Erbin and ErbB2 Play Roles in the Sexual Differentiation of the Song System Nucleus HVC in Bengalese Finches (*Lonchura Striata Var. domestica*)

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**ABSTRACT:** Song control nuclei have distinct sexual differences in songbirds. However, the mechanism that underlies the sexual differentiation of song nuclei is still not well understood. Using a combination of anatomical, pharmacological, genetic, and behavioral approaches, the present study investigated the role of erbb2 (a homolog of the avian erythroblastic leukemia viral oncogene homolog 2) and the erbb2-interacting gene, erbin, in the sexual differentiation of the song nucleus HVC in the Bengalese finch. We first found that both erbin and erbb2 were expressed in the developing HVC at posthatch day (PHD) 15 in a male-biased fashion using qRT-PCR and *in situ* hybridization. Following the addition of a pharmaceutical inhibitor of the ErbB2 signaling pathway to the culture medium, cell proliferation in the cultured ventricle zone (VZ) that overlies the developing HVC decreased significantly. After the injection of erbin- or erbb2-interfering lentiviruses into the HVC and its overlying VZ at PHD 15, the cell proliferation in the VZ at PHD 24, the number of the differentiated neurons (Hu+/BrdU+ or NeuN+/BrdU+) in the HVC at PHD 31 or PHD 130, and the number of RA-projecting cells at PHD 130 all decreased significantly. Additionally, the adult songs displayed serious abnormalities. Finally, 173 male-biased genes were expressed in the developing HVC at PHD 15 using cDNA microarrays, of which 27.2% were Z-linked genes and approximately 20 genes were involved in the Erbin- or ErbB2-related signaling pathways. Our results provide some specific genetic factors that contribute to neurogenesis and sex differentiation in a song nucleus of songbirds.

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**Keywords:** Songbirds; cell proliferation; cell differentiation; sexual difference; Z-linked genes

**INTRODUCTION**

In many oscine species, only male birds sing highly stereotyped songs. Accordingly, male birds possess dimorphic and highly interconnected song control nuclei (Nottebohm and Arnold, 1976; Tobari et al., 2005). Therefore, songbirds provide a model to study the neural mechanism that underlies the sexual dimorphism of brain areas.
During mammalian embryonic development, the undifferentiated gonad develops into a testis rather than an ovary under the action of the testis-determining gene, the sex-determining region of the Y-chromosome (Sry) (Sinclair et al., 1990; Kashi-mada and Koopman, 2010). The testes secrete steroid hormones, directing somatic cells to differentiate in a male fashion (McCarthy, 2009). However, this mode of sexual differentiation does not extend to all vertebrates, including avian species with homogametic males (ZZ) and heterogametic females (ZW). First, unlike in mammals, there is an incomplete sex-reverse of masculinization in female birds following estradiol treatment and an unsuccessful prevention of masculine development in male birds after blocking testicular hormones (Wade and Arnold, 1996). Second, beyond the lack of a gene that is homologous to the mammalian Sry, chromosome-wide dosage compensation akin to mammalian X-inactivation is largely lacking in birds (Ellegren et al., 2007; Itoh et al., 2007; Naurin et al., 2011). Studies of gynandro-morphic zebra finches and chickens reveal that sexual differentiation of somatic cells (including cells in the song control nuclei) is the result of hormonal and genetic factors (Agate et al., 2003; Zhao et al., 2010). Thus, the sexual differentiation of the song control nuclei seems to depend both on sex steroids and on the steroid-independent differential expression of genes. To our knowledge, the possible mechanisms that underlie the sexual differentiation of song control nuclei are still not clear.

We recently identified male-biased genes in the telencephalon of the Bengalese finch, including a Z chromosome gene erbin (ErbB2 interacting protein) and ErbB2 (a homolog of the avian erythroblastic leukemia viral oncogene homolog 2) (Sun et al., 2010). Both Erbin and ErbB2 have been reported to be involved in cell proliferation, survival, and differentiation through signaling pathways mediated by Erbin (mitogen-activated protein kinases, MAPK; transforming growth factor β, TGF-β; nuclear factor-κB, NF-κB) or by ErbB2 (extracellular regulated protein kinases, ERK or Ras-Raf-Mek-Erk; protein kinase C, PKC) (Borg et al., 2000; Rangwala et al., 2005; Citri and Yarden, 2006; Zhou et al., 2012; Roskoski, 2014; Tao et al., 2014). Given the reported roles of erbin and erbb2, we further wanted to know: (1) whether erbin and erbb2 expressions have sexual differences in the developing HVC, a song control nucleus in the Bengalese finch; (2) whether cell proliferation, and neuronal differentiation are affected after injections of erbin- or erbb2-interfering lentiviruses into the developing HVC and its overlying VZ (after erbin- or erbb2 knock-down). Based on these investigations, we wanted to know whether the two genes are involved in the sexual differentiation of HVC.

To address the issue, we first investigated the sexual dimorphism in the expression of erbin and erbb2 in the developing HVC of the Bengalese finch. We then examined whether erbin and erbb2 affect cell proliferation in the ventricular zone overlying the developing HVC at PHD 15 using a pharmaceutical inhibitor for the ErbB family. Next, we injected erbin- or erbb2-interfering lentiviruses into the developing HVC and its overlying VZ and further investigated how cell proliferation, neuronal differentiation and adult song organization are affected. Finally, using cdNA microarrays, we examined sex-biased genes in the developing HVC, specifically those in the Erbin- or ErbB2-involved signaling pathways.

METHODS

Animals

The Bengalese finches (Lonchura striata var. domestica) used in this study were originally purchased from a local supplier (Beijing Guanyuan Flowers and Birds Market, Beijing Haidian District, Beijing, China) and then raised in the breeding colony at Beijing Normal University. Nestling (PHD 15), young (PHD 45) and adult birds (PHD >120) were used in this study. The birds were maintained in cages (50 cm × 62 cm × 38 cm) equipped with perching sites and nest boxes in a room under a 14/10 h light/dark cycle at 20–30°C. Seeds and fresh water were provided at all times, while green vegetable supplements were provided occasionally. Each cage contained 4–7 adult birds or 3–4 nestling birds that lived with foster parents. All experiments that were performed in this study were conducted in accordance with the Beijing Laboratory Animal Welfare & Ethics Review guidelines. Furthermore, all of the protocols were reviewed and approved by the Animal Management Committee of the College of Life Sciences of Beijing Normal University.

Sex Genotyping, RNA Extraction, in Situ Hybridization and Quantitative Real-Time PCR (qRT-PCR)

To identify the sex of nestling finches (PHD 15), genomic DNA was extracted from axillary venous blood using a TIANamp Genomic DNA Kit (TIANGEN Biotech, Beijing). The PCR primers used in sex determination were previously described in detail (Wang and Zhang, 2009; Wang et al., 2010), and listed in Table 1. PCR products were electrophoresed in a 1.5% agarose gel. There was a 0.18-kb Z-linked fragment in both sexes and a 0.22-kb W-linked fragment in the female.
For qRT-PCR

erbin
Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer’s instructions. The qualified RNA was used in qRT-PCR or cDNA microarray analysis.

The primers for the RNA probes used in the in situ hybridization protocol were designed with the Primer3 output program and shown in Table 1. The Erbin probe length was 234 bp, ranging from chromosome location 6300 to 6533, with an 18-bp sense and anti-sense primer at the two ends of zebra finch XM_004177159.1 (Chr. Z). The ErbB2 probe length was 466 bp, ranging from chromosome location 1185 to 1650, with 20-bp sense and 18-bp anti-sense primers at the two ends of chicken NM_001044661.1 (Chr. 27). Reverse transcription was performed with M-MLV (Promega). The target bands were then cloned into the pGEM-T Easy vector (Promega) and sequenced to confirm the accuracy of the probes. The Erbin fragment shared 99% identity with zebra finch XM_004177159.1, and the ErbB2 fragment shared 98% identity with chicken NM_001044661.1. The fragment of ErbB2 cloned from the Bengalese finch was deposited into GenBank (KT932702). The sense and anti-sense RNA probes were transcribed using a digoxigenin (DIG) RNA labeling kit (Roche) according to the manufacturer’s protocol. The corresponding sense probes were used as negative controls.

For in situ hybridization, birds of both sexes at PHD 15, PHD 45 and adult (PHD > 120) were euthanized as described above. The hearts were then perfused with ice-cold 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by ice-cold 4% paraformaldehyde (PFA, in 0.1 M PB, pH 7.4). After fixation with 4% PFA for 24 h and cryoprotection in 30% sucrose (in 0.1 M PB, pH 7.4) at 4°C, the brains were cut into 10-µm-thick sections using a cryostat (Leica). Every sixth sagittal section was mounted on a poly-lysine-coated slide, and a total of six sets of sections were collected for each hemisphere. The sections were stored at −80°C until use.

