Development/Plasticity/Repair

Adult Neurogenesis Leads to the Functional Reconstruction of a Telencephalic Neural Circuit

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Seasonally breeding songbirds exhibit pronounced annual changes in song behavior, and in the morphology and physiology of the telencephalic neural circuit underlying production of learned song. Each breeding season, new adult-born neurons are added to the pallial nucleus HVC in response to seasonal changes in steroid hormone levels, and send long axonal projections to their target nucleus, the robust nucleus of the arcopallium (RA). We investigated the role that adult neurogenesis plays in the seasonal reconstruction of this circuit. We labeled newborn HVC neurons with BrdU, and RA-projecting HVC neurons (HVC_{RA}) with retrograde tracer injected in RA of adult male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) in breeding or nonbreeding conditions. We found that there were many more HVC_{RA} neurons in breeding than nonbreeding birds. Furthermore, we observed that more newborn HVC neurons were back-filled by the tracer in breeding animals. Behaviorally, song structure degraded as the HVC-RA circuit degenerated, and recovered as the circuit regenerated, in close correlation with the number of new HVC_{RA} neurons. These results support the hypothesis that the HVC-RA circuit degenerates in nonbreeding birds, and that newborn neurons reconstruct the circuit in breeding birds, leading to functional recovery of song behavior.

Key words: adult neurogenesis; HVC; plasticity; regeneration; songbird; testosterone

Significance Statement

We investigated the role that adult neurogenesis plays in the seasonal reconstruction of a telencephalic neural circuit that controls song behavior in white-crowned sparrows. We showed that nonbreeding birds had a 36%–49% reduction in the number of projection neurons compared with breeding birds, and the regeneration of the circuit in the breeding season is due to the integration of adult-born projection neurons. Additionally, song structure degraded as the circuit degenerated and recovered as the circuit regenerated, in close correlation with new projection neuron number. This study demonstrates that steroid hormones can help reestablish functional neuronal circuits following degeneration in the adult brain and shows non–injury-induced degeneration and reconstruction of a neural circuit critical for producing a learned behavior.

Introduction

Neurogenesis in the adult brain is a striking form of plasticity. The contribution of newborn neurons to functional plasticity of neural circuits is an interesting topic that is challenging to address in the leading mammalian models of adult neurogenesis. New neurons added to the olfactory bulb in mammals are interneurons (Lledo et al., 2006; De Marchis et al., 2007), and those added to the dentate gyrus synapse locally (Toni et al., 2008). The avian song control system, however, provides a unique model for investigating the incorporation of new projection neurons into long-range neural circuits in the telencephalon.

New neurons are added to the telencephalic nucleus HVC (acronym used as a proper name, Reiner et al., 2004) of adult songbirds (e.g., Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Tramontin and Brenowitz, 1999; Absil et al., 2003; Balthazart and Ball, 2016). Most of these new HVC cells likely project at least 4 mm to synapse on neurons in the robust nucleus of the arcopallium (RA; Fig. 1*a*) (Alvarez-Buylla et al., 1990; Kirn and Nottebohm, 1993; Scotto-Lomassese et al., 2007). One study showed that new interneurons are also added to HVC (Scott and

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Lois, 2007). The HVC-RA circuit is necessary for the production of stereotyped song, a learned sensorimotor behavior used to attract mates and defend territories (Ölveczky et al., 2011). The functional significance of the addition of new projection neurons to the HVC-RA circuit in adult songbirds remains unclear, however (Brenowitz and Larson, 2015). Songbirds that breed seasonally and show pronounced seasonal plasticity of the song system provide an excellent model for investigating this topic.

Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) show pronounced seasonality of their reproductive physiology, song behavior, and the underlying neural circuitry for song production (Marler and Tamura, 1964; Wingfield and Farner, 1978; Smith et al., 1995). Circulating testosterone (T) levels increase at the onset of breeding and this induces a trophic cascade (largely mediated through BDNF) (Wissman and Brenowitz, 2009), which leads to an increase in HVC volume and total neuron number from ~90,000 to 160,000 cells (Tramontin et al., 2000; Brenowitz and Larson, 2015) (see Fig. 7). This increase results from increased incorporation and survival of newborn neurons in HVC (Larson et al., 2014). In nonbreeding birds, T concentration falls to basal levels, trophic support is withdrawn, HVC volume decreases, and neuron number rapidly decreases back to ~90,000 cells. This loss of neurons results from caspase-mediated apoptosis (Thompson et al., 2007; Thompson and Brenowitz, 2008; Larson et al., 2014). Changes in song behavior parallel these changes in the song system. Males sing stereotyped song at high rates while breeding and produce less stereotyped and fewer songs during the nonbreeding season (Smith et al., 1995; Meitzen et al., 2009).

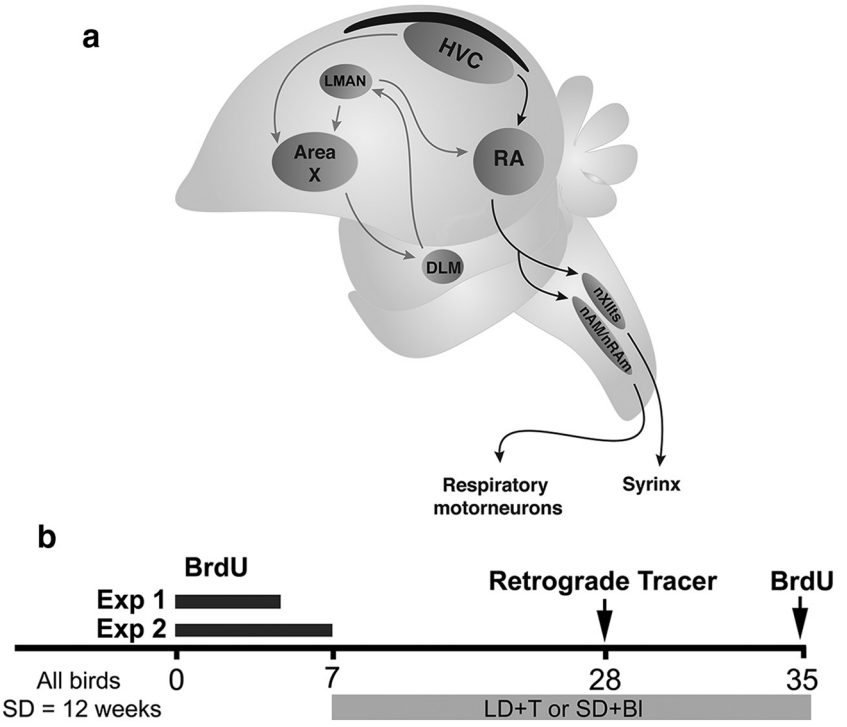

Figure 1. ***a***, Schematic diagram of the avian song control system. Black arrows indicate the song motor pathway. Gray arrows indicate the anterior forebrain pathway. DLM, Dorsolateral nucleus of the medial thalamus; nAM/nRAm, nucleus ambiguous/nucleus retroambigualis; nXIIts, tracheosyringeal portion of the hypoglossal nucleus. ***b***, Timeline of experimental procedures. All animals received 5 d of BrdU injections (Experiment 1) or 7 d of continuous infusion (Experiment 2) before hormone and photoperiod manipulations, as well as a single BrdU injection 2 h before death. Retrograde tracer was injected into RA 1 week before death.

