Dynamic transcriptome landscape in the song nucleus HVC between juvenile and adult zebra finches

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Abstract
Male juvenile zebra finches learn to sing by imitating songs of adult males early in life. The development of the song control circuit and song learning and maturation are highly intertwined processes, involving gene expression, neurogenesis, circuit formation, synaptic modification, and sensory-motor learning. To better understand the genetic and genomic mechanisms underlying these events, we used RNA-Seq to examine genome-wide transcriptomes in the song control nucleus HVC of male juvenile (45 d) and adult (100 d) zebra finches. We report that gene groups related to axon guidance, RNA processing, lipid metabolism, and mitochondrial functions show enriched expression in juvenile HVC compared to the rest of the brain. As juveniles mature into adulthood, massive gene expression changes occur. Expression of genes related to amino acid metabolism, cell cycle, and mitochondrial function is reduced, accompanied by increased and enriched expression of genes with synaptic functions, including genes related to G-protein signaling, neurotransmitter receptors, transport of small molecules, and potassium channels. Unexpectedly, a group of genes with immune system functions is also developmentally regulated, suggesting potential roles in the development and functions of HVC. These data will serve as a rich resource for investigations into the development and function of a neural circuit that controls vocal behavior.

KEYWORDS
circuit development, gene expression, HVC, RNA-Seq, vocal communication, zebra finch

1 | INTRODUCTION

Zebra finches (Taeniopygia guttata) use songs and calls to communicate with members of their species. Male juvenile zebra finches learn to sing during a developmentally restricted period early in life by imitating songs of conspecific adult males. Typically, juveniles begin to vocalize at around 30 days of age (30 d). Initially, their songs are highly variable. Through a sensory-motor learning process that spans about 2 months, juveniles hear an adult tutor’s song, practice singing, and use auditory feedback to match their own vocal output with the tutor song. By 90 days of age, male zebra finches reach sexual maturity, and their song matures into a stereotyped adult song. This newly acquired adult song, although displaying some variability, resembles the tutor song, and the bird sings this adult song throughout life.2-6

Zhimin Shi, Zeyu Zhang, and Lana Schaffer contributed equally to the work.

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Song behavior is controlled by a group of interconnected brain nuclei commonly referred to as the song system (Figure 1A). The song system consists of two distinct pathways: the motor pathway, which controls song production, and the anterior forebrain pathway (AFP), which is necessary for song learning.7-10 HVC is a cortical nucleus at the junction of these two pathways.11-13 Neurophysiological, lesion, and imaging studies suggest that HVC encodes the spectral features and temporal patterns of a song.10,14-17 It is also known that before 45 days of age, when juveniles sing a poorly structured subsong, HVC is not required for singing, and singing is controlled by the anterior forebrain nucleus lMAN. After 45 d, song control gradually transfers from lMAN to HVC.16 The process of shifting functional dominance is accompanied by structural development in HVC. HVC begins to establish functional synapses with its downstream nucleus RA at about 35 d,18 and the volume of HVC and the number of neurons in HVC continue to increase19,20 in parallel with changes in electrophysiological firing properties of HVC neurons.5

In recent years, applying molecular and genomics tools such as in situ hybridization and cDNA microarrays to birdsong research, progress has been made in understanding gene expression programs relevant to gender, age, brain regions, learning experiences, and song behavior.21-30 However, the dynamic transcriptional landscapes in HVC that enable its structural and functional changes as juveniles mature to adulthood remain enigmatic. In this study, we profiled transcriptomes in the HVC of male zebra finches using RNA-Seq; we focused on two age groups: 45 d, when motor control of song begins to transfer to HVC, and juveniles sing a highly variable song; and 100 d, when song learning is complete, and the now adult zebra finches sing a mature adult song.3,4,16 Using a combination of bioinformatics analysis and experimental validation, we identified gene repertoires that define molecular signatures of HVC at 45 d and 100 d. We also describe the dynamic changes in gene expression that occur in HVC as zebra finches mature. These results provide unique insights into the transcriptional landscape underlying the development and functional maturation of the neural circuit for vocal communication.