In situ hybridization was routinely carried out as described by Zhu et al. (2008). Briefly, after endogenous AP activity was quenched with 0.2M HCl, proteinase K (GIBCO) digestion was carried out at 37°C, followed by postfixation in 4% freshly depolymerized paraformaldehyde. The sections were

Table 1 Primers for PCR or qRT-PCR

<table>
<thead>
<tr>
<th>Use</th>
<th>Gene name</th>
<th>Primers (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>For sex determination</td>
<td>erbin</td>
<td>SS: CTCCCAAGGATGAAAACTGTGCAAAACAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: CTCCACTTCATTAAAGCTGATCTGGAATTTC</td>
</tr>
<tr>
<td>For RNA probe synthesis</td>
<td>erbin</td>
<td>SS: CAAAGTCAGTCCAAAGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: GACAGGCTTCAGTTGTTA</td>
</tr>
<tr>
<td></td>
<td>erbb2</td>
<td>SS: AAGATCTTTGGAGCTGCGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: CGGGCCCCCAGCAGTTGCC</td>
</tr>
<tr>
<td>For qRT-PCR</td>
<td>erbin</td>
<td>SS: CAGTTAAGAATCGTTCTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: ATGTTACATCATACATGTCAT</td>
</tr>
<tr>
<td></td>
<td>erbb2</td>
<td>SS: ATCAAGTGGATGCGCGTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: TCGGCTTTCCACAGGTTG</td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
<td>SS: TTGGCAATGAAGGTCAGAGTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS: TACGGATGTCCACATCACACT</td>
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SS: Sense sequence; AS: antisense sequence.
then acetylated with 0.25% (v/v) acetic anhydride (Sigma) in a 0.1M triethanolamine (Sigma), and incubated for 1 h with prehybridization solution (50% formamide, 2× SSC, 0.05 g/ml dextran sulfate, 1× Denhardt’s, and 0.1mg/ml salmon sperm DNA). After digoxigenin-labeled RNA probes were diluted in prehybridization buffer (1ng/ml), they were added to the sections for incubation overnight (100μl for each section). The sections were then washed with 2× SSC and 0.05% Tween-20, 0.1M Tris-HCl, and 0.15M NaCl. The sections were incubated with a sheep polyclonal antidigoxigenin antibody F(ab)2 fragment conjugated with alkaline phosphatase (Roche Molecular Biochemicals, 1:1000).

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**Figure 1** In situ hybridization and quantitative real-time PCR show sexual differences in expression levels of erbin and erbb2 mRNA in the HVC of Bengalese finches. A1-D1, Nissl staining for male (A1 and C1) or female (B1 and D1) HVC at PHD 15 (A1 and B1), and 45 (C1 and D1). A2-D3, Labeling for erbin or erbb2 mRNA in male and female HVC at PHD15 (A2-B3) and 45 (C2-D3). E, Sagittal schematic of HVC in the brain. Dorsal is up, and caudal is to the left. Scale bar in D3 = 200 μm for A1-D3. F, G, Labeling for erbin or erbb2 sense probes in male (F) and female (G) HVC at PHD 45. H, I, Total numbers of labeled cells for erbin (H) and erbb2 (I) mRNA in HVC at PHD15, 45 and adult (>PHD120). *p < 0.05; **p < 0.01; ***p < 0.001. Error bars indicate SEM. J, K, Quantitative real-time PCR of erbin (J) and erbb2 (K) at PHD 15 in brain samples or explants that contained the developing HVC and its overlying VZ, which we obtained by cutting along the thick dashed line (shown in E) located at the caudal hyperpallium lamina, and in narrow belts that were outside the HVC (delimited by two dashed lines, E). Bars with different letters are significantly different. [Color figure can be viewed at wileyonlinelibrary.com]
After washed with TTN and Tris (pH 9.4), color was developed in the presence of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) for 48 h. Negative controls were prepared using the corresponding sense probes. At least one set of sections was used for Nissl-staining.

The primers for qRT-PCRs were designed following the aforementioned method, based on the sequences of the studied genes published in the NCBI GenBank: Erbin (ERBB2IP, XM_004177159.1) and β-actin (ACT5C, XM_002192780.2) in zebra finch and ErbB2 (NM_001044661.1) in chicken (not available for zebra finch). The designed primers were shown in Table 1, and the expected fragments for erbin, erbb2 and β-actin were 161 bp, 162 bp and 123 bp, respectively. The amplicons were cloned into the pGEM-T Easy vector (Promega) and sequenced to confirm the consistence with the corresponding accession numbers in the NCBI GenBank mentioned above. Real-time PCR was performed with an ABI 7500 Sequence Detection System (Applied Biosystems), using the SYBR Green Mix kit with 10–20 nM cDNA template and 40 cycles at 95°C, and placed on 25-mm porous culture with a 0.4-
micron membrane. The explants that contained the developing HVC (labeled by BrdU) per mm in the VZ that over-lay the developing male HVC decreased with increased concentrations from 1.25 to 20 

μM and were examined 24 h after inhibitor addition. However, the numbers of cells stained by cresyl violet within HVC (per mm²) were also significantly decreased under the treatment with 20 

μM inhibitor, indicating a toxic effect, but there were no significant changes in the other groups (suggesting no significant toxic effect). Thus, the dose of 10 

μM inhibitor for the ErbB family was chosen. After culturing for 1 day at 37°C and 5% CO₂, the explants were fixed, dehydrated and cut into 10-
micron sections according to the above described protocol. Every sixth sagittal section was mounted on a poly-l-lysine-coated slide and used for immunohistochemistry analysis.

**Brain Slice Culture and the Effect of the ErbB2 Inhibitor on Cultured Brain Slices**

After the nestling male Bengalese finches (PHD 15) were anesthetized, they were perfused with ice-cold HBSS containing 1% penicillin/streptomycin (Shanghai Weike) and 5.5 g/L D-glucose, which was sterilized by filtration using a 0.22-micron membrane. The explants that contained the developing HVC and its overlying VZ were obtained using the above-mentioned protocol. For each hemisphere, 2–3 explants containing HVC were obtained. The explants were then transferred into cold HBSS, incubated for 90 min at 4°C, and placed on 25-mm porous culture with a 0.4-
micron pore size (NUCN, 137060) inserted in a 6-well plate with six explants per well (1.8 ml Dulbecco’s Modified Eagle Media: Nutrient Mixture F-12 in a 1:1 ratio (Sigma), 1% (v/v) penicillin/streptomycin, 1% (v/v) N2 (Sigma), 2% (v/v) B27 (Sigma), 2 mM glutamine (Gibco) and 1% VC (v/v) (Sigma)). A highly selective inhibitor for the ErbB family (EGFR/ErbB2), a 4-(4-benzyloxyanilino)-6,7-dimethoxyquinazoline (Merck Millipore, 324673) stock solution in DMSO, and 5'-bromo-2'-deoxyuridine (BrdU, Sigma) to label proliferating cells were added to the culture medium at a final concentration of 10 

μM for both the inhibitor and BrdU and washed out after 24 h. According to the manufacturer’s protocol, 4-(4-benzoxyanilino)-6,7-dimethoxyquinazoline acts a potent, reversible, and ATP-competitive inhibitor of EGFR and c-erbB2. This drug can effectively inhibit the proliferation of cells expressing EGFR or c-erbB-2.

The chosen doses of the drug were determined in our preliminary experiments. To obtain the dose-response curve, final drug concentrations of 0, 1.25, 2.5, 5.0, 10.0, or 20 

μM were used in the cultured brain explants that contained the developing male HVC at PHD 15, with reference to the protocol and a related report (Yu et al., 2011). The concentration of DMSO in the media was adjusted to 0.1% in all six groups. The numbers of proliferating cells (labeled by BrdU) per mm in the VZ that over-lay the developing male HVC decreased with increased concentrations from 1.25 to 20 

μM and were examined 24 h after inhibitor addition. However, the numbers of cells stained by cresyl violet within HVC (per mm²) were also significantly decreased under the treatment with 20 

μM inhibitor, indicating a toxic effect, but there were no significant changes in the other groups (suggesting no significant toxic effect). Thus, the dose of 10 

μM inhibitor for the ErbB family was chosen. After culturing for 1 day at 37°C and 5% CO₂, the explants were fixed, dehydrated and cut into 10-
micron sections according to the above described protocol. Every sixth sagittal section was mounted on a poly-l-lysine-coated slide and used for immunohistochemistry analysis.