It was unknown whether (1) the pronounced changes in HVC neuron number result in seasonal remodeling of the adult HVC-RA circuit, (2) the degree to which newborn HVC projection neurons (HVC_{RA}) directly contribute to regeneration of the circuit in breeding birds, and (3) increased song stereotypy in breeding birds is related to the incorporation of newborn HVC_{RA} neurons. Our study was designed to address these questions in adult male sparrows. We report here that white-crowned sparrows show pronounced seasonal changes in the total number of HVC_{RA} neurons, as well as the number of newborn neurons integrated to this circuit. Furthermore, the degree to which song stereotypy improves in breeding condition birds is highly correlated with the number of new HVC_{RA} neurons. Our results demonstrate that the addition of new projection neurons regenerates a neural circuit important for the production of a learned sensorimotor behavior.

Materials and Methods

Animals. We collected adult male Gambel's white-crowned sparrows in eastern Washington during their spring and fall migrations. Birds were group housed in outdoor aviaries for at least 12 weeks before being placed in indoor aviaries. Once inside, birds were exposed to a short day photoperiod (SD; 8 h light: 16 h dark) typical of their wintering grounds for at least 12 weeks before the start of experiments, which ensured that the birds were photosensitive and responsive to hormone manipulations (Tramontin et al., 2000). Food and water were available *ad libitum*. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines.

Experimental design. Experiment 1: We gave acute systemic injections of BrdU to label newborn cells, and injected a 3000 MW dextran retrograde tracer (microruby; Invitrogen D7162) in RA to back-label HVC_{RA} projection neurons (Fig. 1*b*) under breeding and nonbreeding conditions. RA neurons receive inputs from both the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and HVC (Fig. 1*a*). However, $LMAN_{RA}$ neurons are not added in adults (Nottebohm, 1985; Alvarez-Buylla et al., 1992); thus, the number of back-filled LMAN neurons is not expected to vary seasonally. The number of $LMAN_{RA}$ neurons therefore provides a good control for any seasonal changes of extraneous factors, such as retrograde uptake or transport of tracer, or survival of tracer-labeled neurons (Kirn and Nottebohm, 1993). We also recorded song and analyzed its structure (see below).

Experiment 2: In Experiment 1, we observed that microruby tended not to spread far from the injection site in RA. Kirn and Nottebohm (1993) made a similar observation when they injected latex microspheres into RA. To determine whether the seasonal differences observed in the total number of HVC_{RA} neurons, and in the number of newborn HVC_{RA} neurons, depended on the specific methods used in Experiment 1, we repeated the study using a different retrograde tracer (Fluorogold; Fluorochrome), as well as a different method of BrdU delivery (continuous release by osmotic pump), and different brain sectioning methods under the same breeding and nonbreeding conditions as Experiment 1.

Labeling new neurons. To mark dividing cells in Experiment 1, we injected birds on SD intramuscularly with bromodeoxyuridine (BrdU, 50 mg/kg dissolved in 7.5% NaCl and 15% DMSO) at the same time

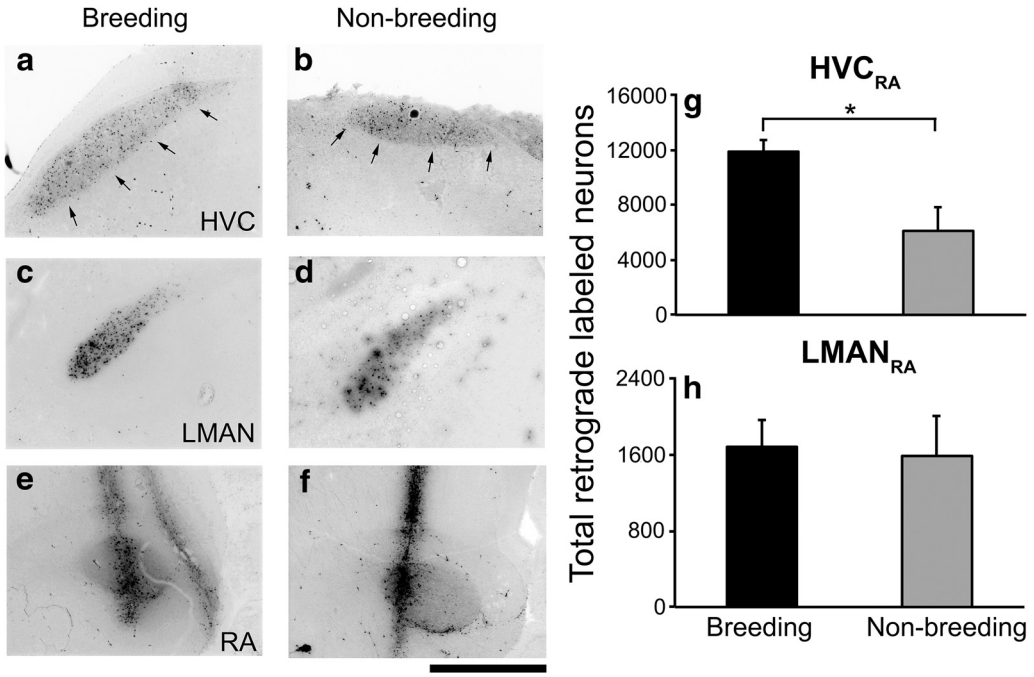

Figure 2. Representative photomicrographs of retrograde labeling (microruby) in HVC (***a***, ***b***) and LMAN (***c***, ***d***), and the injection site in RA (***e***, ***f***). Pictures from a breeding bird are on the left (***a***, ***c***, ***e***) and from a nonbreeding bird in the middle (***b***, ***d***, ***f***). ***a***, ***b***, Arrows indicate the ventral border of HVC. Scale bar, 1 mm. ***g***, HVC of breeding birds has a greater number of HVC_{RA} neurons than nonbreeding birds. ***h***, There is no difference in the number of $LMAN_{RA}$ neurons between breeding conditions. $n = 8$ (Breeding; LD + T) and 4 (Nonbreeding; SD + Bl). $^*p = 0.006$.

relative to "dawn" once per day for 5 d (acute BrdU administration; Fig. 1*b*). In Experiment 2, we labeled dividing cells by infusing BrdU (50 mg/kg dissolved in 7.5% NaCl and 15% DMSO) for 7 d via an osmotic pump (Alzet pump 1007D) implanted subcutaneously between the shoulders. All birds began this regimen of BrdU injections or infusions while on SD to ensure that we labeled the cells that would be incorporated into HVC during subsequent photoperiod and hormone treatments (see below). Two hours before death (in the morning), most birds also received a single intraperitoneal injection of BrdU, which labeled proliferating progenitor cells in the ventricular zone (VZ) of the lateral ventricles (Brown et al., 1993).

Breeding condition manipulation. In both Experiments 1 and 2, breeding and nonbreeding conditions were reproduced in a controlled manner in the laboratory by manipulating photoperiod and hormone levels (Smith et al., 1997; Tramontin et al., 2000). To induce breeding-like conditions, birds were housed on a long day photoperiod typical of their arctic breeding grounds (20 h light: 4 h dark) and given a subcutaneous T implant (LD + T, breeding) placed between the shoulders. This manipulation produces morphological and physiological changes in the song control nuclei, and increases in song stereotypy, similar to those observed in wild birds (Tramontin et al., 1998; Meitzen et al., 2009). To produce nonbreeding-like conditions, birds were maintained on the same SD photoperiod they were exposed to previously and received an empty implant as a control (SD + Bl, nonbreeding). The implants were constructed from SILASTIC tubing (1 mm inner diameter, 2 mm outer diameter, 20 mm length), either filled with 12 mm of T or left empty, and sealed at the ends with silicone. This treatment produces physiological plasma levels of T (Tramontin et al., 2003).

