.8 cells/bird). (*I*) Top 10 highly significant Gene Ontology (GO) terms identified through GO enrichment analysis of individually different expressed genes.

Individual Differences in Transcriptional Characteristics in the Vocal-motor Nucleus. The different behavioral phenotypes observed across individuals within a particular species or subfamily may be attributable to variations in gene expression levels and patterns of anatomically hardwired neural circuits (14, 55, 56). We thus investigated whether the transcriptional characteristics of neural cells contributed to determining the individual variations observed in the vocal acoustic biases of F_1 hybrid songs. To this end, we conducted single-nucleus RNA sequencing (snRNA-seq) in the basal ganglia nucleus Area X and the cortical vocal output nucleus RA of F_1 hybrids at the subsong stage (n = 6 ZOs)(Fig. 4*E*). We chose these two song nuclei, while excluding the smaller LMAN and DLM nuclei in the AFP, to ensure an adequate collection of cells from individual birds for conducting snRNA-seq.

Consistent with previous reports in ZFs (57, 58), we identified marker genes representing specific cell types within the song nuclei and identified Area X medium spiny neurons (MSNs), Area X pallidal-like neurons, RA glutamatergic projection neurons (RAPNs), GABAergic neurons, astrocytes, microglia, oligodendrocytes, oligo precursor cells, and RA surrounding arcopallium glutamatergic neurons (Fig. 4*F* and *SI Appendix*, Fig. S5). By examining single nucleotide polymorphism (SNP) variations in the transcripts of each cell, we associated the cellular transcripts with individual birds, and labeled these "cells" with a color scheme to match individuals in the uniform manifold approximation and projection (UMAP) plot (Fig. 4*F*). Multicolored UMAPs indicated a dispersed transcriptional distribution pattern, representing individual variations in gene expression within the glutamatergic projection neurons in RA (indicated by pink shaded area in Fig. 4 *F*, *Left*). In contrast, other cell types did not exhibit such differences between F_1 individuals in the UMAPs, although slight variations were observed in the transcriptional profiles of MSNs in Area X and astrocytes in RA. Of note, although our initial sampling did not specifically target sibling comparisons (one pair each from two different families), we unexpectedly found that adjacent F_1 individuals within RAPNs (cells colored with pink/dark blue or green/orange in Fig. 4 *F*, *Left*) were indeed sibling pairs originating from the same family. Brain tissues sampled at similar ages may exhibit a tight clustering of cells because the age difference between families A and B was larger (7 d) than between siblings of the same family (1 to 3 d). However, we realized that RAPNs from birds #2 and #5 from different families, killed on different days but at the same age, did not cluster closely in the UMAP (Fig. 4 *F* and *H*). This suggests that multiple factors, including familial genetic diversity and age, could shape unique transcriptional features in RAPNs among individuals.

Next, we identified genes with individually different expressions, defined as those showing significant differences in expression between at least one individual and the remaining birds (adjusted *p*-value by FDR < 0.05, Kruskal–Walls test). Notably, among the cell types in Area X and RA, RAPNs exhibited the highest number of genes with individually different expressions (270 genes) (Fig. 4*G*). These differently expressed genes in RAPNs showed a consistent expression trend among siblings from the same family, although exceptions were observed for certain genes (e.g., GABRG3 expression between birds #1 and #2 and PLXNB2 expression between birds #3 and #4 in Fig. 4*H*). 6.3% (17 genes out of the total 270) of the differently expressed genes in RAPNs were neurotransmitter receptors (e.g., GRIA1, GRIK1, GABAB1, GABRG3, and HTR1F) and ion channels (e.g., KCNIP4, KCNQ3, and KCNQ5). Additionally, 14 genes (5.2%) with different expressions at the individual level code for axon guidance (for instance, SLIT3, PLXNB2, SEMA5B, NTN1, and ROBO2) or cell adhesion molecules (for instance, CDH11, 12, and 18, and PCDH15). GO enrichment analysis further confirmed significant enrichment of GO categories related to neural functions, including gated channel and neurotransmitter receptor activities, cell–cell adhesion, and calcium ion binding among the genes with individually different expressions (Fig. 4*I*). These results indicate that the transcriptional features of RAPNs are distinct among individual F_1 hybrid juveniles but relatively similar within sibling pairs. Moreover, the expression of a specific set of individually different genes may be associated with neural excitability and circuit formation during the initial stage of vocal learning for song acquisition.