**Construction of Lentivirus-Interfering Vectors, Injections of Lentivirus-Interfering Vectors, Song Recording and Neural Tract Tracing**

To obtain an effective interference fragment, two or three different short hairpin RNA (shRNA) fragments were designed to target the erbin (XM_004177159.1) or erbb2 (KT932702) mRNA of the Bengalese finch, respectively. These shRNA fragments were designed online (http://www.genelink.com/sirna/shRNAi.asp). The following linear DNA structure was used to encode the shRNA hairpins: sense-loop-antisense-TTTTT. The sequence of the loop was TTCAAGAGA. The constructs for the expression of the shRNA were cloned into a GU11 vector (pBS-U6-RNAi-dual-CMV-GFP) in which the U6 promoter was used to drive expression of the shRNA. Each hairpin construct was tested for knockdown efficiency in HEK293 cells that were co-transfected with an erbin or erbb2 expression plasmid and an shRNA GU11 vector. Here, we used the same fragments as in the in situ hybridization protocol, which were not expressed in un-transfected HEK293 cells. The hairpins that had the best interference efficiency in
HEK293 cells were synthesized (shErbin targeting sequence: 5'-CCTGAAAGACCACCATCTA-3'; shErbb2 targeting sequence: 5'-CCTGGCCACCTGACATGAA-3') and inserted into a GV248 vector (phU6-MCS-Ubiquitin-EF1A-IRE5-puromycin, Shanghai GeneChem) to generate a recombinant RNAi virus. The expression of shRNA for the target gene was driven by the human U6 promoter and the enhanced green fluorescent protein (EGFP) gene using the Ubiquitin promoter. The recombinant RNAi virus was packaged using the Lentivirus Expression System (Shanghai GeneChem). The scramble sequence (5'-TTTCTCGAACGTGCAGT-3') proposed and provided by Shanghai GeneChem was used as a negative control. The titers were 6–8 × 10^8 TU/ml.

The injection of lentiviral solution was referred to a previous report (Haesler et al., 2007). Before surgery, the nestling birds (PHD 15) were deprived of food and water for one hour. They were then anaesthetized via intramuscular injections of sodium pentobarbital as described above, and fixed in a stereotaxic head holder. Lidocaine (2%) was injected locally at the incision site over the bifurcation of the mid-sagittal sinus. The scalp was dissected along the midline, and the skull was opened by a small fenestra. The stereotaxic point 0.0 was defined as the branch point of the midline, and the skull was opened by a small fenestra. The stereotaxic point 0.0 was defined as the branch point of the sagittal sinus. To keep the developing HVC and its overlying VZ to be well infected with lentiviral viruses, the lentiviral solution was injected at four sites (medial to lateral: 2.0 mm; anterior to posterior: 0.1 and 0.3 mm; dorsal to ventral: 0.4 mm) that was attached to a glass syringe. To keep the same injection number of the three types of lentiviral viruses (erbin RNAi, erbb2 RNAi or negative control) (TU/ml*Volume = 3 × 10^5) into a hemisphere, 100–125 nl lentiviral solution were injected into each site over a course of 10 min. Such injections could cause the developing HVC and its overlying VZ to be well infected with lentiviral viruses [Fig. 4(A1-B4)], and the infected cells were distributed around the injected areas. For each of the birds, the recombinant viruses (erbin RNAi or erbb2 RNAi) were injected into the developing HVC at either side (right or left) of the hemisphere, while the control viruses (control RNAi) were injected into the other hemisphere. In the male birds that were used to study the effects of the lentiviral-mediated RNAi in song behavior, each bird received an injection of the recombinant viruses (erbin RNAi or erbb2 RNAi) or the control viruses in both hemispheres of the brain.

After recovery from the surgery, the birds were returned to the cage and lived with their foster parents. On the seventh day following surgery, they received intramuscular injection of BrdU (50 μg/gbody weight in 0.75% NaCl; Sigma) twice daily (8:30 am and 6:30 pm) for two successive days to label the dividing cells. These birds were killed at 24 h (PHD 24), 7 d (PHD 31) or adulthood (PHD 130) after the final injection of BrdU (Fig. 2(E)).

The song recording was performed in a small soundproof room, and a single male bird (PHD > 120) was placed in a cage of the same size as the housing cages. The songs of each male were recorded using SAP software (Sound Analysis Pro 2011) and a condenser microphone (Behringer C-2) between 7:00 am and 7:00 pm after the bird had adapted to the new cage and began to sing. Recording was continued for at least 100 bouts for each bird.

After the song recording, the adult birds received the injections of a total of 500 nl retrograde tracer (dextran conjugated Alexa Fluor 647, 10,000 MW; Molecular Probes; 5% solutions made in 0.01 M PBS) into the RA at four sites (medial/lateral: 2.2 mm, anterior/posterior: 0.1 and 0.4 mm, dorsal/ventral: 2.2 and 2.6 mm) in both hemispheres using the same methods (including anesthetic method) as described for the injection of lentiviruses.

Immunohistochemistry and Song Analysis

The birds were deeply anesthetized, perfused and fixed using the above-described protocol. The brains were cut into 10-μm-thick sections, and every sixth sagittal section was mounted on a poly-lysine-coated slide. For the BrdU immunohistochemistry, the sections were incubated with 2 N HCl for 3 h and then incubated with Borax buffer (0.1 M, pH 8.4) for 30 min. All sections that were used for immunohistochemistry were first incubated in 3% H2O2 for 15 min to quench the endogenous peroxidase activities (this step was neglected for immunofluorescent staining) and then in a permeabilization/blocking buffer (5% normal donkey, rabbit or goat serum that was soluble in TritonX-100/0.1 M PBS) for 40 min at room temperature. The sections were then incubated with the rat anti-BrdU antibody (AbD Serotec, 1:2000) in blocking buffer overnight at 4°C. After the sections were washed, they were incubated with either fluorescent or biotinylated secondary antibody: Texas Red-conjugated donkey anti-rat (Jackson; 1:400), Alexa Fluor 350-conjugated donkey anti-rabbit (Jackson, 1:600), or Biotin-conjugated rabbit anti-γ-GABA (Pinaud and Mello, 2002), anti-NeuN (Scott and Lois, 2007; Zhao et al., 2009), and anti-GABA (Pinaud and Mello, 2007). The specificity of antibodies in the brain of songbird has been verified in previous reports, including anti-HuC/HuD (Louissaint et al., 2002), anti-NeuN (Scott and Lois, 2007; Zeng et al., 2009), and anti-γ-GABA (Pinaud and Mello, 2007). The antibody specificity was also confirmed from our staining patterns which were similar to related reports (Pinaud and Mello, 2007; Chen et al., 2014). To exclude nonspecific staining, the primary or secondary antibody
was omitted, but all other immunohistochemical procedures were the same as those described above. No staining was observed in these sections.

The recorded songs were quantitatively analyzed using SASLab Pro sound analysis software (Avisoft, Inc.) at the level of (1) the song produced repeatedly during a bout(s), (2) the phrase (or song motif) that appears repeatedly in different songs and composed of serial syllables that always appear in a relatively fixed sequential order, and (3) the syllable (or note) that appears as a continuous, morphologically discrete trace on the sonogram (Catchpole and Slater, 2008).
cDNA Microarray Analysis

Four male or female brain samples that contained the developing HVC and its adjacent parenchyma were obtained as described above [Fig. 1(E)] and combined for RNA extraction.

RNA quality and purity were assessed as described above. RNA was then reverse transcribed and labeled with either a Cy3 (male) or a Cy5 (female) fluorochrome (GE Healthcare Biosciences). The detailed methods for labeling using the two complementary dyes (Cy3 or Cy5) were described in a previous report (Li et al., 2008). Four matched male and female sample pairs were hybridized onto the Agilent Whole Chicken 60-mer oligonucleotide 44K microarray (Agilent Technologies). Each pair included RNA extracted from four male or female brains of nestling birds at PHD 15. Each microarray contained 42,034 probes described in a previous report (Li et al., 2008). Four microarrays for zebra finch compared to those for chicken (Li et al., 2008; Tomaszycki et al., 2009; Sun et al., 2010; Griffiths-Jones et al., 2006). Considering that our main goal in using microarray analysis was to determine the genes that are expressed in a sex-biased fashion in HVC, especially those in erbin- or erbb2-mediated pathways, we did not use the Affymetrix Lund-zf array. We also considered the following in our choice of microarray: 1) avian gene order, genome size, and chromosomal arrangements are highly conserved among avian species (Derjusheva et al., 2004; Backstrom et al., 2006; Dawson et al., 2007), and 2) blast analyses indicate that the similarity of EST sequence was 89.5% between zebra finch and chicken and 92.8% between zebra finch and house finch (Replogle et al., 2008).