Hormone measurements. To measure hormone levels, blood was collected from the alar wing vein of each bird twice: once before hormone pellet implantation and once before death. Blood was collected into heparinized microtainer tubes (BD Biosciences), centrifuged and the plasma was stored at −20°C. We measured the concentration of T in plasma using an enzyme immunoassay kit (Enzo Life Sciences). Samples were run on three different days, and groups were randomly distributed between these days. Each run had an intra-assay coefficient of variation (CV) of 0.076, 0.154, or 0.266, with an interassay CV of 0.165 between the runs.

Song analysis (Experiment 1 only). One week before surgery, Experiment 1 birds were individually housed in sound isolation chambers (Industrial Acoustics or Coulbourn Instruments). Songs were recorded continuously for 4 d using Syrinx (J. Burt) or custom-made sound recording software, with sampling parameters described previously (sampling rate was 22050 Hz) (Meitzen et al., 2009). Song was also recorded from each bird 24 h before death. Depending on when the birds sang during these time periods, 20 song files were randomly selected from either the last day the bird sang before surgery (11 LD + T individuals), or the day before death (1 LD + T and 2 SD + Bl individuals) for a total of 12 LD + T and 2 SD + Bl birds analyzed. Birds in nonbreeding condition normally sing infrequently in captivity, which accounts for the small sample size for the SD + Bl group (Tramontin et al., 2000; Meitzen et al., 2009). Song files were bandpass filtered from 1.5 to10 kHz. Each syllable (whistle, warble, and buzzes; Fig. 2*a*) was extracted from these files. We performed a cross-correlational analysis (i.e., similarity in the energy distribution of two signals) on the spectrograms of whole songs and individual syllables using the batch correlator function in RavenPro 1.4 (Cornell Laboratory of Ornithology) (Larson et al., 2014). Each song or syllable was correlated with other renditions of that same song or syllable from the same bird to determine the percentage of signal similarity across renditions (i.e., stereotypy) for each individual bird. The average percent similarity score was calculated for each syllable and the whole song of all individuals analyzed. We did not analyze the third buzz (Fig. 2*a*) as birds in both reproductive conditions often omitted this syllable.

Surgery. Following anesthetization with isoflurane (2%), we made a small incision in the skin overlaying the skull, and a small hole was made in the skull above RA. A glass pipette filled with retrograde tracer (5% microruby in saline [Experiment 1] or 0.5% Fluorogold in water [Experiment 2]) (Kirn and Nottebohm, 1993; Reiner et al., 2000) was lowered bilaterally into RA using the intersection of the midsagittal and transverse sinuses as the reference point (anterior/posterior, −1.25 mm; medial/lateral, ±2.65 and 2.55 mm; depth, 2.35 and 2.45 mm). Approximately 110 nl of tracer was pressure injected at each of the four sites using a customized pressure delivery system. All injections were performed blind to the bird's treatment. One week following retrograde tracer injection, animals were deeply anesthetized and perfused with saline followed by

4% PFA. Brains were collected, postfixed in 4% PFA, soaked in sucrose, and stored at −20°C until processing.

Tissue was sectioned coronally at 40 μm on a cryostat into six series and thaw-mounted onto gelatin-coated slides (Experiment 1). Slides were stored at −20°C until further processing. Tissue from Experiment 2 was sectioned at 40 μm on a freezing microtome and stored in saline at 4°C until further use. All sections were collected for both experiments.

Immunohistochemistry. We performed immunohistochemistry (IHC) for BrdU on one series (every sixth section) and an IHC for both BrdU and neuron-specific marker (NeuN) on another series. For Experiment 1, IHC was conducted on slide-mounted sections. For Experiment 2, IHC was conducted on free-floating sections that were mounted on slides after the procedure. We rinsed tissue in 0.5% Triton X and 0.5% DMSO in 0.1 M PBS (PDTX) and treated with 2 M HCl at 37°C for 30 min. Tissue was rinsed with 0.1 M boric acid, pH 8.5, followed by PDTX. We blocked with 5% normal goat serum (NGS) for 1 h and incubated overnight at 4°C with primary antibodies (BrdU, 5 μg/ml, rat IgG, AbD Serotec, #MCA2060GA; NeuN, 2 μg/ml, mouse IgG, Millipore Bioscience Research Reagents, #MAB377) in NGS. Tissue was rinsed in PDTX and incubated with secondary antibodies (7.5 μg/ml, 488 goat anti-rat IgG #A11006, 568 goat anti-mouse IgG #A11004; Invitrogen) in 0.1 M PBS for 2 h at room temperature. Tissue was rinsed in PBS and coverslipped with 10% 0.1 M Tris buffer, pH 9.0, and 5 mg/ml *n*-propyl gallate in glycerol.

Cell counts. For both Experiments 1 and 2, every third section containing HVC (8 sections per hemisphere on average) or LMAN (3 sections per hemisphere on average) was examined for retrogradely labeled cells. We confirmed that tracer was present within the borders of RA and retrogradely labeled neurons were present in HVC and LMAN. We counted labeled neurons throughout HVC and LMAN. Axons of individual HVC neurons branch widely in different regions of RA (i.e., are not topographical), whereas individual LMAN neurons do have topographical projections to RA (Johnson et al., 1995; Yip et al., 2012). Labeled cells were therefore sampled throughout the full extent of HVC, but only in the sections of LMAN that contained tracer. To determine the density of backfilled neurons, four boxes (each 125 μm^2) were randomly placed in each sampled section of HVC or LMAN and all labeled cells were counted. Following these counts, the coverslips were removed and the same sections were Nissl-stained. HVC, LMAN, and RA volumes were calculated by tracing the borders of each nucleus as defined by Nissl staining (Tramontin et al., 1998) and volumes from both hemispheres of each nucleus were summed. The number of projection neurons was estimated by multiplying the density by the entire volume of HVC, or the volume of LMAN that contained retrogradely labeled cells, for each bird individually.

To ensure that tracer injections in RA were taken up and retrogradely transported by neurons throughout the full extent of HVC, we also determined HVC volume based on the distribution of labeled HVC_{RA} neurons. HVC volumes from both Nissl and retrograde tracer were compared using paired *t* tests for each treatment separately and combined. There was no difference between HVC volume as defined by Nissl or retrograde tracer in Experiment 1 (breeding only: $t = 0.36$, $p = 0.741$; nonbreeding only: $t = 0.68$, $p = 0.544$; both: $t = 0.095$, $p = 0.927$) or Experiment 2 (breeding only: $t = 0.21$, $p = 0.849$; nonbreeding only: $t = 1.72$, $p = 0.228$; both: $t = 1.28$, $p = 0.247$). These results show that both microruby and Fluorogold injections in RA successfully labeled RA-projecting neurons distributed throughout HVC.

All new neurons (cells that colabeled for BrdU and NeuN), and new HVC_{RA} projection neurons (cells colabeled for BrdU and tracer), were counted throughout HVC on every sixth section (4 sections per hemisphere on average). We also counted the number of BrdU-positive cells that were not colabeled with either tracer or NeuN, presumably glial or ependymal cells.