2 | RESULTS

2.1 | Profiling transcriptomes in HVC by RNA-Seq

To survey gene expression and transcriptome changes in HVC during song development, we collected brain tissues of 45 d and 100 d male zebra finches obtained from our breeding colony. Juveniles were reared with their parents in breeding cages until the collection time. The newly adult birds were separated from their parents at around 60 to 70 d, and subsequently kept in group cages until brain collection. It is known that moment-to-moment sensory-motor experiences, such as singing or hearing songs, induce changes in gene expression in song related brain regions.31,32 To obtain a gene expression profile in HVC at basal levels that reflects developmental stages, we monitored the birds in the morning for one hour prior to brain collection to verify that they did not sing and did not hear songs. We dissected out HVC tissue from sagittal brain sections under a dissecting microscope. We isolated total RNAs, and pooled RNA samples from HVC tissues of four animals for making each cDNA library (see the methods section for details). To identify genes with enriched expression in HVC relative to the rest of the brain, we also made cDNA libraries from whole brain tissue (WB) of male zebra finches at 45 d and 100 d. We sequenced these cDNA libraries using the Illumina GAII platform, which produced over 30 million raw sequence reads of 79 nt per library.
After filtering and trimming adaptor sequences, we obtained a total of 150 million high quality sequence reads from all libraries combined. We mapped these reads to the zebra finch genome assembly (3.2.4/taeGut1) using the Eland (Illumina) software package. Typically, we obtained 25-30 million high quality sequence reads for each library; among them, 50% to 60% were mapped to the zebra finch genome. About 15% to 25% of the mapped reads were mapped to exons of annotated genes, while the remaining reads were mapped to intronic, intergenic regions, and/or regions without annotation. Altogether, mapped genes represent about 50% of the total number of annotated zebra finch genes. The general characteristics of library samples, sequencing, and mapping results, including the read counts of each library, their mapping rates to the genome, and the number of genes covered by each sequenced sample, are summarized in Table S1.

### 2.2 RNA-Seq analysis reveals distinct gene expression patterns in juvenile and adult HVC

We first compared gene expression profiles in HVC with those in the whole brain samples, which allowed us to identify a large number of genes with enriched expression in the HVC of juvenile and adult finches. Using a cutoff of $q < 0.1$ (FDR-adjusted $p$-value), at 45 d, 1577 genes were differentially expressed in HVC relative to the whole-brain sample, and at 100 d, 811 genes were differentially expressed. Among these, less than half, 659 and 365 genes, respectively, showed enriched expression in HVC in juveniles and adults (Figure 1B and Data S1 and Data S2). These HVC gene expression patterns, distinct from the average gene expression in the whole brain tissue, define the transcriptional programs in the HVC at 45 d or 100 d. The larger number of genes with enriched expression in HVC at 45 d indicates higher transcriptional activities at 45 d, and suggests that gene expression at 45 d is not synchronized with the rest of the brain. The asynchronicity and transcriptional activity in HVC are gradually reduced as birds mature to adulthood, suggesting that chronological age plays a determinative role in regulation of gene expression in HVC.

The enriched expression of many genes in adult HVC, especially those expressed at high levels (e.g., ALDH1A2, CCNB, CADPS2, CRHB, GLAR2, MUSTN1, NTS, PVBL, RELN, etc.), have been reported previously using various experimental platforms including differential display, cDNA microarray, and/or in situ hybridization, supporting the robustness of the present study. We focused on a few genes showing enriched expression in 45 d HVC, and performed in situ hybridization to verify their expression. Consistent with the RNA-Seq results, in situ hybridization revealed enriched expression of ISG12, LDB3, LY6E2, and THBS4 in HVC compared to other brain regions at 45 d (Figure 1C). These results further show the suitability of our dissection method for isolating HVC-specific tissue. The in situ hybridization results also revealed gene expression patterns in areas in addition to HVC. For example, on a sagittal brain section, ISG12, LDB3, and THBS4 also showed enriched expression in RA, a song nucleus acting downstream from HVC, which, together with HVC, controls song related motor activities. These expression patterns suggest that gene expression in the song control circuit might be functionally segregated. In an analysis of cDNA microarray gene expression data in several song-related brain regions, Lovell et al observed that each song-related region has a distinct gene expression pattern compared to its adjacent region, and their data also suggest that HVC and RA share a large number of co-expressed genes. Together, these data hint at the ontogeny and evolutionary history of the song control circuit.