Image Acquisition, Cell Counting and Statistical Analyses

Bright-field images were captured with a digital camera (Spot Enhance 2e; Diagnostic Instrument, Corp., USA) attached to a BH-2 microscope (Olympus), and fluorescent images were captured with a monochromatic digital camera (AxioCam MRm, Zeiss). Adobe Photoshop was used to process TIFF files. All labeled cells in the examined areas were counted manually using Image J 1.44 software (NIH Image program).

The boundaries of song nuclei were outlined, and their areas were obtained using Image-Pro Plus 5.2. The nuclei volumes were calculated by summing the areas in the series of sections in which song nuclei appeared and multiplying by six (sampling interval) and 10 µm (section thickness). Some examined measurements, such as the labeling for erbin and erbb2 mRNA in song nuclei, were assessed by the total numbers of positive cells in the song nuclei, which were obtained using the densities of the labeled cells (the ratios of the total numbers of positive cells to the sizes of their examined areas, numbers/mm³) multiplied by the nuclei volumes (mm³).

Song nuclei are generally characterized by some anatomical features including the relative sizes of cells and the density of cells to their adjacent areas. The outlines of song control nuclei studied in the present report could readily be distinguished in Nissl-stained sections, including the developing HVC at PHD 15. According to our previous study to inject a neural tract tracer (Dil) into the Area X in 600-µm sagittal brain slices at posthatch day 15 (Chen et al., 2014), the sizes of HVC determined by Area X-projecting cells were largely consistent with those obtained by Nissl-staining. The borders of HVC were thus determined with the aid of Nissl-staining in the present study. However, the boundaries of some song nuclei, including male/female LMAN and female Area X at PHD 15 or HVC at earlier ages than PHD 15, could not be clearly identified in Nissl-stained sections. The volumes of these nuclei were thus not obtained. These nuclei were not included in this report. For in situ hybridization sections for erbin and erbb2 mRNA, the boundaries of some song nuclei such as female HVC were not clearly identified, and the positions or sizes of these nuclei were determined by referencing those in the interval sections stained for cresyl violet.

The present study chose 15 days old birds as subjects, as this age is within the critical period for sexual differentiation, which is generally during the first few posthatch weeks, and not beyond 30 days after hatching (Konish and Developmental Neurobiology
Akutagawa, 1988). At the age of 15 days, a significant number of HVC cellular cohorts were reported to generate (Alvarez-Buylla et al., 1994; Burek et al., 1994). They are incorporated into HVC 1–3 weeks after they are produced within the VZ (Burek et al., 1994; Kirn et al., 1999).

Once the outline of the HVC was determined, BrdU+ cells along the VZ overlying the HVC (approximately 500 μm for males, and 300 μm for females) were counted at PHD 15 and expressed as the number of proliferating BrdU+ cells per mm. During the analyses, the experimenters were blind to sex and experimental treatments. The birds that did not survive to the scheduled ages or whose injection sites were outside the targeted areas were excluded from the study (not included in the sample data).

Statistical analyses were performed using the SPSS 11.5 software package. Two-way ANOVA was used to examine the effect of gender and different ages, and one-way ANOVA was conducted to compare the differences among the groups exposed to several treatments. Student’s t-test was used to compare the differences between the two groups under the same experimental conditions. Before ANOVA, the distributions of independent variables were tested for normality, and homogeneity of variance was assessed for equality of error variance (Levene’s test). The data for labeling density were averaged for each experimenters.

Results indicated that the sex differences in the number of HVC cellular cohorts were reported to generate (for erbin- or erbb2-mRNA expression, Fig. 1(A2-D3)). However, the labeling was negative for the sense probes of erbin- or erbb2-mRNA [Fig. 1(F,G)]. At PHD 15, some weakly stained small cells were observed to be scarcely distributed along the VZ overlying HVC [VZ is pointed by arrow heads, Fig. 1(A2-B3)]. The adult group was very similar to that at PHD 45 and the labeling is thus not shown in Figure 1. The labeled cells for erbin or erbb2 mRNA within HVC were counted, and the density or the total number of the labeled cells were obtained and compared among the studied groups (the sizes of female HVC were determined with reference to those in the interval sections for Nissl-staining, and the data for the density were not shown). By using two-way ANOVA with sex and age as factors, there was significant interaction between the effects of sex and age on the total number of the labeled cells for erbin mRNA and erbb2 mRNA within HVC [erbin mRNA: \(F(2, 135) = 35.45, \ p < 0.001; \) erbb2 mRNA: \(F(2, 14) = 41.76, \ p < 0.001\)]. Differences in the total number of the labeled cells in HVC were significant between the two sexes [erbin mRNA: \(t = 50.54, \ p < 0.001; \) erbb2 mRNA: \(t = 56.71, \ p < 0.001; \) Fig. 1(H1)].

We also examined the densities (cell numbers per mm³) of the labeled cells for erbin mRNA and erbb2 mRNA in the area (200 μm × 200 μm) just underneath the ventral bottom of HVC in each section containing HVC, and no significant changes were found between the males and females \((n = 5 \text{ for each sex})\) at PHD 15 [erbin: males: 90.0 ± 16.2/mm³; females: 80.8 ± 8.47/mm³; \(t = 1.13, \ p = 0.29; \) erbb2: males: 111.2 ± 22.6/mm³; females: 95.2 ± 19.0/mm³; \(t = 1.28, \ p = 0.26\)] and PHD 45 [erbin: males: 71.2 ± 13.3/mm³; females: 67.8 ± 10.17/mm³; \(t = 2.24, \ p = 0.13; \) erbb2: males: 93.6 ± 25.6/mm³; females: 84.8 ± 21.7/mm³; \(t = 2.59, \ p = 0.06\)].

Using qRT-PCR, we compared sex differences in the expression levels of erbin- or erbb2-mRNA in the brain slice samples that contained the developing HVC and its overlying VZ, and the areas adjacent to HVC (but not contain HVC), the long narrow brain belts at PHD 15 [approximately 300 μm wide and just under the above collected brain samples containing HVC, Fig. 1(E), delimited by two dashed lines]. The results indicated that erbin and erbb2 mRNA expression was significantly higher in the collected brain samples that contained the male HVC than in the collected brain samples that contained the female HVC [for erbin, \(t = 7.54, \ p = 0.012; \) for erbb2, \(t = 6.17, \ p = 0.031\)], or than in the brain belts that did not contain male or female HVC [for erbin, \(t = 6.35, \ p = 0.027; \) for erbb2, \(t = 5.98, \ p = 0.039\)]. There were no significant differences in the expression...
levels of erbin- or erbb2-mRNA between the male and female brain belts that did not contain HVC [for erbin, t = 0.86, p = 0.754; for erbb2, t = 0.64, p = 0.351; Fig. 1(J,K)].

**Effects of ErbB Family Inhibitor on Cell Proliferation in the VZ Overlying the Developing HVC**

Following the addition of the ErbB family inhibitor 4-(4-benzyloxyanilino)-6,7-dimethoxyquinazoline, and BrdU into the culture medium (both 10 μM) at PHD 15, the number of proliferating cells (BrdU⁺) per mm in the VZ that overlies the developing male HVC was examined 24 h after inhibitor addition. The number of BrdU⁺ cells per mm decreased by 63.11% compared to that observed for the culture medium that contained only DMSO [t = 34.61, p < 0.001; Fig. 2(A-C)].

**Effects of Erbin or erbb2 Knock-down on the Sexual Differentiation of the HVC and Adult Birdsongs**

Cultured brain slices have several limitations, including a short survival time (not beyond 7 days) and an inability to represent the in vivo brain completely. To resolve these problems, we constructed erbin- and erbb2-interfering lentiviruses, both of which contained the enhanced green fluorescent protein (EGFP) reporter gene downstream of the interfering fragment. Using qRT-PCR, we observed significant changes in the interference rates (the decreased percentage of the target mRNA) 7 days after erbin- or erbb2-interfering lentiviruses were injected into the male HVC at PHD 15, compared to injections of control lentiviruses [for erbin RNAi, the interference rates = 46.03 ± 5.78%, t = 13.58, p = 0.001; for erbb2 RNAi, the interference rates = 54.86 ± 4.76%, t = 9.75, p = 0.009; Fig. 2(D)]. As ERBIN or ERBB2 antibodies were not available for avian species, the changes in protein levels were not examined. In addition, EGFP could be detected in all experimental groups, including the adult group [PHD 130, Fig. 7], which indicated that lentivirus-mediated RNAi could be persistently produced in the whole HVC (not only limited to the adjacent areas of injection sites) in all the studied groups. In addition, we compared the density of EGFP cells (per mm²) in the male HVC at PHD 24 (9 days after injection of erbin, erbb2 or control lentiviruses), and there were no significant differences among the three groups (erbin, erbb2 and control RNAi) [n = 5 for each group, F(2,12) = 3.78, p = 0.43; Fig. 3]. These data suggested that different types of lentiviruses had similar infection activities within the HVC. The schematic of studies performed after the injections of erbin- or erbb2-interfering lentiviruses into the male HVC at PHD 15 is shown in Figure 2E.