Association between Gene Modules and Vocal Acoustic Features. The finding that transcriptional characteristics representing individual variations and kinship within the specific cell types, such as RAPNs, inspired us to examine whether siblings from the same families exhibited similar vocal acoustic biases at the subsong stage. We assessed the resemblance of acoustic bias among subsongs produced by pairs of F_1 siblings from different families without exposing them to passive tutor model songs (n = 22 ZOs from 11 different families) (Fig. 5*A*). Consequently, we found a significant similarity in vocal acoustic biases within these songs between siblings originating from the same families (r = 0.964, P = 0.0023, Spearman's rank correlation) (Fig. 5*B*). In addition, individual (familial) differences in vocal acoustic biases persisted even in the absence of song tutoring experience, suggesting the existence of heritable vocal acoustic biases. These results support the idea that there is a potential link between individual differences in transcriptional features of RAPNs and vocal acoustic biases at the initial stage of song sensorimotor learning.

The neurophysiological importance of RAPNs resides in their involvement in controlling vocal activity by regulating the brainstem regions responsible for maintaining the contraction of the syringeal and respiratory muscles during vocalization (59, 60) (*SI Appendix*, Fig. S1). Thus, we further investigated a potential association between vocal acoustic features and the transcriptional characteristics within RAPNs at the subsong stage, using a cohort of 6 ZO F_1 hybrids. To accomplish this, we performed a single-cell weighted gene coexpression network analysis (scWGCNA) of transcripts encompassing the individually different genes and identified 8 Gene Modules as coexpressed gene clusters within metacells representing multiple RAPNs from individual birds (Fig. 5 *C* and *D*) (61). Subsequently, employing the identified Gene Modules, we conducted a correlation analysis between the average of Module Eigengene of each Gene Module and the discriminant score of vocal acoustics, along with 11 associated acoustic parameters. As a result, we found that 3 out of the 8 Gene Modules (Modules 1, 2, and 7) exhibited significant correlations with the discriminant scores of vocal acoustics (Fig. 5 *D*–*F*). Moreover, 6 of the 11 acoustic parameters utilized for calculating vocal acoustic bias correlated with at least one of the 8 Gene Modules. However, no Gene Modules correlated with the discriminant score of vocal acoustics in GABAergic neurons in RA (*SI Appendix*, Fig. S6). Furthermore, although we identified seven subtypes of GABAergic neurons and two subtypes of astrocytes in RA, as well as four subtypes of MSNs in Area X, only one significant correlation was found between one Gene Module #5 in RA astrocyte subtype 1 and the discriminant score of acoustics (*SI Appendix*, Figs. S7 and S8). These results suggest the possibility that Gene Modules in other cell types (subtypes) in the AFP song nuclei may also contribute to generating vocal acoustic bias; however, RAPNs could still be a major cell type associated with vocal acoustic biases.

Discussion

Vocal learning in songbirds and humans develops through sound perception, memorization, and vocal-motor practice (22). Predisposed learning biases can influence at any point in these processes, generating inter- and intraspecies variation in learned vocalization (25, 26, 32, 37). However, how and when these individual differences in learning bias arise is not fully elucidated. Our results indicate that a vocal production bias is one of the origins of individuality in vocal learning. Furthermore, we found that the cortical-basal ganglia circuits generated vocal acoustic bias in the absence of HVC inputs, and this process was associated with individually unique transcriptional characteristics of glutamatergic RAPNs and potentially other cell types in the AFP. Vocal babbling is an early motor behavior generated by primate and bird juveniles (22, 62, 63). Although subsong singing is considered a highly variable unstructured