To determine the rate of proliferation of progenitor cells in the VZ, BrdU-positive cells were counted in all sections that exhibited arching of the VZ (Scott and Lois, 2007; Larson et al., 2014). New HVC neurons are born in this region of the VZ (Scott et al., 2012). BrdU-positive cells were only counted when they were on the ventral side of the ventricle, adjacent to HVC (12 sections per hemisphere on average).

Table 1. Testosterone levels and brain morphology across treatments[a]

	Breeding condition (LD + T)	Nonbreeding condition (SD + Bl)
T levels (ng/ml)		
Pre	0.42 ± 0.13	0.36 ± 0.11
Post	17.57 ± 2.38*	0.22 ± 0.07*
HVC volume (mm^3)		
Experiment 1	0.70 ± 0.03*	0.53 ± 0.05*
Experiment 2	2.12 ± 0.14*	1.10 ± 0.09*
RA volume (mm^3)		
Experiment 1	0.30 ± 0.02*	0.21 ± 0.01*
BrdU-positive cell no. in HVC		
Experiment 1	463 ± 95	535 ± 158
Experiment 2	797 ± 449	65 ± 27
BrdU-positive cell no. in VZ		
Experiment 1	2034 ± 129	1890 ± 287
HVC_{RA} density ($\times 10^2$ cells/mm^3)		
Experiment 1	173.22 ± 8.54*	100.74 ± 27.41*
Experiment 2	504.61 ± 44.63	592.01 ± 81.05

[a]Data are mean ± SEM. BrdU-positive cells are cells that did not colabel with other markers.

*Treatment groups differ significantly ($p \leq 0.008$, *t* test).

Statistics. After performing Levene's test for variance equality, we used independent *t* tests to determine whether there were differences between breeding and nonbreeding birds in Experiments 1 and 2 (SPSS Statistics, IBM). If the Levene's test revealed that the data did not meet the assumptions of the independent *t* test, the data were log-transformed before further statistical analysis (Kempermann et al., 2006). Pearson's correlations were used to determine whether there was a relationship between retrograde labeling in LMAN and HVC, as well as whether there was a relationship between the number of new neurons (total and retrogradely labeled) and song stereotypy or between the number of new neurons (total and retrogradely labeled) and T levels (Experiment 1 only). Final sample sizes are indicated in the figure legends, and all values reported are mean ± SEM. Cohen's *d* was used to calculate effect size (*d* values >0.8 indicate large effects) (Cohen, 1988).

Results

Confirmation of breeding condition

Our hormone and photoperiod manipulations were effective in inducing physiological and behavioral states typical of breeding (LD + T) and nonbreeding (SD + Bl) condition birds in both experiments.

Before hormone and photoperiod manipulation, there was no difference in plasma T concentration between animals in Experiments 1 and 2 that were later used in LD + T or SD + Bl conditions ($t_{(28)} = 0.27$, $p = 0.789$, $d = 0.30$; Table 1). At the time of death, birds exposed to LD + T had significantly higher T levels than those exposed to SD + Bl ($t_{(17.03)} = 7.28$, $p < 0.001$, $d = 3.53$).

Changes in HVC volume and neuronal number, and in behavioral song stereotypy, described below, also confirm the effectiveness of the hormonal and photoperiod manipulations.

Experiment 1

In Experiment 1, mean HVC volume was greater in birds in breeding compared with nonbreeding condition ($t_{(19)} = 3.04$, $p = 0.007$, $d = 1.39$; Table 1). RA volume was also larger in breeding compared with nonbreeding individuals ($t_{(11)} = 3.85$, $p = 0.003$, $d = 2.32$).

Breeding birds had more HVC_{RA} neurons (Experiment 1)

To quantify the number of HVC neurons that project to RA in breeding and nonbreeding conditions, we injected the retrograde tracer microruby into RA and counted the number of cells that were labeled in HVC (HVC_{RA} neurons). The number of retro-

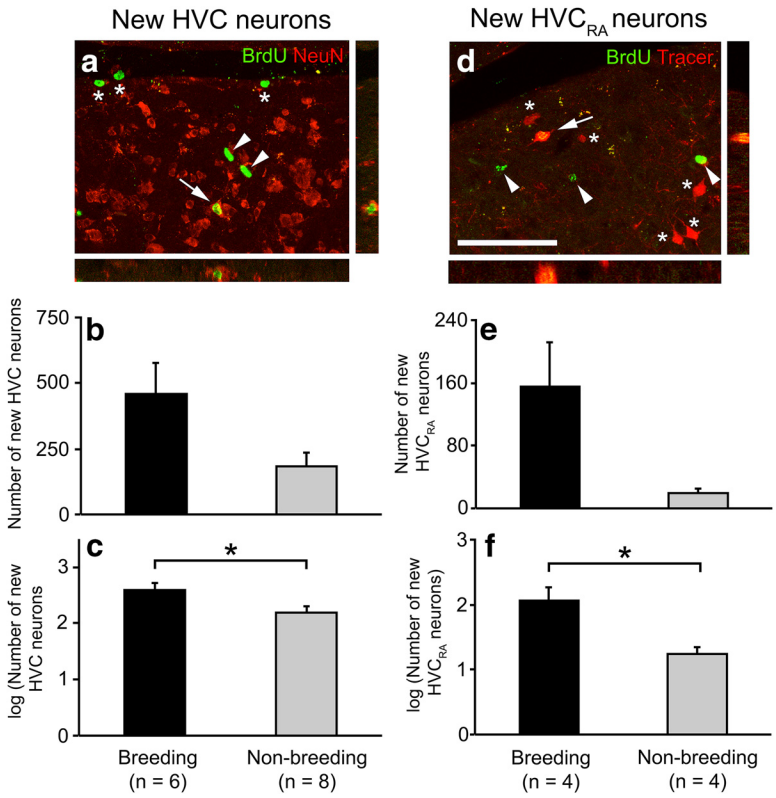

Figure 3. Neuronal addition to HVC (***a–c***) and HVC$_{RA}$ projection neuron addition (***d–f***) is higher in breeding than nonbreeding birds. ***a***, Cells labeled for NeuN (red) and BrdU (green). Arrow indicates a new HVC neuron that was double-labeled and is shown in *xz* and *yz* planes. Arrowheads indicate BrdU-positive cells that did not label for NeuN. Asterisks indicate BrdU-positive cells in the ventricular zone. ***b***, Raw data of the number of new neurons in HVC. ***c***, Log transformations revealed that there were significantly more new HVC neurons in breeding than nonbreeding birds ($n = 6$ breeding and 8 nonbreeding). ***d***, Labeling for retrograde tracer (microruby; red) and BrdU (green). Arrow indicates a new HVC$_{RA}$ projection neuron that was double-labeled, shown in *XZ* and *YZ* planes. Arrowheads indicate BrdU-positive cells that were not projection neurons. Asterisks indicate HVC$_{RA}$ neurons that were not BrdU-positive. ***e***, Raw data of the number of new HVC$_{RA}$ projection neurons. ***f***, Log transformations revealed that there were significantly more new HVC$_{RA}$ neurons ($n = 4$) in breeding compared with nonbreeding birds. Scale bars: ***a***, ***d***, 100 μm. ***c***, ***f***, *$p <$ 0.05.

gradely labeled neurons in HVC was greater in breeding compared with nonbreeding birds, with 49% fewer RA-projecting neurons in nonbreeding birds (Fig. 2*a*,*b*,*g*; $t_{(10)} = 3.47$, $p = 0.006$, $d = 2.20$). HVC$_{RA}$ neuron density was also greater in breeding compared with nonbreeding birds (Table 1; $t_{(10)} = 3.27$, $p = 0.008$, $d = 2.07$).