**Effects of Erbin or erbb2 Knock-down on Cell Proliferation at PHD 24**

BrdU⁺ cells along the VZ overlying the developing HVC were examined 24 h after the final injection of BrdU (at PHD 24) in birds previously injected with lentiviruses with the interfering or control constructs [as in Fig. 2(E)]. At this time point, the proliferating cells (BrdU⁺ labeled) do not migrate out of the ventricular zone and do not begin to differentiate (Chen et al., 2014). As shown in Figure 4A1-B4, the cells infected with lentiviruses (GFP⁺) were distributed within the HVC, as well as its overlying VZ. However, no GFP⁺ cells were found outside the injected areas. The number of BrdU⁺ cells (per mm) along the VZ overlying the developing HVC significantly decreased in males [Fig. 4(A1-B2), 4(C); erbin: t = 8.72, p = 0.007, erbb2: t = 5.11, p = 0.02] or females [Fig. 4(A3-B4), 4(D); erbin: t = 6.79, p = 0.01, erbb2: t = 10.82, p = 0.005] after injection of erbin- or erbb2-interfering lentiviruses into the HVC at PHD 15, compared to the injection of control lentiviruses. To assess the effect scope of lentivirus injection to cell proliferation, we counted the numbers of BrdU⁺ cells along the VZ (for approximately 500 μm long, in which GFP⁺ cells were not seen) next to the injection regions (GFP⁺, overlying the developing HVC) in males, and found that the numbers (cells per mm) did not differ significantly between the groups receiving injections of erbin- or erbb2-interfering lentiviruses and the groups receiving injection of control lentiviruses [erbin vs control RNAi: n = 5, t = 0.58, p = 0.43; erbb2 vs control RNAi: n = 5, t = 0.78, p = 0.51].

**Effects of Erbin or erbb2 Knock-down on Early Neuronal Differentiation at PHD 31**

The numbers of BrdU-labeled, Hu-labeled (Hu is a marker expressed in early differentiated neurons, Barami et al., 1995) or BrdU and Hu double-labeled cells were examined in the HVC at PHD 31 (7 d after the final injection of BrdU). As the HVC is quite small in female Bengalese finches, the following inhibition studies were only performed in males. As shown in Figure 5A1-B2', the cells double-labeled for BrdU and Hu were observed in the developing HVC after the injection of erbin or erbb2-interfering...
lentiviruses into the HVC. When compared to the control groups, a significant decrease was observed in the total numbers of BrdU$^+$, Hu$^+$ or BrdU$^+$/Hu$^+$ cells within the HVC [for erbin RNAi, BrdU$^+$: $t = 43.87$, $p < 0.001$. Hu$^+$: $t = 23.11$, $p = 0.024$. BrdU$^+$/Hu$^+$: $t = 25.98$, $p = 0.031$. Fig. 5(A1-A2), 5(D-F); for erbb2 RNAi, BrdU$^+$: $t = 19.24$, $p = 0.035$. Hu$^+$: $t = 37.65$, $p < 0.001$; BrdU$^+$/Hu$^+$: $t = 20.06$, $p = 0.027$. Fig. 5(B1-B2), 5(D-F)]. We also examined the densities (cell numbers per mm$^3$) of BrdU$^+$, Hu$^+$ or BrdU$^+$/Hu$^+$ cells in the area (200 $\mu$m $\times$ 200 $\mu$m) just under the bottom of the HVC (no GFP$^+$ cells were seen) in the groups receiving injections of erbin- or erbb2-interfering lentiviruses [for erbin RNAi, BrdU$^+$ $2.31 \pm 0.46 \times 10^4$/mm$^3$; Hu$^+$ $7.65 \pm 1.98 \times 10^3$/mm$^3$; BrdU$^+$/Hu$^+$ $1.32 \pm 0.65 \times 10^3$/mm$^3$; for erbb2 RNAi, BrdU$^+$ $2.87 \pm 0.89 \times 10^4$/mm$^3$; Hu$^+$ $7.13 \pm 1.75 \times 10^3$/mm$^3$; BrdU$^+$/Hu$^+$ $1.87 \pm 0.64 \times 10^3$/mm$^3$] and the groups receiving control lentiviruses [$n = 4$ for each group; for erbin vs control RNAi, BrdU$^+$ $t = 0.32$, $p = 0.37$; Hu$^+$: $t = 0.55$, $p = 0.45$. BrdU$^+$/Hu$^+$: $t = 0.72$, $p = 0.57$. for erbb2 vs control RNAi: BrdU$^+$ $t = 0.51$, $p = 0.49$. Hu$^+$: $t = 0.89$, $p = 0.61$. BrdU$^+$/Hu$^+$: $t = 0.91$, $p = 0.77$].

**Effects of Erbin or erbb2 Knock down on Adult HVC and Birdsongs**

As in the zebra finch, song nuclei and song behavior are mature after PHD 120 in the Bengalese finch (Okanoya and Yamaguchi, 1997; Tobari et al., 2005). To study the effect of erbin or erbb2 knock-down on the adult HVC sizes and birdsongs, we examined changes in the sizes of the HVC and one of its targeting nucleus-RA, the number of BrdU-labeled cells that also project to the RA and the number of double-labeled cells for NeuN and BrdU or GABA and BrdU in the HVC at PHD 130.

After the injection of erbin- or erbb2-interfering lentiviruses into the HVC at PHD 15, the volumes of the HVC significantly decreased by 40.74% for erbin RNAi [$t = 35.47$, $p < 0.001$] and 49.42% for erbb2 RNAi [$t = 43.87$, $p < 0.001$].
RNAi \( t = 20.41, p = 0.008 \) in contrast to the control groups (both decreases in the sizes of HVC sections and the lengths along medial-lateral axis) [Fig. 6(A1-D1)]. Accordingly, the volumes of the RA were also significantly reduced following the injection of erbin- or erbb2-interfering lentiviruses into the HVC [erbin RNAi: \( t = 8.21, p = 0.02 \); erbb2 RNAi: \( t = 13.78, p = 0.004 \). Fig. 6(A2-D2)].

Following a neuronal tracer (dextran conjugated Alexa Fluor 647) injection into RA in which the neuronal tracer-filled cells were distributed in most of RA (>2/3) [Fig. 7D] (if not covered most of RA, the cases were not included), HVC\textsubscript{RA}-projecting neurons (HVC\textsubscript{RA} PNs) were observed in the HVC [Fig. 7(A1-A3, B1-B2, C1-C2)]. Some of these neurons were also labeled with BrdU (injected at PHD 22 and 23). We compared the percentages of BrdU-labeled cells that also project to the RA (double-labeled cells) to the total number of retrograde-labeled RA-projecting cells in the HVC and found that they decreased significantly after injection of erbin- or erbb2-interfering lentiviruses into the HVC compared to the control groups [erbin: \( t = 14.54, p = 0.007 \); erbb2: \( t = 9.32, p = 0.03 \); Fig. 7(F)]. In addition, following the injection of erbin- or erbb2-interfering lentiviruses into the male HVC, we compared the total number [Fig. 7(G,H)] and the density (cell numbers per mm\(^3\)) of NeuN\(^+/\)BrdU\(^+\) and GABA\(^+/\)BrdU\(^+\) cells with those in the groups receiving control lentiviruses [for NeuN\(^+/\)BrdU\(^+\) density, erbin RNAi: 1262 ± 103.12; control RNAi: 2364 ± 184.86; erbb2 RNAi: 1700 ± 153.09; control RNAi: 2481 ± 175.16; GABA\(^+/\)BrdU\(^+\) density, erbin RNAi: 205 ± 25.03; control RNAi: 254 ± 22.67; erbb2 RNAi: 160 ± 9.75; control RNAi: 179 ± 13.41]. There were significant decreases in the total number or density of NeuN\(^+/\)BrdU\(^+\) [for total number, erbin: \( t = 48.72, p < 0.001 \); erbb2: \( t = 36.78, p < 0.001 \); for density, erbin: \( t = 76.79, p < 0.001 \); erbb2: \( t = 68.45, p < 0.001 \); Fig. 7(G)] or in the total number, but not
in the density, of GABA+/BrdU+ cells [for total number, \( t = 7.23, p = 0.02; \) erbb2: \( t = 29.76, p < 0.001; \) for density, \( t = 3.45, p = 0.43; \) erbb2: \( t = 2.79, p = 0.67. \) Fig. 7(H)] in the HVC, compared to the groups receiving control lentiviruses. For normally reared Bengalese finches, a typical complete song generally began with “introductory” notes, followed by two phrases “A” and “B,” and ended with another phrase “C” (phrases “A”, “B,” or “C” each consist of syllables “a”, “b” or “c”, respectively) [Fig. 8(A)]. Introductory (i) notes and syllables “a”, “b” or “c” could be distinguished on the sonogram on the basis of various sound parameters, including duration (“i” < “a” < “b” < “c”), harmonic waves (two main frequencies in “a”, more than two frequencies in “b”, no dominant frequency in “c”), and frequency modulation (frequencies in “i” or “a” are highly modulated).