To gain more insight into the biological functions of the differentially expressed genes, we performed REACTOME pathway enrichment analysis of genes with enriched expression in HVC at both 45 d and 100 d. The term axon guidance is most highly enriched in 45 d HVC and to a lesser extend in 100 d HVC. This group includes genes coding for calcium channel, potassium channel, NMDA receptors, endocytosis proteins, extracellular adhesion proteins, RELN signaling, ROBO and Slit signaling, and many ribosomal and proteasome proteins. The term metabolism of RNA is also highly enriched in 45 d HVC. This group includes many genes with functions related to mRNA splicing, processing of capped intron-containing pre-mRNA, cleavage of growing transcript in the termination region, and mRNA 3’-end processing. Other terms enriched in 45 d HVC include metabolism of lipids, aryl hydrocarbon receptor signaling, respiratory electron transport, and ATP synthesis (Figure 2A). Fewer terms are significantly enriched in adult HVC than in juvenile HVC. The most highly enriched term in adult HVC is transport of small molecules. This term includes a large group of genes related to ion transporters or ion exchangers for sodium, potassium, or chloride and amino acid transporters. Other terms enriched in adult HVC are retinoid metabolism and G protein coupled signaling events (Figure 2A). Details of genes associated with each term can be found in Data S3. Interestingly, gene network analysis indicated that terms related to RNA metabolism and axon guidance were connected (Figure 2B), suggesting that posttranscriptional mRNA processing such as RNA splicing may play a role in regulating axon guidance or growth.

### 2.3 Developmentally regulated gene expression in HVC

We examined gene expression changes in HVC between 45 d and 100 d. At a significance level of $q < 0.1$, 113 genes changed their expression between 45 d and 100 d. Among them, 70 genes increased expression and 43 genes decreased expression in HVC as juveniles matured into adulthood (Figure 3A). When we lowered the significance threshold to $p < 0.01$, 330 genes changed expression; 189 genes increased expression, and 141 genes decreased expression (Data S4 lists the genes, including their annotations, chromosomal locations, and read counts). These findings indicate that large-scale reorganization of the transcriptome landscape occurs in HVC during the day 45 and day 100 interval when the song circuit maturation and song learning occur.
To verify the RNA-Seq gene expression results, we performed quantitative real-time PCR (RT-qPCR) for a handful of genes that met with the $q < 0.1$ cutoff to assess their expression levels in a separate set of HVC samples. This group of genes included CD74, CD200, CHRM4, DLK1, IFRD1, IL16, LY6E-2, PHF15, RGS10-2, and RASGRP1. All these genes showed significant expression changes between 45 d and 100 d in HVC (Figure 3C). In the RNA-Seq experiment, expression levels of these genes were represented with read counts ranging from 15 to several thousand, and fold-changes from 1.7-fold to 13-fold. Despite these wide ranges and different analysis platforms, regression analysis indicated a high level correlation between the RNA-Seq and RT-qPCR results ($R^2 = 0.8318$, Figure 3B). We further performed RT-qPCR to test eight differentially expressed genes that did not meet with the stringent $q < 0.1$ cutoff, but had $p < 0.01$ in the RNA-Seq experiment. This second group, which included CAMK2B-1, CRIP1, FAM19A1, GABRB1-2, GAD2, NEUROD6, RAPGEF1, and UBE2A, also showed significant expression changes between 45 d and 100 d HVC by RT-qPCR, and the RNA-Seq and RT-qPCR results showed a good correlation ($R^2 = 0.69$, Figure 3B). For all RT-qPCR experiments, $n = 4$-9 animals per age group were used. The RT-qPCR data are also shown in Data S5). Together, these results provide an independent validation of our RNA-Seq approach to identifying differentially expressed genes in HVC during song development.