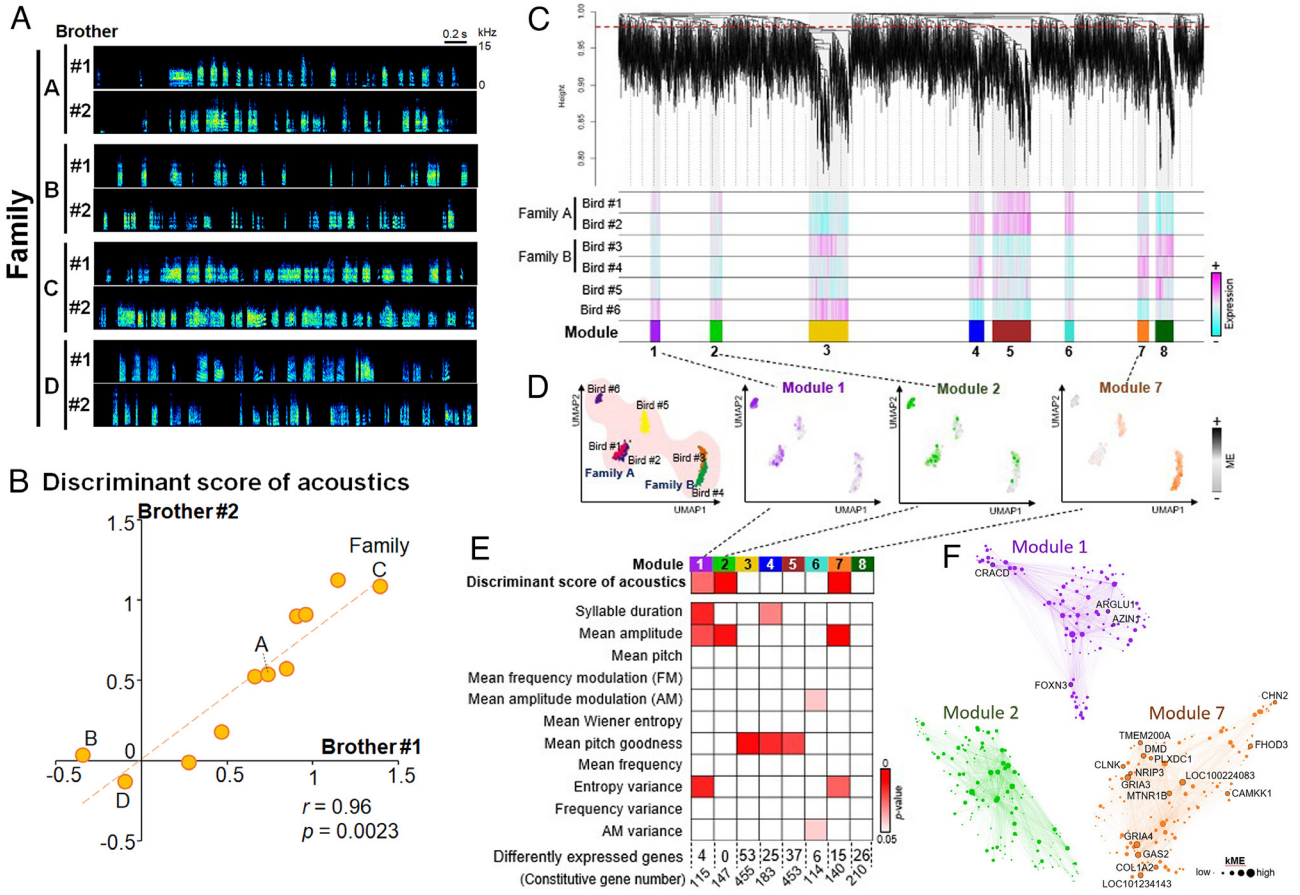

Fig. 5. Gene coexpression network analysis reveals correlations between gene modules and vocal acoustic features. (*A*) Examples of subsongs generated by a pair of ZO F_1 hybrid juvenile brothers from four families (n = 8 birds). (*B*) Correlation of vocal acoustic biases between ZO F_1 hybrid siblings (11 families; r = 0.96, P = 0.0023, Spearman's rank correlation). The birds from families A–D are the ones illustrated in panel A. The initial subsongs were produced at an average age of 43.4 ± 4.8 phd. (*C*) The dendrogram generated through average linkage hierarchical clustering of genes identifies 8 Gene Modules of coexpressed genes in RAPNs. The pink- and blue-colored bands indicate positive and negative correlations, respectively, with the expression levels in individual ZO F_1 hybrids. The red line in the dendrograms indicates the height at which the tree was cut. (*D*) Representative UMAPs depicting Gene Modules 1, 2, and 7. (*E*) Heat map displaying the correlations between Gene Modules and vocal acoustic features. The numeric values at the bottom indicate the number of genes with individually different expressions in each Gene Module. (*F*) Coexpression relationships among individually different expressed genes (labeled with their gene names) and the remaining genes in Gene Modules 1, 2, and 7 of RAPNs in ZO F_1 hybrids. The individually different genes were also included within the top 30 genes based on the highest module membership (kME).

vocalization observed at the initial stage of sensorimotor learning (53), we found that vocal production bias was subsumed in the acoustic feature of subsongs and varied widely across F_1 individuals (Fig. 2). When dividing F_1 hybrids into ZO and OZ groups, we did not observe an evident influence of cross-breeding genotype or early auditory experience with the paternal species' songs on determining individual variations in vocal acoustic biases (Figs. 1 and 3, and *SI Appendix*, Fig. S1). Rather, the emergence of the vocal acoustic bias could be largely genetically predisposed.