The total number of birds originally used for the analysis of song behavior was 20 (\( n = 5 \) for each group). However, three of these died from inadequate surgical operations (significant blood loss), and four birds did not survive to the scheduled age (unexpected death). We analyzed at least 25 songs (complete typical songs as described above for control birds, and relatively complete songs for treated birds with interfering lentiviruses). These birds included normally reared birds (\( n = 5 \)), birds receiving the injection of control lentiviruses (\( n = 3 \)), erbin- (\( n = 3 \)) or erbb2- (\( n = 2 \)) interfering lentiviruses at PHD 15. Analyzing more songs (\( > 25 \)) did not alter the results significantly for each studied bird. The numbers of repeating syllables “a”, “b,” or “c” are shown in Table 2. In all the birds that received injections of control lentiviruses, song organization was similar to that observed in the normally reared birds [Fig. 8(A,B)]. Additionally, the numbers of repeating syllables “a”, “b,” or “c” were within the range of normal birds. These data indicated that the surgical operation and control lentiviruses did not
substantially affect song learning and production. However, no audible songs were heard from two birds that received an injection of erbin- or erbb2-interfering lentiviruses, respectively. Song organization was abnormal in the other three birds that received the injection of erbin (n = 2) or erbb2 (n = 1)-interfering lentiviruses, compared to the normally reared birds or birds receiving control lentiviruses [erbin RNAi, Fig. 8(C); erbb2 RNAi, Fig. 8(D)]. These abnormalities were similar among the three birds, including the deletion of “i” notes and the syllable “a” (lost in all the recorded songs) as well as a decrease in the number of repeating syllables “b” or “c” in both erbin- and erbb2- RNAi groups [Fig. 8(C,D)]. Before statistical analysis, the data from the above three birds were combined and compared with the control lentivirus group. As shown in Table 2, the numbers of syllables “b” and “c” both significantly decreased after the injection of erbin- or erbb2-interfering lentiviruses into HVC at PHD 15 [for syllable “b”, \( t = 27.42, p = 0.02 \); for syllable “c”, \( t = 18.92, p = 0.03 \)].

cDNA Microarray to Identify Sex-Biased Genes in the Developing HVC of the Bengalese Finch

Finally, we used the Agilent Whole Chicken Genome Oligo Microarray, which contains approximately 40,000 probes or 28,000 genes (covering almost all protein-coding genes in the chicken genome), to examine the sex-biased genes during the sex differentiation of HVC, especially those in erbin- or erbb2-related signaling pathways. A total of 19,996 genes (including 569 on chromosome Z) were expressed in the developing HVC of nestling Bengalese finches at PHD 15. Of these, 2,098 genes were found to be significantly sex-biased (\( p < 0.05 \), one sample \( t \)-test, SPSS), including 1,672 genes that were located on chromosomes. These genes are summarized in Table 3 with different fold changes (FC) in expression level (FC >1.3, 1.5 or 2). A total of 103 sex-biased genes were mapped onto the chicken Z chromosome and did not show obvious valley or peak distributions along the chromosome [Fig. 9(A)].

The distribution of 367 sex-biased genes (FC >1.5; males: 173, and females: 194) in the chromosome is shown in Figure 9B. Overall, 27.17% of the male-biased genes and 5.15% of the female-biased genes were found on chromosome Z [Fig. 9(C)]. Of the sex-biased genes (FC > 1.5), 35 genes were classified into the category of “metabolic process” (i.e., protein metabolic process, RNA biosynthetic process and small molecule metabolic process), and 31 genes were classified into a “single-organism process” (i.e., signal transduction, single-organism cellular process and system development) [Fig. 9(D)]. In addition, 81 genes were classified into the category of “binding” (i.e., ion binding, protein binding and organic cyclic compound binding), 26...
Changes in double-labeled cells for BrdU and RA-projecting cells, BrdU and NeuN, and BrdU and GABA in the adult HVC at PHD 130 after injection of erbin- or erbb2-interfering lentiviruses into the developing HVC at PHD 15. A1-C1, BrdU-positive cells (white) and RA-projecting cells (HVCRAPNS, blue) in the adult HVC. Some RA-projecting cells were also labeled for BrdU (pointed by a small arrow). A2-C2, BrdU-positive cells (white), NeuN-positive neurons (red) and RA-projecting cells (blue) in the adult HVC. Cells that were labeled for NeuN and BrdU are indicated by small arrows. A3, BrdU-positive cells (white), GABA-positive neurons (red) and RA-projecting cells (blue) in the adult HVC. Green fluorescent protein (GFP) produced by the erbin- or erbb2-interfering lentiviruses can be observed in A1-A3. D, Injection site of neural retrograde tracer into RA. The dashed circle indicates the outline of RA, and the asterisk marks the approximate injection center. E, Schematic for the injection site of RA and retrograde-labeled cells in HVC. F-H, Changes in the percentage of cells labeled for both BrdU- and RA-projecting cells to RA-projecting cells (F), total numbers of cells labeled for both BrdU and NeuN (G) or GABA (H) in the adult HVC. *p < 0.05; **p < 0.01. ***p < 0.001. Error bars indicate SEM. Scale bar in D = 150 μm for A1-C1, 100 μm for A2-A3, 350 μm for D and 50 μm for inserts in A1-C1. [Color figure can be viewed at wileyonlinelibrary.com]
genes were classified into “catalytic activity”, and 8 genes were classified into “receptor activity”, according to the gene product’s molecular function.

Twenty-one genes in Erbin- or ErbB2-located signaling pathways were found to be expressed in a sex-biased fashion (Table 4). However, no significant gene alterations were detected in the ErbB2-interacting NF-κB pathway. These altered genes, including their names, GenBank accession number, chromosomal location, fold change in their expression levels between the two sexes and p-values (one sample t-test, SPSS), are listed in Table 5.

Table 2 The Numbers of Repeating Syllable “a”, “b,” and “c” in the Songs of Adult Bengalese Finches after the Injection of the Lentiviruses into the Developing HVC at PHD 15

<table>
<thead>
<tr>
<th>Groups</th>
<th>Syllable “a”</th>
<th>Syllable “b”</th>
<th>Syllable “c”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
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<tr>
<td>Normal (n = 5)</td>
<td>8.42 ± 1.50</td>
<td>6–12</td>
<td>11.48 ± 2.21</td>
</tr>
<tr>
<td>Control lentivirus (n = 3)</td>
<td>8.70 ± 1.01</td>
<td>7–10</td>
<td>12.19 ± 1.24</td>
</tr>
<tr>
<td>erbin-interfering lentivirus (n = 3)</td>
<td>-</td>
<td>-</td>
<td>3.15± ± 1.10</td>
</tr>
<tr>
<td>erbb2-interfering lentivirus (n = 2)</td>
<td>-</td>
<td>-</td>
<td>6.84± ± 2.30</td>
</tr>
</tbody>
</table>

*The mean difference is significant at the 0.05 level. The two groups of erbin or erbb2-interfering lentivirus (n = 5) were combined before statistical analysis with the control lentivirus group.

Developmental Neurobiology
DISCUSSION

In situ hybridization analyses indicated that the Z-linked gene erbin and its binding partner erbb2 were expressed within the HVC from PHD 15 to adulthood, and its overlying VZ at PHD 15, which was consistent with qRT-PCR analysis of the brain samples containing male and female HVC. Although some genes have been reported to be expressed in a sexually dimorphic manner in song nuclei through experiments that included suppression subtraction hybridization and cDNA microarray (Wade et al., 2004; Tomaszycki et al., 2009; Kato and Okanoya, 2010), the expression of erbin or erbb2 in male-biased ways is reported here for the first time.