To examine the functions of the developmentally regulated genes, they were sorted into groups according to their functional annotations. The relative distribution of these groups is summarized in Figure 4A. These groups include genes with functions related to cytoskeleton and microtubule, extracellular matrix and cell adhesion, G protein-coupled neuromodulator receptors, immune system function, mitochondrial function, RNA processing, and signal transduction (Figure 4B). The cell adhesion and extracellular matrix proteins have important roles in neuronal migration and maturation, while the cytoskeletal and microtubule proteins represent essential structural components for establishing and modifying neuronal dendritic and synaptic morphology. Within these groups, some genes were upregulated and others downregulated between the two ages. Among the signal transduction genes, FZD10 is known to promote sensory neuron development in Xenopus and was observed here as upregulated at 45 d. In contrast, genes encoding proteins for synaptic functions, including G-protein coupled neuromodulator receptors, were mostly upregulated at 100 d compared to 45 d. These patterns are consistent with and reflect the highly orchestrated processes of circuit maturation and synaptic reorganization, particularly, the transition from synaptogenesis at 45 d to mature synaptic functions as zebra finches mature into adulthood. The downregulation of genes related to mitochondrial function may indicate the shifting of energy demand during this process. Dysfunction of many of these genes has been associated with a wide range of neurodevelopmental disorders (Table 1).

Unexpectedly, a group of genes known for their functions in the immune system was found as developmentally regulated in HVC (Table 2). This group includes genes encoding the major histocompatibility complex class II invariant chain (CD74), the immune inhibitory molecule CD200, the brain-specific chemokine (FAM19A1), interferon-related developmental regulator 11 (IFRD1), interleukin IL16, interferon inducible protein ISG12, and Lymphocyte antigen complex 6 (LY6E-2). Expression changes of these genes were validated using RT-qPCR (see Figure 3C, D). In addition, at least some of them (e.g., ISG12 and LY6E-2) exhibited enriched expression specifically in HVC and other song related brain regions (Figure 1C). Together, these observations argue against the possibility that their expression patterns were due to pathogen-activated immune responses, although that cannot be entirely excluded.

We performed REACTOME analysis of genes displaying developmentally regulated expression in HVC. The most highly enriched genes include:

- Axon guidance
- Transport of small molecules
- Metabolism of RNA
- Retinoid metabolism and transport
- Metabolism of lipids
- Platelet homeostasis
- Aryl hydrocarbon receptor signalling
- Respiratory electron transport, ATP synthesis, and heat production
- G alpha (i) signalling events

These enriched pathways reflect the biological processes occurring during song development.
term among genes with higher expression in 45 d HVC is metabolism of amino acids and derivatives. This group includes many genes related to ribosomal proteins, proteasome proteins, and translation initiation and elongation. Interestingly, terms related to cell division such as DNA replication and repair, G1/S phase transition, cell cycle, and mitosis are highly enriched. Genes with functions related to laminin interactions such as extracellular matrix organization, integrin cell surface interactions, and crosslinking of collagen fibrils are enriched as well. Other enriched terms include mitochondrial protein synthesis. Network analysis further reveals interconnections among these terms (Figure 5A,B and Data S6). These interactions suggest that, instead of singular and independent events, well-orchestrated and well-coordinated gene expression changes collectively play critical roles during HVC development. As juveniles mature to adults, genes with higher expression in 100 d HVC are enriched with terms pertaining to neuronal system functions, these terms include genes encoding for calcium channels, potassium channels, sodium channels and glutamate transporters, various amine ligand binding receptors (including dopamine receptors, adrenergic receptors, and serotonin receptors), and G protein signaling. Other enriched terms include transport of small molecules, transcriptional regulation of MECP2, and effects of PIP2 hydrolysis. These changing enrichment patterns in HVC reflect orchestrated gene expression changes occurring as the growth and formation of circuit connections in juveniles switches to the mature functional state in adults.
These RNA sequencing based experiments have enabled us to generate a comprehensive genome-wide inventory of gene expression in the song control nucleus HVC in juvenile and adult male zebra finches. While a large portion of the genome is active in HVC during its development, we noted that a sizable fraction of the high-quality sequence reads (~40%) failed to map to the zebra finch genome. Of the reads mapped to the genome, a large fraction mapped to regions without annotation. The incomplete assembly and annotation of the zebra finch genome may have contributed to these observations. However, in recent years, as the sensitivity of detection techniques improves, mounting evidence has indicated that, in addition to known genes, a large portion of mammalian genomes is transcriptionally active. This