Currently, the neural substrates regulating individual differences in learned behaviors remain unknown, especially at the cellular transcriptional level. Here, we revealed that input from the premotor nucleus HVC to the AFP is unnecessary for generating individual variations in subsong acoustic biases. Instead, a particular transcriptional profile in glutamatergic RAPNs was associated with individual differences in the vocal acoustics biases. The latter finding is intriguing, as RAPNs in songbirds are analogous to the human cortical layer V projection neurons sitting within the laryngeal motor region of the brain (64). Moreover, the RA is a crucial area, responsible for syllable production (40, 41, 65). RAPNs topographically project to the brainstem: The dorsal RAPNs connect to the expiratory- or inspiratory-regulating neurons in the medullary respiratory regions, whereas the ventral RAPNs extend to the tracheosyringeal hypoglossal nucleus for mediating the coordination of vocal and respiratory muscle activity during singing (59, 60). Our study identified axon guidance and cell adhesion molecules as the functions of prominent genes showing individual variation among transcripts of RAPNs (Fig. 4). Thus, individual differences in the expression of genes related to circuit formation may contribute to the development and maintenance of individually unique RA–brainstem connections. Furthermore, individual variability in the expressions of neurotransmitter/neuromodulator receptors and ion channels in anatomically hardwired and conserved circuits could also contribute to individual differences in neural circuitry excitability (14, 55, 66, 67). Accordingly, receptors for glutamate, GABA, serotonin, acetylcholine, and ion channels were differently expressed in RAPNs of F_1 hybrids. In the ZF, intrinsic neuronal properties mediated by ion channels of HVC neurons projecting to Area X are functionally associated with song learning (68). Thus, transcriptional differences among F_1 individuals are likely regulators of the different excitatory properties of glutamatergic RAPNs in each bird. Moreover, transmission from RAPNs to the targeted sites in the brainstem could, in turn, contribute to generating individually unique vocal acoustic biases.