Following the addition of the ErbB family inhibitor to the culture medium, we found that cell proliferation in the VZ that overlies the developing male HVC decreased significantly, which was further confirmed by injecting erbin- or erbb2-interfering lentiviruses into the developing HVC. We also observed decreases in the number of differentiated neurons (Hu+/BrdU+ or NeuN+/BrdU+) in the HVC, indicating that it overlies the developing male HVC decreased significantly. In addition, we found an abnormal organization of adult songs following the injection of erbin- or erbb2-interfering lentiviruses into the developing HVC at PHD 15. Finally, using Genome Oligo Microarray, 19,996 genes were found to be expressed in the developing HVC of nesting Bengalese finches, including 367 sex-biased genes and 173 male-biased genes (of which 27.2% were Z-linked genes, and 21 genes were located in Erbin- or ErbB2-related signaling pathways).

The following point is noteworthy: The boundaries of HVC, especially female HVC, were difficult to determine in brain slices (approximately 400 μm thick) without any staining, even under dark-field illumination (however, LH could be reliably identified). For RNA extraction from HVC, we used separate male or female brain samples containing HVC (but not exact HVC) in qRT-PCR and DNA microarray analysis. Thus, the extracted RNA was not only from the HVC and its overlying VZ but also from other areas in the samples. Notably, the HVC is larger in males than in females, and the relative contributions of the HVC and its adjacent tissue to the reported expression levels of genes including erbin or erbb2 mRNA vary between male and female samples. However, according to our qRT-PCR data from the narrow brain belts [just under the chosen samples containing the HVC, but the HVC was not contained, Fig. 1(F,G)], the expression levels of erbin and erbb2 mRNA did not differ significantly between the two sexes. Thus, if the distribution of a studied gene, like erbin or erbb2, had no significant sex difference in the areas adjacent to the HVC, the reported variations in the expression levels in the examined brain samples could also reflect, to some degree, those existing between male and female HVC. Actually, our in situ hybridization indicated that erbin- or erbb2-mRNA-positive cells were both located within the HVC in a male-biased way, reflecting by higher densities or more total numbers of the labeled cells for erbin- or erbb2-mRNA in male HVC [Fig. 1]. In contrast, the densities of the labeled cells for erbin mRNA and erbb2 mRNA in the area just underneath the ventral bottom of HVC did not show any significant changes between the males and females at PHD 15. These data strongly indicate that erbin or erbb2 mRNA expression in the HVC differ significantly between the two sexes.

It is known that lentiviral vectors can be stably incorporated into the genomic DNA of dividing or nondividing cells (Park, 2007). This is very important for birds, as it is not as easy as to create transgenic birds compared to mammals. Although lentiviral knockdown is often used in mammals, it has also been used in oscine species (Haesler et al., 2007; Schulz et al., 2010). Following the injection of lentivirus-mediated RNAi for the Forkhead box protein 2 (Foxp2) into Area X, both mRNA and protein levels are reduced without observed side effects in the zebra finch (Haesler et al., 2007). Our study indicated that after transferring the erbin- or erbb2-interfering lentiviruses into the developing HVC, lentivirus-mediated RNAi could be persistently

Table 3  The Number of Sex-Biased Genes Expressing in the Developing HVC of Bengalese Finch at Different FC Levels (p < 0.05)

<table>
<thead>
<tr>
<th>Mapped genes</th>
<th>FC</th>
<th>Sex-biased</th>
<th>Male-biased</th>
<th>Female-biased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1672</td>
<td>1.3&lt;FC&lt;1.5</td>
<td>547 (32.72%)</td>
<td>178 (10.65%)</td>
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<tr>
<td></td>
<td>1.5&lt;FC&lt;2</td>
<td>310 (18.54%)</td>
<td>137 (8.19%)</td>
<td>173 (10.35%)</td>
</tr>
<tr>
<td></td>
<td>FC&gt;2</td>
<td>57 (3.41%)</td>
<td>36 (2.15%)</td>
<td>21 (1.26%)</td>
</tr>
</tbody>
</table>

*All the sex biased genes could be localized to the chromosomes.*
produced in the HVC even at adulthood [Fig. 7A1-A3]. Positive cells for GFP (reporter gene for lentiviruses) were seen not only within HVC but also in its overlying VZ at PHD 24 [Fig. 4A1-B4]. However, GFP+ cells were not seen in the areas outside the injection regions. These data indicated that

**Figure 9** DNA microarray to identify sex-biased genes expressed in the developing HVC of Bengalese finches at PHD 15. A, Male/female expression ratios as a function of Z chromosome position (×107 bp) in chicken. There are a total of 103 genes on the Z chromosome that are expressed in the developing HVC in a significant sex-biased fashion. B, Distribution of male- and female-biased genes (with fold-change in expression greater than 1.5, p < 0.05 significant level) in the chromosomes. C, Percentages of sex-biased genes in autosomes and sex chromosomes (Z/W). D, Functional classification of sex-biased expression genes according to GO biological processes (left) and GO molecular function (right). [Color figure can be viewed at wileyonlinelibrary.com]
lentiviruses were successfully infected the cells within HVC and its overlying VZ but did not spread to wider areas through the vessels or other unknown pathways. As shown above, erbin and erbB2 were both expressed within the HVC from PHD 15 to adulthood, and in the overlying VZ at PHD 15.

### Table 4: Sex-Biased Genes Expressing in the Developing HVC of Bengalese Finch in Erbin Interacted Pathways at 1.3 FC level ($p < 0.05$)

<table>
<thead>
<tr>
<th>Signaling pathways</th>
<th>Male-biased genes</th>
<th>Female-biased genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC signaling pathway</td>
<td>ESR1, DAG1</td>
<td>-</td>
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<tr>
<td>PI3K-Akt signaling pathway</td>
<td>FRAP1, GSK3B</td>
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</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>MAP4K4, HSPA5, MAPK8IP3, MAPK8(PAK-JNKK-JNK)</td>
<td>CRK(PAK-JNKK-TNK); HSPB, MAPK14 (P38); HRAS, ATF4(Ras-Raf-MEK-ERK)</td>
</tr>
<tr>
<td>TGF-β signaling pathway</td>
<td>INHBA(ligand); SMAD1, SMAD2, SMAD3(R-Smads); CREBBP (coactivator family); ZNF423, RUNX3(cofactor)</td>
<td>SPP1(repressor)</td>
</tr>
<tr>
<td>NF-κB signaling pathway</td>
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<td>-</td>
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### Table 5: Details of Sex-Biased Genes in the Developing HVC of Bengalese Finch in Erbin Interacted Pathways

<table>
<thead>
<tr>
<th>GeneName</th>
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<th>Chromosome allocation</th>
<th>Fold Change (M/F)</th>
<th>p-value</th>
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<tr>
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</tr>
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(osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
Following the injection of erbin- or erbb2-interfering lentiviruses, the expression level of erbin or erbb2 significantly decreased in the HVC and its overlying VZ, which might cause changes in cell proliferation or cell differentiation (Olayioye et al., 2000; Nie and Chang, 2006; Roskoski, 2014; Tao et al., 2014). However, our data also indicated that the cell proliferation and differentiation towards Hu cells in the areas (no GFP cells) adjacent to the injection regions did not differ between the groups receiving injections of erbin- or erbb2-interfering lentiviruses with the groups receiving injection of control lentiviruses. Thus, the effect scope of lentivirus injection was limited, and it might be judged by the presence of GFP cells.

Although the different types of lentiviruses (erbin, erbb2 and control RNAi) had similar infection activities in the HVC, the total number of NeuN+/BrdU+ cells in HVC decreased more significantly than in GABA+/BrdU+ cells after injection of erbin- or erbb2-interfering lentiviruses into the male HVC at PHD 15 (erbin-interfering lentiviruses: NeuN+/BrdU+ decreased by 72.84% vs GABA+/BrdU+ decreased by 37.96%; erbb2-interfering lentiviruses: NeuN+/BrdU+ decreased by 66.67% vs GABA+/BrdU+ decreased by 57.69%). This was caused by significant decreases in the density of NeuN+/BrdU+ cells in HVC, but no significant changes in the density of GABA+/BrdU+ cells. As shown above, following the injection of erbin- or erbb2-interfering lentiviruses, the sizes of the HVC decreased by 40–50%, resulting in decreases in the total number of GABA+/BrdU+ cells in the HVC. However, we did not further examine whether the total number of cells in HVC also decreased (although neurons decrease, various types of glia cells may increase). The above inconsistent results concerning GABA+/BrdU+ or NeuN+/BrdU+ in the HVC might be ascribed to the fact that these cells have different birth sites or times (Alvarez-Buylla et al., 1994; Scott and Lois, 2007; Schulz et al., 2010). For example, GABA cells originate from the VZ in the subpallium, but other types of cells originate in the VZ of the pallium (Co bos et al., 2001). As a result, there were different effects of erbin- or erbb2-interfering lentiviruses on the production or differentiation of these cells.