3 | DISCUSSION

These RNA sequencing based experiments have enabled us to generate a comprehensive genome-wide inventory of gene expression in the song control nucleus HVC in juvenile and adult male zebra finches. While a large portion of the genome is active in HVC during its development, we noted that a sizable fraction of the high-quality sequence reads (~40%) failed to map to the zebra finch genome. Of the reads mapped to the genome, a large fraction mapped to regions without annotation. The incomplete assembly and annotation of the zebra finch genome may have contributed to these observations. However, in recent years, as the sensitivity of detection techniques improves, mounting evidence has indicated that, in addition to known genes, a large portion of mammalian genomes is transcriptionally active. This
phenomenon, now known as “pervasive transcription,” could give rise to long non-coding, intronic, intergenic, and/or antisense transcripts.36,37 The biological functions of these novel “dark matter” transcripts have yet to be explored. Our data add evidence to the suggestion that similar pervasive transcription occurs from non-annotated regions of the zebra finch genome as well.

The RNA-Seq results reveal complex and dynamic gene expression profiles in HVC of juvenile and adult zebra finches. Although gene expression patterns in HVC at 45 d and 100 d share many similarities, they are both clearly distinct from the remainder of the brain. Many genes with general cellular functions are highly enriched in juvenile HVC. For example, gene pathways related to electron transport and ATP synthesis are highly expressed in 45 d HVC, highlighting the energy metabolism demand at this age. A large number of genes related to transcription and RNA processing are also highly enriched in 45 d HVC. These groups include components for RNA polymerase, mRNA splicing, cleavage of growing transcript in the termination region, and mRNA 3′-end processing. Many neuronal genes use alternative splicing to increase gene product diversity, and via regulating gene products and functions, RNA splicing can affect many aspects of neural development and physiology.

| TABLE 1 | Genes developmentally regulated in HVC that have been implicated in nervous system related disorders |
| --- | --- | --- |
| Gene symbol | Gene name | Disorders |
| CLCN2 | Chloride channel 2 | Epilepsy |
| COL4A1 | α1 chain of type IV collagen | Cerebral small vessel disease, epilepsy, developmental delay |
| CRIPT | CXXC repeat containing Interactor of PDZ3 domain | Microcephaly, developmental delay |
| IFRD1 | Interferon related developmental regulator 1 | Sensory/motor neuropathy with ataxia (SMNA) |
| RAPGEF1 | Rap guanine nucleotide exchange factor 1 | Cerebral palsy, communication difficulties, cognitive impairment |
| TLL2 | Zinc-dependent metalloprotease | Non-Syndromic intellectual disability, ADHD |
| UBE2A | Ubiquitin-conjugating enzyme E2A | Intellectual disability, seizure, poor speech |
| UPF3A | Regulator of nonsense mediated mRNA decay | ASDs |

Abbreviation: ASDs, autism spectrum disorders.

*Note all genes listed met with q < 0.1 cutoff in RNA-Seq or have been validated by RT-qPCR. Mutations of some of these genes have been implicated in multiple diseases, but only nervous system disorders are listed.

| TABLE 2 | Immune system related genes that are developmentally regulated in HVC |
| --- | --- | --- |
| Gene symbol | Descriptive name | Log2 FC |
| CD74 | MHC class II invariant chain 1 | -1.690 |
| CD200 | Type I membrane glycoprotein | 2.720 |
| FAM19A1 | Brain-specific chemokine | -0.863 |
| IFRD1 | Interferon-related developmental regulator 1 | -1.125 |
| IL16 | Interleukin 16 | 2.172 |
| ISG12 | Interferon inducible protein | -3.758 |
| LY6E-2 | Lymphocyte antigen complex 6 | -2.634 |

*Note positive values indicate increased expression and negative values indicate decreased expression in HVC from 45 d to 100 d. The fold changes are based on RNA-Seq results, all have been validated by RT-qPCR with p < 0.05 (see Figure 3).
of the Split-ROBO signaling pathway have been shown to be expressed in HVC and to be developmentally regulated in RA, the downstream nucleus to which HVC connects. Other members of the axon guidance term enriched in 45 d HVC include genes coding for calcium channel, potassium channel, NMDA receptors, and endocytosis proteins. We have noted that many genes enriched in 45 d HVC are also enriched in adult HVC, but to a lesser extent. Their continued expression in adult HVC, reported as well by Lovell et al, is expected, since many of these signaling molecules (eg, Slit-ROBO), channels, and receptors are the very components that define neuronal properties and functions.