To determine whether there was a difference between treatment groups in either the injection of tracer or its retrograde transport, the number of retrogradely labeled neurons was also measured in the song nucleus LMAN. LMAN projects to RA but does not add new RA-projecting neurons in adulthood (Kirn and Nottebohm, 1993). There was no effect of reproductive condition on the number of labeled LMAN$_{RA}$ neurons (Fig. 2*c*,*d*,*h*; $t_{(10)} = 0.66$, $p = 0.523$, $d = 0.42$) or density ($t_{(10)} = 0.98$, $p = 0.351$, $d = 0.62$). We tested whether the number of labeled neurons in LMAN was related to the number of labeled neurons in HVC for all Experiment 1 animals using a Pearson's correlation. There was no correlation between these two variables for each breeding condition analyzed separately ($r^2_{(8,4)} \leq 0.77$, $p \geq 0.123$), or combined ($r^2_{(12)} = 0.04$, $p = 0.561$), indicating that there was no relationship between the numbers of retrogradely labeled HVC$_{RA}$ and LMAN$_{RA}$ neurons. Together, these data suggest that there was no systematic difference between treatment groups in the retrograde transport of tracer from RA to afferent HVC neurons.

Breeding birds had more new neurons in HVC (Experiment 1)

To quantify the number and type of new adult-born neurons, we counted the cells that were labeled for BrdU (a marker of cell division), NeuN (a neuron-specific marker), and/or retrograde tracer (an HVC$_{RA}$ neuron). We quantified the numbers of new HVC neurons (BrdU + NeuN), new HVC$_{RA}$ neurons (BrdU + tracer), and new non-neuronal HVC and VZ cells (BrdU only).

We found that there were more new HVC neurons in birds in breeding compared with nonbreeding conditions (Fig. 3*a–c*; $t_{(12)} = 2.57$, $p = 0.025$, $d = 1.48$). We also detected more new HVC$_{RA}$ neurons in breeding compared with nonbreeding birds (Fig. 3*d–f*; $t_{(6)} = 3.48$, $p = 0.013$, $d = 2.84$). The number of new non-neuronal cells in HVC ($t_{(12)} = 0.38$, $p = 0.708$, $d = 0.22$; Table 1) or VZ ($t_{(12)} = 0.62$, $p = 0.547$, $d = 0.36$) did not differ between breeding and nonbreeding condition birds.

Song stereotypy correlated with new HVC$_{RA}$ neurons (Experiment 1)

We used cross-correlational analysis for individual song and syllable files to determine the similarity of each syllable or the entire song across renditions for each bird. Whole song had a higher percent similarity in breeding than nonbreeding birds ($t_{(10)} = 4.42$, $p = 0.001$, $d = 2.80$; Fig. 4). Analysis of individual syllables revealed that each syllable also had a higher percent similarity in breeding than nonbreeding individuals (all $t \geq 3.07$, $p \leq 0.012$, $d \geq 1.942$). These behavioral results further confirm that the two treatment groups differed in physiological condition and are similar to previous work examining song stereotypy in white-crowned sparrows (Meitzen et al., 2009; Larson et al., 2014).

We performed a Pearson's correlation analysis to examine the relationship between new neurons and song stereotypy. Interestingly, we found that there was a strongly positive correlation between whole song stereotypy (from cross-correlations) and the number of new HVC$_{RA}$ neurons ($r^2_{(5)} = 0.98$, $p = 0.001$; Fig. 5*a*). This relationship was also true for whistle percent similarity ($r^2_{(5)} = 0.996$, $p < 0.001$; Fig. 5*c*) and warble percent similarity ($r^2_{(5)} = 0.83$, $p = 0.032$; Fig. 5*e*). There was no relationship between the number of new HVC$_{RA}$ neurons and other syllables ($r^2_{(5)} \leq 0.69$, $p \geq 0.082$; data not shown), or the total number of new neurons (BrdU + NeuN) with any measure of song quality ($r^2_{(6)} \leq 0.58$, $p \geq 0.078$; Fig. 5*b*,*d*,*f*). We also did not detect a correlation between the total number of retrogradely labeled HVC$_{RA}$ neurons and song stereotypy (all $r^2_{(9)}$ or $r^2_{(8)} \leq 0.37$, $p \geq 0.108$; data not shown).

Hormone levels correlated with new neurons (Experiment 1)

We performed a Pearson's correlation analysis to examine the relationship between T levels and neurogenesis. We found that there was a positive relationship between circulating T levels at

the time of death and both the number of new HVC_{RA} neurons ($r^2_{(8)} = 0.75$, $p = 0.005$; Fig. 5*g*) and the total number of new HVC neurons ($r^2_{(13)} = 0.41$, $p = 0.018$; Fig. 5*h*). There was no relationship between T levels and the number of new non-neuronal cells in HVC or VZ (all $r^2_{(13)} \leq 0.09$, $p \geq 0.325$).

Experiment 2

As in Experiment 1, we found that breeding condition birds had a larger HVC volume than nonbreeding birds ($t_{(8)} = 6.027$, $p < 0.001$, $d = 4.26$; Table 1), and these volumes were similar to those observed in previous studies (Larson et al., 2014). The results of Experiments 1 and 2 agree in showing that HVC increased in size in breeding condition birds, defined either by Nissl staining or the distribution of labeled HVC_{RA} neurons.

Experiment 2 used a chronic BrdU infusion to label new cells, a different retrograde tracer (Fluorogold), and different tissue processing methods (IHC on free-floating freezing microtome sections) in a different set of birds. The results from this experiment confirmed those from Experiment 1. Similar results were observed, such that there were 36% more HVC_{RA} neurons in breeding than nonbreeding individuals ($t_{(8)} = 2.66$, $p = 0.029$, $d = 1.88$; Fig. 6*a*,*d*,*i*), although there was no difference in HVC_{RA} neuron density (Table 1; $t_{(8)} = 0.95$, $p = 0.373$, $d = 0.67$). We did not find a difference between conditions in $LMAN_{RA}$ neuron number ($t_{(8)} = 0.07$, $p = 0.947$, $d = 0.05$; Fig. 6*b*,*e*,*j*) or density ($t_{(8)} = 0.31$, $p = 0.767$, $d = 0.22$). We observed more new neurons in HVC (log-transformed: $t_{(8)} = 3.92$, $p = 0.004$, $d = 2.77$; Fig. 6*g*,*k*,*l*) and more new HVC_{RA} neurons (log-transformed: $t_{(7)} = 3.13$, $p = 0.017$, $d = 2.36$; Fig. 6*h*,*m*,*n*) in breeding compared with nonbreeding individuals. There was no difference in new non-neuronal HVC cells between treatment groups ($t_{(8)} = 1.63$, $p = 0.142$, $d = 1.03$; Table 1).

We examined whether there was a relationship between circulating T levels and neurogenesis in Experiment 2 using a Pearson's correlation analysis. Similar to Experiment 1, we found a positive correlation between circulating T levels at the time of death and the number of new HVC neurons (log-transformed; $r^2_{(10)} = 0.55$, $p = 0.014$). Additionally, we did not detect a correlation between T levels and the number of new non-neuronal HVC cells ($r^2_{(10)} = 0.08$, $p = 0.436$). The correlation between T levels and new HVC_{RA} neurons approached significance (log-transformed; $r^2_{(9)} = 0.43$, $p = 0.055$).