Despite the challenges in identifying the mechanisms underlying familial clustering based on transcriptional similarity in specific cell types like RAPNs, the observed segregation of transcriptional

profiles by families might have been influenced not only by genetic similarity within families but also by differences in age and other unknown factors. In addition, considering other possibilities, the observed individual differences in the transcriptional features of RAPNs may not be the direct cause of generating a variety of vocal acoustic biases. This could be supported as a Gene Module of astrocyte subtype 1 in RA also correlated with vocal acoustic biases (*SI Appendix*, Fig. S7). Moreover, critical sites for generating subsongs with highly variable vocal acoustics include LMAN and DLM in the AFP (53, 69). Hence, it is essential to explore the possibility that other cell types in different AFP song nuclei could exhibit transcriptional differences among families and individuals, potentially influencing vocal acoustic biases. Further research is necessary to thoroughly examine these hypotheses utilizing in vivo gene manipulation techniques, despite the existing technical challenges in manipulating multiple genes in a specific cell type. Also, selective cell type–specific cell ablation for the candidate cell types in F_1 hybrids could be a realizable procedure to elucidate neural mechanisms underlying vocal acoustic biases (70).

Future research on snRNA-seq and scWGCNA using song nuclei from parental species will provide valuable insights into understanding the relationship between transcriptional states of specific cell types and vocal acoustic biases. Analyzing the transcriptional distribution patterns of RAPNs in ZFs and OFs will be crucial. If the RAPN distributions of ZFs, OFs, and F_1 hybrids in UMAP overlap each other, it suggests that the variations in RAPN transcriptional features among F_1 hybrids do not directly correspond to species-typical acoustic features. Conversely, if the RAPN distributions of ZFs and OFs are separated into different cell clusters and do not overlap with the RAPN clusters of F_1 hybrids, which may be located in an intermediate area; it further supports a potential association between vocal acoustic features and the transcriptional characteristics within RAPNs. In this scenario, we speculate that using scWGCNA with transcriptional data from F_1 hybrids and both parental species will help identify more specific Gene Modules associated with vocal acoustic biases and other acoustic parameters.

Nature via nurture integration has been proposed as a basis for individuality in learned behaviors (71). In the tutoring experiment using songs from the single-parental species (Fig. 3), we observed that some F_1 individuals efficiently learned tutor songs, whereas others developed unique songs structured with untutored cross-parental species traits. Notably, when we compared songs from F_1 pupils as groups tutored with only ZF or OF songs, we found a clear tutoring effect observed as a significant difference in the average measure of song learning tendencies in the adult stage (Fig. 3*C*). This result is reminiscent of the educational effects of schooling in humans, such as the relationship between teacher efficacy and student achievement. Even when educated by the same teacher, students in a classroom usually differ in their academic performance. Simultaneously, the average grades between classes vary depending on the teachers (72, 73). However, the biological understanding of such learning tendencies affecting individual and group average differences in schooling is still challenging, despite the recent identification of specific genomic loci associated with educational attainment (3, 74–76). As humans, songbird species show genetic heterogeneity. Interspecies hybridization further expands genetic heterogeneity, which could increase the phenotypic variation of their parental species (77–80), although, in some cases, genetic incompatibilities in F_1 hybrids can produce variants with cognitive impairments (81). Thus, interspecies F_1 hybrid songbirds could be a potential model system informing this research field for biological individuality at the molecular and neural circuit levels, and could also develop insights into the educational/tutoring impacts of both genetics and the environment on personalized learning.

Materials and Methods

Animals. F_1 hybrid chicks were raised by both parents in breeding cages until 10 to 20 phd, and then, the father was removed by 15 to 25 phd from the cage to prevent juveniles from listening to their father's song. After observation of fledging and independent feeding, F_1 hybrid juveniles were individually housed in sound-attenuation boxes. See *SI Appendix* for details.