As HVC matures, enriched gene expression shifts to components of mature functional neural circuits. We noted that a group of genes related to retinoid metabolism are enriched in adult HVC, a complement to previous findings that \textit{ALDH1A2} plays critical roles in HVC function and song learning. By analyzing earlier cDNA microarray data, Lovell et al report a large number of genes differentially expressed in adult HVC compared to the adjacent shelf. Although that study and the present study used different methods (cDNA microarray vs RNA-Seq) and different references for gene comparison (HVC vs shelf opposed to HVC vs whole brain tissue), the two studies share many concordant observations. For example, both find enriched expression of potassium channel genes in HVC. These findings, together with an earlier study reporting a large number of potassium channel genes in HVC, strengthen the roles potassium channels play in defining the complex electrical firing properties of HVC neurons, whose bursting patterns are linked to the temporal pattern of a song. Another common theme between the two studies is the enriched expression of genes related to G protein signaling and many receptors for neural modulators in adult HVC. The latter group includes adrenergic receptors, dopamine receptors, serotonin receptors, and cholinergic receptors. These diverse modulatory mechanisms of G protein-coupled receptors provide many possibilities for HVC structure and function to be tuned in response to physiological and environmental events. The massive gene expression change during extended postnatal development is not limited to HVC. London et al have shown that gene expression in auditory regions of zebra finches at 20 d is vastly different from that in adults, even though the gross morphology of these regions does not significantly differ between juveniles and adults. It appears that age plays important roles in regulating gene expression in song related brain regions, and the extended period of development would allow the structure and function of these brain regions to be modulated by sensory-motor and/or perceptual learning experience via gene expression changes.

Some genes developmentally regulated in HVC have been implicated in neural developmental disorders. COL4A1 encodes collagen type IV α1 chain, an extracellular matrix protein. Mutations in COL4A1 have been found to cause cerebral vasculature defects, migraine, stroke, and epilepsy. Other members of the collagen family, COL12A1 and COL21A1, are also developmentally regulated in HVC, highlighting the roles of this gene family in HVC development. \textit{RAFGEF1} encodes Rap guanine nucleotide exchange factor 1, which modulates GTPase signaling pathways. \textit{RAFGEF1} has been linked to Autism Spectrum Disorders (ASDs), cerebral palsy, communication difficulties, and cognitive impairments. \textit{TLL2} encodes the zinc-dependent metalloprotease tolloid-like protein 2, a protease required for the development of the adult nervous system.
for organizing extracellular matrix. Diseases associated with TLL2 include autosomal recessive non-syndromic intellectual disability.\textsuperscript{54,55} UBE2A encodes a member of the E2 ubiquitin-conjugating enzyme family, which catalyzes the covalent attachment of ubiquitin to substrate proteins. Disorders associated with UBE2A include mental retardation, seizures, poor speech, and aggressive behavior.\textsuperscript{56-58} UPF3A encodes the regulator of nonsense mediated mRNA decay (NMD), a process cells use to eliminate faulty mRNAs. UPF3A and its paralog UPF3B antagonistically regulate NMD, and both have been implicated in ASDs, intellectual disability, and schizophrenia.\textsuperscript{59-62} The links between these genes and neurodevelopmental disorders are often established by genome-wide screening for mutations and/or copy number variations in human patients. Further investigation of these genes in a neural circuit for vocal communication will likely provide insight into the molecular and cellular mechanisms underlying these developmental disorders in humans.