We found that Fluorogold spread considerably further from the injection site in RA and labeled more HVC_{RA} neurons than did microruby. The differential spread of these tracers, however, did not alter any of the fundamental results of our study. Breeding birds had larger HVC as defined by tracer labeling, and a greater number of both newborn and total HVC-RA neurons than did nonbreeding birds, regardless of the tracer injected in RA. Replication of these results using different tracers, different ways of delivering BrdU, and different tissue processing methods for IHC increases confidence in the results.

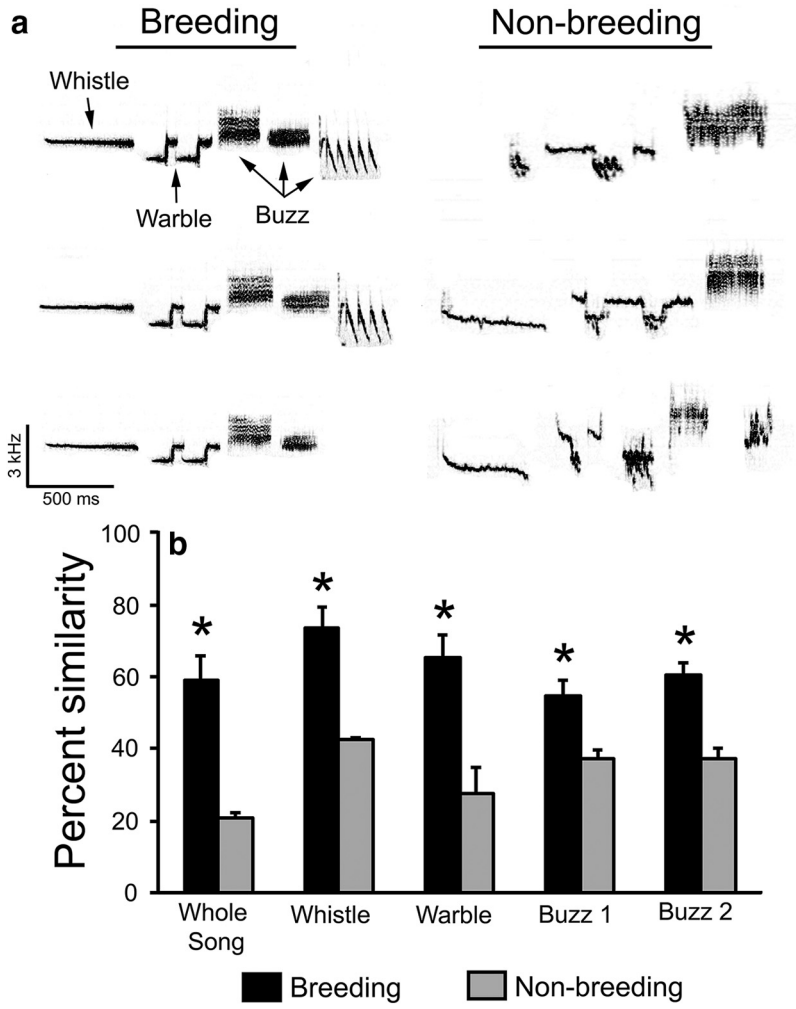

Figure 4. Birds in breeding condition sing more stereotyped song than those in nonbreeding condition. ***a***, Three song exemplars from one bird in breeding condition (left) and a different bird in nonbreeding condition (right). ***b***, The percent similarity between different renditions of whole song and individual syllables for breeding and nonbreeding individuals. The terminal buzz is sometimes omitted from song and therefore was not included in analysis. *$p < 0.05$. $n = 10$ (LD + T) and 2 (SD + BI). Some birds in nonbreeding condition do not sing in isolation from other birds, which accounts for the small sample size in that group.

Effect sizes

In both Experiments 1 and 2, the magnitude of the differences between breeding and nonbreeding condition birds in the numbers of new HVC and new HVC_{RA} neurons, and the number of all HVC_{RA} neurons, is quite large. The Cohen's *d* values for all statistically significant comparisons are >1.48. As a reference, a *d* value of 1.5 indicates that the mean values for breeding birds are >93% of the values for nonbreeding birds (Sullivan and Feinn, 2012). This analysis indicates that the seasonal change in the HVC-RA circuit is a robust phenomenon.

Discussion

The birth of new neurons and their integration into circuits in the adult brain has been the focus of intensive research. The functional significance of adult neurogenesis, however, continues to be unclear. Here, we demonstrate that new neurons contribute to the regeneration of a telencephalic long-range neural circuit in

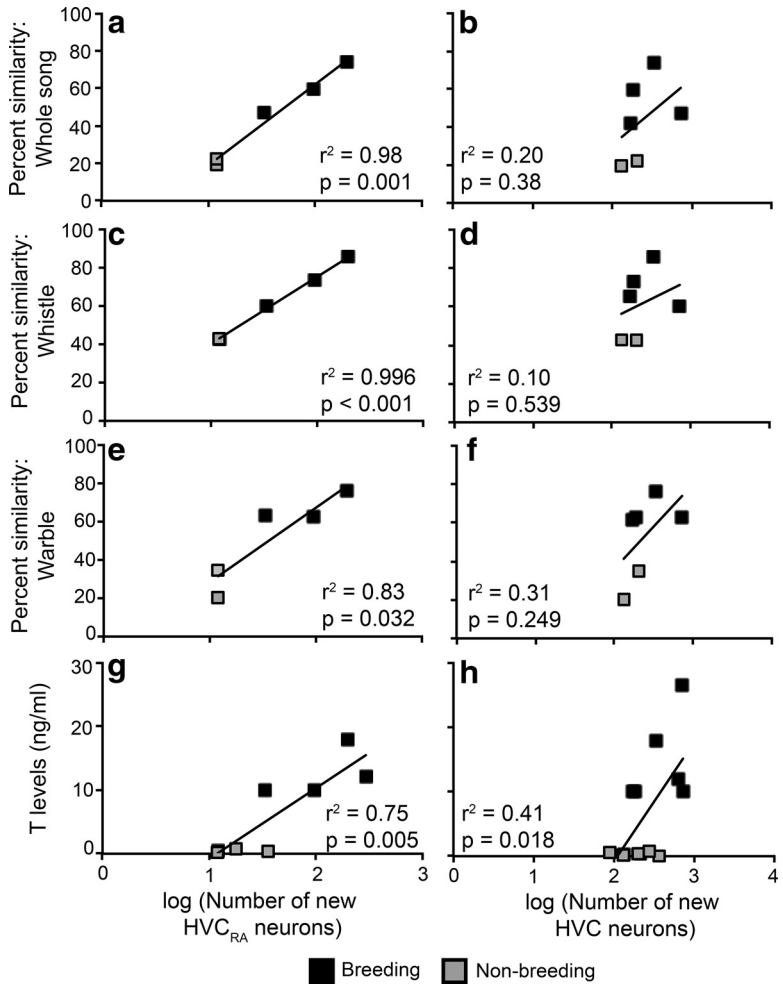

Figure 5. The integration of new projection neurons to HVC is correlated with song quality and T levels. The number of new HVC_{RA} neurons is correlated with the percent similarities of whole song (***a***), whistle (***c***), and warble (***e***), and is also correlated with circulating T levels (***g***). The total number of new HVC neurons did not correlate with song measures (***b***, ***d***, ***f***) but is correlated with T levels (***h***). Log-transformed data were used for correlational analysis.

adult songbirds, and this leads to improved performance of learned song.