The data from gene microarrays were largely consistent with a previous report that used the Affymetrix Chicken Genome Array, which includes 19,734 genes expressed in the brain, heart and gonads of E18 chicks, including 286 sex-biased genes in the brain (Ellegren et al., 2007). Using the same Genome Array, another report has shown that 14,548 probe sets appear in the anterior primitive streak region of chicken embryos, of which 246 genes are male-biased (Zhang et al., 2010). In addition, some reports have shown that the male-to-female ratios of Z chromosome gene expression are similar across tissues and ages between the two bird species (chicken and zebra finch) (Itoh et al., 2010), and male-biased or Z-linked genes are extensive in the brain of two species of songbirds, zebra finch (Taeniopygia guttata) and whitethroat (Sylvia communis) (Naurin et al., 2011). However, unlike the chicken Z chromosome, in which there is a reduced male-to-female ratio of gene expression near MHM (male hypermethylated) (Melamed and Arnold, 2007), no obvious valley values or peak values in the male-to-female ratio of gene expression occur along Chromosome Z for the studied sex-biased genes in the Bengalese finch [Fig. 9A] or zebra finch (Itoh et al., 2010). These data suggest that the mechanisms for dosage compensation may differ among avian taxa. A detailed study of dosage compensation in the developing HVC of Bengalese finch is underway in our lab. We are attempting to use RNA-seq analysis to study the male/female expression ratios of Z-linked genes to autosomal genes, as shown in a previous report (Graves, 2014).

Erbin does not affect the total level of erbb2 mRNA (Liu et al., 2008) or ErbB2 tyrosine phosphorylation (Huang et al., 2003). However, Erbin inhibits the ubiquitination of ErbB2. Its PDZ domain specifically binds to ErbB2 to increase its protein levels, and the expression of Erbin is elevated in some tumors, activating the ErbB2-dependent ERK pathway (Yao et al., 2015). Deletion of the PDZ domain (or Erbin silencing) hinders ErbB2-dependent tumorigenesis or tumor growth and decreases the differentiation of mature myelinating Schwann cells (Tao et al., 2009, 2014). In addition, by interacting with Ras-Raf in the MAPK pathway, Erbin can suppress ERK activation (Dan et al., 2010). Thus, Erbin can either promote or suppress ERK signaling through various mechanisms, resulting in varied effects such as the inhibition of NGF (nerve growth factor)-induced neuronal differentiation in PC12 (Huang et al., 2003; Dai et al., 2006, 2007) and the promotion of keratinocyte differentiation (Harmon et al., 2013). Through its involved signaling pathways (Ras-Raf-Mek-Erk, PI3K and PKC), ErbB2 plays roles in cell proliferation, migration and cell survival in cancer formation and in early vertebrate embryogenesis (Olayioye et al., 2000; Nie and Chang, 2006). Our results indicated that both cell proliferation in the VZ overlying the developing HVC and the number of differentiated neurons decreased significantly in HVC after down-regulation of erbin or erbb2, consistent with the above-mentioned reports. However, we did
not determine whether our results were caused by the interaction of Erbin and ErbB2 (through ErbB2-dependent ERK pathway) or the signal pathways directly mediated by Erbin (TGF-β, MAPK or NF-κB) or ErbB2 (Raf-Mek-Erk, PI3K or PKC). These issues should be further investigated in future studies.

The results of the cDNA microarray indicated that, of 21 sex-biased expression genes in Erbin and ErbB2 or their interacting signal pathways, most were located in the MAPK (including Pak-Jnkk-Jnk and Ras-Raf-Mek-Erk) and TGF-β signal pathways. In the MAPK pathway, several genes that are expressed in a male- or female-biased fashion could be involved in the sexual differentiation of HVC through different mechanisms, as mentioned above. However, we did not examine further whether phosphorylation levels changed in Erbin- or ErbB2-involved signal pathways following the down-regulation of erbin or erbb2.

Studies in breast cancer cells indicate that the nuclear estrogen receptor (ER) can be phosphorylated by the activated MAPK in Erbin- or ErbB2-mediated signal pathways, resulting in increased ER-related transactivation of genes, including those playing roles in cell proliferation and cell differentiation (Levin, 2003). Notably, such action occurs independently of estrogen. If the above action through MAPK pathway is also confirmed to be involved in the sexual differentiation of the HVC, it will explain, to some degree, why the sexual differentiation of song nuclei is not solely dependent on steroid hormones (Wade and Arnold, 1996; Agate et al., 2003; Zhao et al., 2010).

No significant differences were found in the song organization between normally reared birds and birds that received an injection of control lentiviruses, suggesting that the surgical operation and control lentiviruses did not substantially affect song learning and production (Haesler et al., 2007). However, no audible songs were produced, and song organization suffered from large changes in birds that received an injection of erbin- or erbb2-interfering lentiviruses into the HVC. In the present study, some birds did not survive to the scheduled age, and thus we did not obtain enough data to address the mechanisms underlying changes in song behavior. However, there was a significant decrease in the number of differentiated neurons in the HVC, including those projecting to the RA, in birds receiving erbin- or erbb2-interfering lentiviruses. This might be one reasons for the changes in song behavior.

It is needed to point out that among the song control nuclei with sexually dimorphic sizes, only HVC or Area X neurons are produced in the ventricular zone with markedly greater numbers in males than in females before the critical period (around 30 days after hatching) (Kirm and DeVoogd, 1989; Böttjer et al., 1994; Chen et al., 2014). Unlike HVC or Area X, almost all the neurons have been generated before hatching in other song nuclei such as RA and LMAN, and the neurons are similar in males and females prior to the critical period. However, the numbers of neurons and their volumes and sizes decrease after the critical period to a much greater extent in females than in males (Böttjer et al., 1986; Nixdorf-Bergweiler, 1996). Although the mechanism of this neuronal reduction remains unclear, unilateral HVC lesions at PHD 20 increased cell death and decreased neuron number and soma size within the ipsilateral RA (Akutagawa and Konishi, 1994; Chen et al., 2014). Thus more cell loss in females in other song nuclei might be primarily caused by the sexual differences in cell production of newborn cells in HVC or Area X. In addition, among song control nuclei, only HVC contains estrogen receptors, and HVC is thus most probably involved in estrogen mediated sexual differentiation of song control system. Considering the above findings, we chose HVC to study the mechanisms under which the song control nucleus is sexually differentiated.

In the present study we first indicated that both erbin and erbb2 have male-biased expressions in the developing HVC of the Bengalese finch by using qRT-PCR and in situ hybridization. Following the injection of erbin- or erbb2-interfering lentiviruses into the HVC and its overlying VZ at PHD 15, the cell proliferation in the VZ at PHD 24, the number of the differentiated neurons (Hu+/BrdU+ or NeuN+/BrdU+) in the HVC at PHD 31 or PHD 130, and the number of RA-projecting cells at PHD 130 all decreased significantly. These data indicated that both erbin and erbb2 are involved in cell proliferation and neuronal differentiation during the developing HVC. These data combined with the male-biased expressions of erbin and erbb2 in the developing HVC suggest that erbin and erbb2 are involved in the sexual differentiation (including cell proliferation and neuronal differentiation) of HVC. By using cDNA microarrays, we further found that approximately 20 genes were involved in the Erbin- or ErbB2-related signaling pathways. However, the details of Erbin- or ErbB2-involved signal pathways involved in the sexual dimorphism of HVC are required to be further clarified in future study.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTION

All authors had full access to all data in the study and took responsibility for data integrity and data analysis. Study concept and design: SJZ, XWZ, XJC, ZMH. Acquisition of data: YLZ, XBJ, RW, FW, YTZ. Analysis and interpretation of data: YLZ, XBJ, RW. Drafting of the article: SJZ, YLZ. Critical revision of the article for important intellectual content: SJZ, YLZ. Statistical analysis: YLZ, XBJ, FW. Administrative, technical, and material support: SJZ, XWZ. Administrative supervision: SJZ, XWZ.

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