It is puzzling that a group of genes known to function in immune regulation and inflammatory responses are developmentally regulated in HVC. The role they may play in HVC is unclear. Emerging research has implicated microglia in regulating neurogenesis and neuronal cell numbers during neural development by initiating neuronal apoptosis followed by cleaning cellular debris through phagocytosis.\textsuperscript{63} The phagocytic microglia also modulate wiring of the neural circuits by regulating the number, maturation, and plasticity of synaptic connections.\textsuperscript{64,65} Recent studies have revealed that many microglia functions are mediated by components of the classical complement cascade or cytokine signaling pathways. For example, the complement cascade components C1q and C3 as well as C3 receptor (CR3) have been shown to mediate synaptic pruning in the developing retinogeniculate system in an activity-dependent manner.\textsuperscript{66-68} Similarly, neuronal expression of MHC Class I genes has been implicated in activity-dependent regulation of ocular dominance plasticity during the critical period of visual system development.\textsuperscript{69,70} During song circuit development, extensive neurogenesis and neuronal cell death as well as formation and modification of synaptic connections occur in HVC.\textsuperscript{15,20} The regulation of major histocompatibility complex class II invariant chain (CD74) and various cytokines and their receptors (eg, FAM19A1 and IL16) in HVC suggests that they may play a role in the development and maturation of HVC. The HVC tissue we sequenced presumably contained multiple cell types, including projection neurons, interneurons, glia, and microglia. Each of these cell types could have contributed to the gene expression profiles. Further cell type specific gene expression analysis will help delineate in which cell types these genes express, and help understand their contribution to HVC development.

The RNA-Seq data obtained in this study provide new insights into the molecular and genomic underpinning in HVC as juveniles mature to adulthood. The datasets will also join other resources to benefit future studies investigating functions of individual genes and/or interactions among genes during song circuit development and vocal learning in songbird. RNA-seq, with its high throughput capacity, higher sensitivity, and unbiased nature, has increasingly become a standard approach to large scale quantitative gene expression analysis, and allows for cross comparison of data from different studies, from different laboratories, and even from different animal species. As exploration of transcriptomes has become an important approach to understanding circuit development and brain diseases, these datasets can serve as a reference for further transcriptome analysis relevant to a wide range of physiological and/or experimental conditions.

\section{METHODS}

\subsection{Animals, tissue collection, and RNA isolation}

Animal usage was approved by the Louisiana State University Health School of Medicine Institutional Animal Care and Use Committee. The juvenile and adult zebra finches were obtained from 10-15 breeding cages in our breeding colony at the LSU School of Medicine animal care facility with birth dates documented. The juveniles were raised with their parents in family cages until 45 days of age. To ensure that gene expression patterns reflect developmental stages, and were not confounded by behavioral factors (eg, singing), birds were monitored in the morning for one hour individually and brains were collected from birds that did not sing and did not hear songs. For HVC tissue collection, birds were euthanized at a specified age, their brains were collected and snap frozen in liquid nitrogen or dry ice. Brains were cut into 80 μm sagittal sections, HVC tissue was dissected out using a syringe needle under a dissection microscope, and quickly transferred into a lysis buffer for RNA isolation. We tried not to include the adjacent tissue on the medial/lateral and ventral sides of HVC. The ventricular zone lies immediately on the top of HVC, and it is difficult to separate it from HVC. Thus, the HVC samples may have contained portions of the ventricular zone. We stained sections especially of juveniles to define the medial/lateral edges of HVC. If there was even a slight doubt, that section was not used. Total RNA was isolated using Trizol reagents (Invitrogen). Residual contaminating genomic DNA was removed by DNase I digestion. RNA concentration was determined using the Nanodrop spectrometer, and RNA quality was examined using the Agilent Bioanalyzer.

\subsection{cDNA library preparation and sequencing}

Total RNAs from HVC of four birds per age group were pooled and prepared into one sequencing library using a NuGEN Ovation RNASeq v1 kit following the manufacturer’s instructions. An additional step of S1 nuclease treatment of cDNA was used as described.\textsuperscript{71} Sequencing libraries were size selected on a 2% agarose gel to obtain libraries in a size range of 350 to 400 bp corresponding to inserts lengths of 230 to 280 bp. Libraries were also made from whole brain tissues of male zebra finches at 45 d and 100 d by pooling two brains per library. We sequenced each library on an Illumina Genome Analyzer II (GAIIx) sequencer, which produced \textasciitilde20 million sequence reads of 79 nt per lane. Library construction and high-throughput sequencing were performed by the Next Generation Sequencing core, The Scripps Research Institute. For more detailed methods description, see.\textsuperscript{71}
4.3 | RNA-Seq analysis