Plasticity of a neural circuit in an adult brain

We found that the premotor HVC-RA circuit in the song system of adult white-crowned sparrows is seasonally degraded and reconstructed. As many as 46% of HVC neurons that project to RA die in nonbreeding birds due to caspase-dependent apoptosis (Thompson and Brenowitz, 2008) (Fig. 7). In breeding birds, this circuit is regenerated as newborn neurons are added to HVC and grow axons to innervate RA neurons. These seasonal changes in the number of HVC_{RA} neurons are correlated with changes in circulating T levels and with changes in song duration and stereotypy.

Our results agree with previous research in adult male canaries (*Serinus canaria*), which showed that HVC_{RA} projection neurons are replaced over time (Kirn et al., 1991; Kirn and Nottebohm, 1993). Sparrows differ from canaries, however, in the extent and seasonal timing of turnover in HVC_{RA} neurons. Kirn et al. (1991) reported that there were no seasonal differences in the volume of HVC, the number or proportion of HVC_{RA} neurons, or the total number of HVC neurons in canaries. Other studies reported a small, transient reduction in HVC volume in early fall, and that the density of newborn HVC neurons, and the density and percent of newborn HVC_{RA} neurons, were somewhat greater in the fall in canaries (Alvarez-Buylla et al., 1990; Nottebohm et al., 1994). By contrast, we found that the number of all HVC neurons, newborn HVC neurons, all HVC_{RA} projection neurons, and newborn HVC_{RA} projection neurons were greater in breeding white-crowned sparrows than nonbreeding birds. The effect sizes for all comparisons in sparrows are large, showing that the seasonal changes in the HVC-RA circuit are robust in this species. It is not clear why canaries and sparrows differ; this may reflect adult song learning in canaries but not sparrows, or more pronounced reproductive seasonality in the arctic breeding sparrows than in the subtropical canaries (Bentley et al., 2003; Leitner et al., 2003).

An alternative explanation of our results might be that seasonal change in HVC_{RA} neurons was not due to seasonal loss and replacement of these neurons but to axon retraction (Luo and O'Leary, 2005) and regrowth. Several observations are inconsistent with this scenario. First, many HVC_{RA} neurons in breeding birds are labeled by BrdU and therefore likely newborn. Second, the decrease in HVC_{RA} neurons we observed is consistent with previous measurements showing that total HVC neuron number rapidly decreases by 40% in nonbreeding birds (Tramontin et al., 1998; Thompson et al., 2007; Larson et al., 2014). Third, the heavily myelinated HVC-RA fiber tract is visible under polarized light in unfixed tissue from nonbreeding sparrows, albeit of reduced size (E.A.B., unpublished observation). Together, these observations indicate that seasonal changes in the number of tracer-labeled HVC_{RA} neurons result from the death and replacement of projection neurons. Further support for this conclusion is the lack of seasonal change in the number of $LMAN_{RA}$ neurons, which suggests that our results cannot be explained by changes in the uptake or transport of the tracer injected in RA (see also Kirn and Nottebohm, 1993).

Previous research has shown that most, if not all, new HVC neurons project to RA (Kirn et al., 1991; Scott and Lois, 2007; Scotto-Lomassese et al., 2007). Not all new HVC neurons in our study were backfilled with tracer, similar to previous work showing that only approximately half of new HVC neurons were labeled by tracer injected in RA of adult zebra finches (*Taeniopygia guttata*) (Walton et al., 2012). One possible explanation for unlabeled new neurons is that 4 weeks after BrdU injection may not be long enough for all new neurons to grow axons to RA. Research in canaries showed an increase in the number of new retrogradely labeled HVC neurons over 240 d after birth (Kirn et al., 1991), suggesting that some new HVC neurons require >4 weeks to synapse on RA neurons. It is also possible that some new HVC neurons that were not labeled could be interneurons (e.g., Scott and Lois, 2007).