Song Tutoring and Recording. Song playback tutoring started on the same day we observed the fledging of juvenile hybrids (mean ± SD = 38.7 ± 5.9 phd) and continued until 150 phd. Birds did not produce subsongs before fledging, as confirmed by continuous 24-h recordings following the removal of the father bird. Juvenile hybrids were tutored under one of three conditions: two parental species tutoring of ZF and OF songs, single species tutoring with ZF songs, or with OF songs. For two parental species tutoring of ZF and OF songs, 17 ZOs from 8 families and 4 OZs from 2 families were used. For the single species tutoring with ZF songs, 7 ZOs from 3 families and 3 OZs from 2 families were utilized as the pupils. Similarly, 7 ZOs from 3 families and 4 OZs from 2 families were applied for the single species tutoring with OF songs. For each song tutoring condition, a total of 14 times of song playback were scheduled each day, with 7 times in the morning (8 AM to 12 PM) and 7 in the afternoon (1 PM to 6 PM). The tutor songs were played passively with an onset probability of 0.0025/s and intervals of more than 20 s using Sound Analysis Pro version 1.04. A song file was randomly selected from 3 to 5 stocked files spanning 6.0 to 11.8 s duration (*SI Appendix*, Fig. S1) and played back at 55 to 75 dB from a speaker (SRS-M30, SONY). A pair of ZO siblings from 11 families (n = 22 birds) were kept isolated after fledging to record their subsongs without hearing experience of model songs. See *SI Appendix* for details.

Song Analysis. Songs files in a day were randomly selected to obtain a total of 500 syllables for analysis at each developmental time point per bird. Song developmental stages of producing subsongs and plastic songs were confirmed by the probability density distribution of syllable duration in songs (53, 82).

Subsongs were analyzed using song files produced within 3 d after the onset of subsong production (82). Crystallized songs were used from songs produced at over 150 phd. Analysis of acoustic features of syllables was performed using Sound Analysis Pro (83). Eleven phonological parameters were measured; syllable duration, mean amplitude, mean pitch, mean frequency modulation (FM), mean amplitude modulation square (AM^2), mean Wiener entropy, mean pitch goodness, mean frequency, entropy variance, frequency variance, and AM variance. Three song structural parameters were measured to analyze the song sequence: motif and repetition indexes and the number of syllables in a song bout. The motif and repetition indexes were calculated as syllable transition type I and II, respectively, by the song similarity matrix (SSM) method (49, 70, 84). The number of syllables in a song bout was calculated as the mean value of the number of syllables in 25 song bouts of an individual bird. Linearized discriminant function analyses (LDA) were performed to reduce the eleven-dimensional features of syllable acoustics and the three-dimensional features of syllable sequences to a single dimension, respectively. Two linearized equations called the discriminant function give the best distinction between the species-specific song traits of the parental species ZF and OF. The two discriminant functions were derived using songs of each 10 birds of parental species. The discriminant scores of ZF and OF songs were given positive and negative weights in these discriminant functions. The LDA was performed at each developmental time point using the two linearized equations to separate ZF and OF. The discriminant scores of the song acoustics were shown as the median for 500 syllables for each developmental time point. See *SI Appendix* for details.

Bilateral Lesions of HVC. Juvenile ZO F_1 hybrids (n = 5, mean ± SD = 37.8 ± 2.9 phd) were first recorded for their subsongs for 1 to 3 d and then performed bilateral lesions of HVC. For surgery, birds were anesthetized with isoflurane and injected 120 nL/hemisphere of 1 % ibotenic acid dissolved in 1 M NaCl using a Nanoject 2 injector (Drummond Scientific). For the evaluation of lesion % of HVC, standard immunohistochemistry was performed using NeuN antibody (GeneTex, GTX132974). See *SI Appendix* for details.