We used Casava 1.7 for demultiplexing and base calling to generate FASTQ files containing the sequence reads. After quality filtering and adaptor trimming, high quality reads were mapped to the zebra finch genome (3.2.4/taeGut1) using the ELAND2 (Illumina) software to define gene structure and alternative splicing events, and to quantify transcript abundance. We used R Bioconductor package EdgeR to compare gene expression between the two age groups. EdgeR provides statistical routines for digital gene expression data and ranks genes by statistical significance, taking into account the total read number, sample reproducibility, and fold changes. Comparing the normalized read frequencies allowed identification of genes with enriched expression in HVC and genes regulated in HVC during development.

4.4 | REACTOME pathway enrichment analysis

Gene lists were constructed as follows: For comparing HVC and WB at 45 d and 100 d, HVC enriched (p < 0.01) gene symbols were extracted from EdgeR output and merged into one file with two separate columns: 45 d HVC-enriched and 100 d HVC-enriched. Similarly, for comparing 45 d and 100 d HVC samples, upregulated (p < 0.05) gene symbols were extracted from EdgeR output and merged into one file with two columns: 45 d HVC and 100 d HVC. The gene lists were subjected to Metascape for REACTOME enrichment analysis in CUSTOM option with default parameters. Note, since the REACTOME database does not include zebra finch genes, human orthologous genes were used as background in this analysis. Pathway terms with p < 0.01 were considered significant. Network analysis was also performed using Metascape in CUSTOM option with default parameters, and the resulting layouts were manually modified with Cytoscape (3.3.0) using approaches as described in References 73, 74.

4.5 | RT-qPCR

RT-qPCR was performed as described previously. Briefly, total RNA was isolated from HVC tissue using Trizol reagent (Invitrogen), and quantified using a Nanodrop spectrophotometer. Reverse transcription was performed using 50 ng of total RNA using an iScript Reverse Transcription Supermix kit (Bio-Rad) following the manufacturer’s instructions. qPCR was performed using the IQ SYBR Green Supermix (Bio-Rad) following the manufacturer’s instructions. GAPDH was used as a reference gene after determining that its expression did not change during development. Relative gene expression levels were determined using the comparative Ct (2−ΔΔCt) method after normalizing to GAPDH. N = 4-9 animals per age group were used in the RT-qPCR experiment. For all samples, RT-qPCR was performed in triplicate twice. Results shown in Figure 3 and Data S5 are from one set of experiment. Dissociation curve analysis was performed to confirm a single peak PCR product for each gene, indicating the specificity of PCR reactions. All primers were obtained from IDT (Integrated DNA Technology); their sequences are listed in Data S7.

4.6 | In situ hybridization

In situ hybridization was performed as described previously. Briefly, fresh frozen zebra finch brains were cut into 10 μm sagittal sections and kept at −80°C until use. Brain sections were fixed in 4% PFA for 10 minutes followed by acetylation for 10 minutes. PCR amplified cDNA fragments containing the probe sequences (200-300 bp, sequence-verified) were cloned into pBluescript plasmid vectors, and sequences were verified. 32P-labeled ribo-probes were made by in vitro transcription using T7 or T3 RNA polymerase (PerkinElmer Kit). For hybridization, 10⁶ cpm probes in 40 μL hybridization buffer were added to each brain section and hybridized at 65°C overnight. After hybridization, brain sections were washed two times, 30 minutes each, at 65°C with wash solution (50% formamide, 1xSSC, and 0.1% Tween 20), followed by washing 2 times with 0.2 X SSC at 65°C. Slides were exposed to X-films for 1-7 days, depending on signal intensity.

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AUTHOR CONTRIBUTIONS

Zhimin Shi: Data collection, analysis, and manuscript preparation; Zeyu Zhang: Data analysis and manuscript preparation; Lana Schaffer: Data analysis; Zhi Huang: Data collection; Lijuan Fu: Data collection; Steven Head: data collection; Terry Gaasterland: Experimental design, data analysis, and manuscript preparation; Xiu-Jie Wang: Experimental design, data analysis, manuscript preparation, and project management.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The RNA-Seq data are available in the Gene Expression Omnibus (GEO) repository under accession number GSE140470 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140470.

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