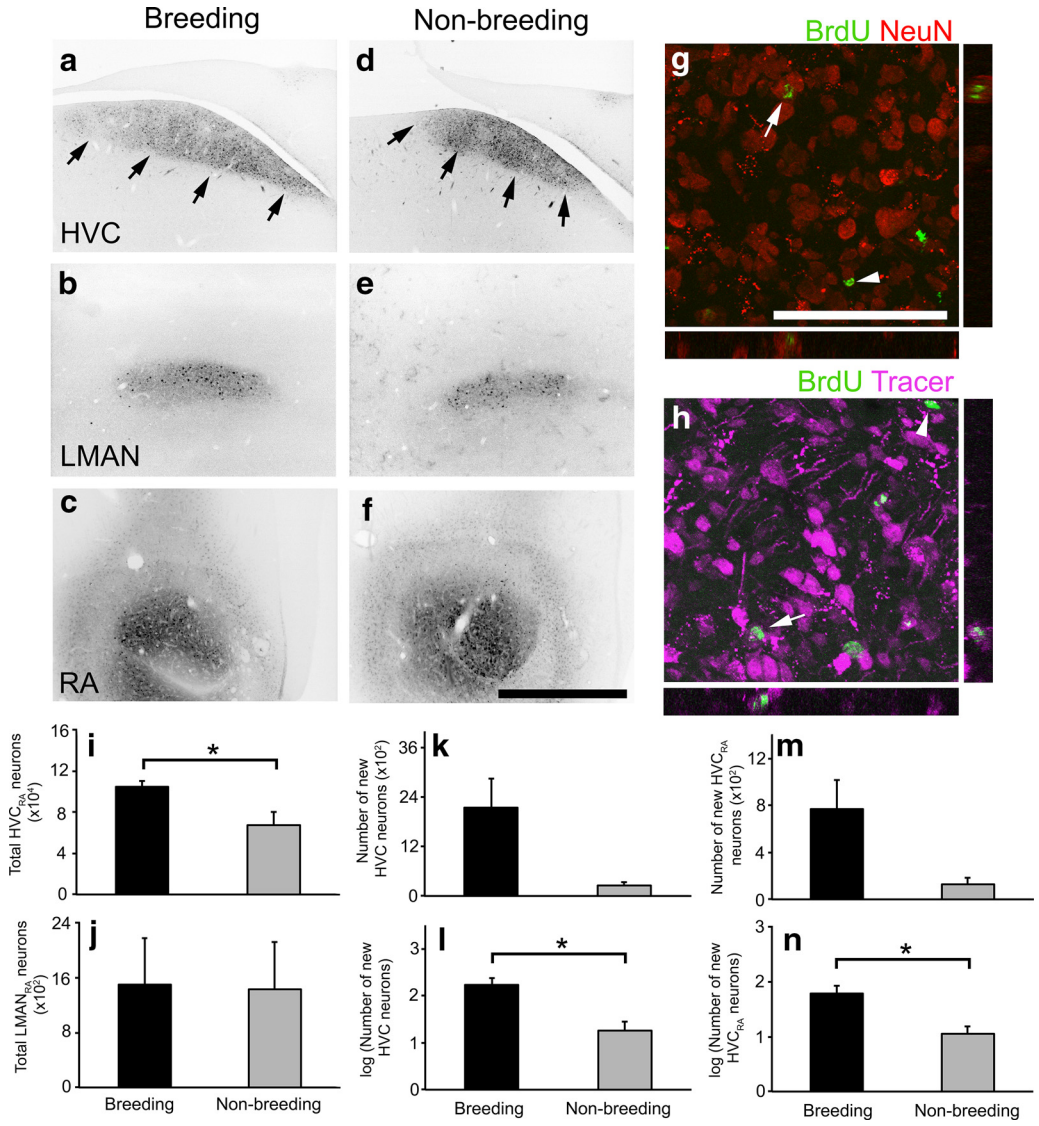

Figure 6. Results from Experiment 1 were confirmed using a chronic BrdU infusion with an osmotic pump and a different retrograde tracer (Fluorogold) in Experiment 2. Representative photomicrographs are shown of retrograde labeling in HVC (***a***, ***d***) and LMAN (***b***, ***e***), as well as the injection site in RA (***c***, ***f***). Pictures from a breeding bird are on the left (***a–c***) and from a nonbreeding bird in the middle (***d–f***). ***a***, ***d***, Arrows indicate the ventral border of HVC. Scale bar, 1 mm. Representative photomicrographs of (***g***) new neurons (NeuN, red and BrdU, green) and of (***h***) new retrogradely labeled neurons (Fluorogold, magenta and BrdU, green). ***g***, ***h***, Arrows indicate a double-labeled cell that is shown in the *XZ* and *YZ* plane. Arrowheads indicate a BrdU-positive cell that did not label for NeuN (***g***) or tracer (***h***). Scale bars: ***g***, ***h***, 100 μm. ***i***, There were more HVC_{RA} neurons in birds in breeding compared with nonbreeding conditions, with no effect on the number of $LMAN_{RA}$ neurons across conditions (***j***). There were more new HVC neurons (***k***, ***l***) and new HVC_{RA} neurons (***m***, ***n***) in breeding compared with nonbreeding conditions. ***k***, ***m***, Raw data. ***l***, ***n***, Log-transformed data. $n = 5$. *$p < 0.05$.

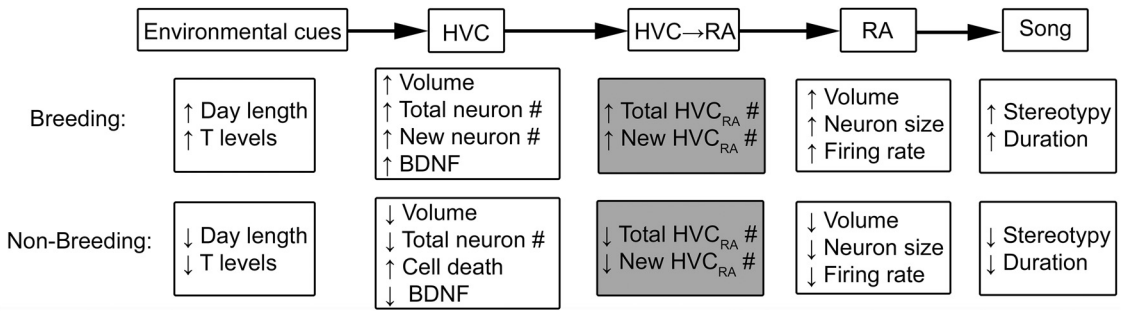

Figure 7. Summary of seasonal changes in the adult white-crowned sparrow song system. As day length increases early in the breeding season, testosterone (T) levels increase. T upregulates expression of BDNF mRNA in HVC cells. T and BDNF increase the survival of new neurons, and total neuron number and volume of HVC increase. Here we demonstrate that many of these new neurons send axons to RA and the HVC-RA circuit is reconstructed. The size and activity of neurons in RA increase, and song becomes more stereotyped. In the nonbreeding season, T concentration drops to basal levels, BDNF expression is silenced, and mature and newborn HVC neurons, including many that project to RA, die from the loss of trophic support. The HVC-RA circuit degrades, electrical activity and size of RA neurons decrease, and song becomes less stereotyped. Shaded boxes represent new information from the current study.

It is interesting that the HVC-RA circuit was not completely eliminated when it degenerated in nonbreeding birds. Approximately half of HVC_{RA} neurons persisted in the degraded circuit. It may be that adult-born HVC neurons can only grow axons to RA by following existing pathways, as shown for adult-born neurons in the mammalian olfactory system (Ma et al., 2014). On a functional level, wild white-crowned sparrows sing in the fall and winter, albeit with less stereotypy and less often. They forage in flocks during the day and roost communally at night, and song may play a role in social cohesion in these contexts (Brenowitz, 1981). Without some remaining connectivity between HVC and RA, nonbreeding birds would not be able to sing (Brainard and Doupe, 2013).

Circuit reconstruction and song behavior

We confirmed that breeding song was more stereotyped than nonbreeding song (see also Smith et al., 1995; Meitzen et al., 2009). The degree of stereotypy, measured as the cross-correlation coefficient, was highly correlated with the number of new HVC_{RA} neurons. The introductory whistle is the dominant cue for learning and recognizing conspecific song in white-crowned sparrows (Soha and Marler, 2000; Nelson, 2007), and its stereotypy was strongly correlated with new HVC_{RA} neuron number. These high correlations suggest that integration of newborn HVC_{RA} neurons to reconstruct the circuit enables males to produce high-quality songs to attract females and defend territories. Similarly, in zebra finches, the number of new neurons in HVC is correlated with song quality (Pytte et al., 2011, 2012). In contrast, the total number of new HVC neurons in our study did not correlate with song stereotypy. New neurons that are not labeled with retrograde tracer may not yet have made connections with RA or may be interneurons (i.e., neurons not directly involved in song production).

Hormones support circuit regeneration

Annual changes in circulating T levels in adult white-crowned sparrows drive the seasonal changes in HVC and RA summarized in Figure 7 (for an extended discussion of this model, see Brenowitz and Larson, 2015). Briefly, early in the breeding season, circulating T levels increase and trigger a trophic cascade. With this trophic support, more newborn HVC neurons survive and total neuron number increases. In the present study, we showed that many of these new neurons grow axons to RA and reconstruct the HVC-RA projection, which is associated with the frequent production of stereotyped, longer song. When the breeding season ends, plasma T drops to basal levels, trophic support is withdrawn, and many newborn and mature HVC neurons undergo apoptosis. We showed in the current study that up to half of the HVC_{RA} neurons are lost and this circuit degenerates. Song becomes less stereotyped, shorter, and less frequent. This cycle repeats annually in adult sparrows. Similarly, steroid hormones support the survival of new neurons in the mammalian dentate gyrus (Mahmoud et al., 2016). In canaries, T increases the survival of new HVC neurons, and this is mediated by BDNF (Rasika et al., 1994, 1999).

In conclusion, we showed that there is extensive plasticity of a telencephalic long-range neural circuit in adult white-crowned sparrows. The HVC-RA circuit degenerates in nonbreeding birds following the death of up to half of its neurons, and is reconstructed in breeding birds by the integration of newborn HVC_{RA} neurons. This circuit is important in the motor production of learned song, and the stereotypy with which birds sing changes as the HVC-RA circuit degenerates and regenerates seasonally. The reconstruction of this circuit in breeding birds correlates with plasma T levels, requires the integration of adult-born projection neurons, and contributes to birds' ability to produce stereotyped song. This study demonstrates that steroid hormones can help reestablish functional neuronal circuits following degeneration in the adult brain.

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