Single-Nuclei RNA Sequencing (snRNA-seq). The brains of male juvenile ZO F_1 hybrids were used for snRNA-seq experiment (each $n = 6$ birds for Area X and RA, 34 to 49 phd: mean ± SD = 43 ± 6.0 phd). Brothers from one family were killed on different days with a 3-d interval, while brothers from another were killed on the same day. The age difference between brothers within the same family ranged from 1 to 3 d, while the age gap between different families was 7 d. The bird was placed in a sound-attenuating box overnight under silent and dark conditions. The next morning before light onset, brain tissues were sampled from birds in dark, silent, and nonsinging conditions and embedded in OCT Compound (Sakura Finetek Japan) on powder dry ice. Frozen brain sections were cut at a thickness of 300-μm in the sagittal plane with a cryostat microtome (Leica Biosystems). Area X and RA were punched out with Miltex Biopsy Punch (0.5 to 1.0 mm diameter; Ted Pella Inc.) and stored at −80 °C until nuclei isolation. Tissue-punching procedures were performed on each bird individually on separate days. The punched tissues from different birds were mixed based on their respective song nuclei regions into single tubes, homogenized in 750 μL of ice-cold Nuclei PURE Lysis Buffer, centrifuged at 500× *g* for 10 min at 4 °C, and washed with 1 mL of Nuclei Wash and Resuspension Buffers. After centrifugation, the nuclei were suspended in 120 μL of Nuclei Wash and Resuspension Buffers with DAPI and filtered with 40 μm cell strainers. Isolated cell nuclei were purified with a cell sorter (SH800, SONY) using DAPI fluorescence. According to the manufacturer's protocol, the 10× Chromium libraries were prepared using Chromium NEXT GEM Single Cell Library Kit v3.1 (PN-1000269, 10× Genomics). GEM formation was conducted using one 10x chip well for each brain region. For identifying transcripts from each bird, a singularity container souporcell was used for cell demultiplexing by an individual (85). See *SI Appendix* for details.

Cell Cluster Analysis. The R package Seurat v.4 was used for data filtering and analyses (86). Filtering criteria was applied to the data, using "CreateSeuratObject," included min.cells = 3 and min.features = 200. After filtering, a total of 7,311 and 7,466 cells in Area X and RA, respectively, were left for further analysis. Principal component analysis was performed using the top 2,000 variable genes. UMAP was performed on 37 principal components for visualizing the cells. The Kruskal–Wallis test was performed to identify the genes with individually different expressions at each cell type with normalized expression values. For calculation of the number of genes with individually different expressions, the number of cells in each cell type was kept uniform to avoid the influence of differences in cell number among cell types on statistical power (15 cells per individual were randomly selected). Individually different genes were defined as corrected *P*-value less than 0.05. See *SI Appendix* for details.

Single-Cell Weighted Gene Coexpression Network Analysis (scWGCNA). Metacells were constructed by grouping together cells with similar gene expression patterns. During metacell computation, we pooled cells belonging to the same individuals to retain this metadata for scWGCNA. WGCNA identifies modules of densely interconnected genes by hierarchical clustering based on the topological overlap, a biologically meaningful measure of similarity of expression patterns among all pairs of genes across all individuals, and by assigning each gene to a "Module" based on shared expression patterns. The first principal component of each Module, referred to as the Module Eigengene (ME), was computed across all cells of glutamatergic RAPNs and RA GABAergic neurons, respectively. To investigate the association between variabilities of the expression within the Gene Modules and vocal acoustics, correlations were computed between the average ME per individual for each Module and the corresponding trait, and *P*-values were calculated for each correlation. See *SI Appendix* for details.

GO Analysis. GO analysis for individually-different genes in RAPNs was performed using Metascape (https://metascape.org/gp/index.html). The enrichment of GO terms associated with 270 individually different genes in RAPNs was statistically calculated. The output terms with redundant elements were clustered by Metascape's algorithm, and the smallest *P*-value of the terms in each cluster was taken as the representative *p*-value of the cluster.

Data, Materials, and Software Availability. Single-cell RNA-seq data are available in the Gene Expression Omnibus for Area X (GEO accession No. GSE217340) (87) and RA (GEO accession No. GSE217341) (88).

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