Postsynaptic neural activity regulates neuronal addition in the adult avian song control system

Tracy A. Larson[a], Tsu-Wei Wang[b,1], Samuel D. Gale[c], Kimberly E. Miller[b,d], Nivretta M. Thatra[a,b], Melissa L. Caras[c], David J. Perkel[a,d], and Eliot A. Brenowitz[a,b,2]

Departments of [a]Biology, [b]Psychology, and [d]Otolaryngology, and [c]Graduate Program in Neurobiology and Behavior, University of Washington, Seattle, WA 98195

A striking feature of the nervous system is that it shows extensive plasticity of structure and function that allows animals to adjust to changes in their environment. Neural activity plays a key role in mediating experience-dependent neural plasticity and, thus, creates a link between the external environment, the nervous system, and behavior. One dramatic example of neural plasticity is ongoing neurogenesis in the adult brain. The role of neural activity in modulating neuronal addition, however, has not been well studied at the level of neural circuits. The avian song control system allows us to investigate how activity influences neuronal addition to a neural circuit that regulates song, a learned sensorimotor social behavior. In adult white-crowned sparrows, new neurons are added continually to the song nucleus HVC (proper name) and project their axons to its target nucleus, the robust nucleus of the arcopallium (RA). We report here that electrical activity in RA regulates neuronal addition to HVC. Decreasing neural activity in RA by intracerebral infusion of the GABA_A receptor agonist muscimol decreased the number of new HVC neurons by 56%. Our results suggest that postsynaptic electrical activity influences the addition of new neurons into a functional neural circuit in adult birds.

birdsong | testosterone | songbird

The ongoing birth and incorporation of neurons into functional neural circuits in the central nervous system of higher vertebrates was first demonstrated conclusively in songbirds and subsequently in rodents, nonhuman primates, and humans (reviewed in ref. 1). Because new neuron generation continues throughout adulthood, the fundamental importance and the clinical implications of neurogenesis are clear. The mechanisms by which new neurons integrate into functional neural circuits, however, are not well understood.

Songbirds provide a tractable model for understanding the mechanisms that regulate new neuron addition into functional circuits. Song is a learned sensorimotor behavior that is important for songbird reproduction. Song learning and production are regulated by a discrete, well-characterized neural circuit that includes HVC (proper name) and its target nucleus, the robust nucleus of the arcopallium (RA), both located in the avian forebrain (Fig. 1A) (2). In the adult Gambel's white-crowned sparrow (WCS), the song control system shows extreme seasonal neuroplasticity (reviewed in ref. 3). Early in the breeding season, HVC and RA of WCS nearly double in volume. The increase in HVC volume results largely from an increase in new neuron incorporation, whereas the increase in RA volume results from increases in neuron size and spacing, but not number. RA neurons also show increased spontaneous electrical activity in the breeding season (4, 5). WCS typically produce only one song type that is longer and more stereotyped in structure during the breeding season (6, 7).

HVC contains three types of neurons: HVC→RA and HVC→area X projection neurons and interneurons. During seasonal growth, most, if not all, neurons incorporated into HVC project to RA (ref. 8, but see ref. 9). Neural progenitor cells are born at the dorsal and ventral portion of the lateral ventricle and migrate from the ventricular zone (VZ) to HVC within 1 wk after birth (10). Over the next 2–3 wk, new neurons send axonal projections to RA (11). These new HVC→RA projection neurons are functional; they can fire action potentials in response to sound stimuli (12).

Environment and experience play important roles in both brain development and adult neurogenesis. For example, during embryonic development, neural activity in the visual cortex is required for target selection by axons from the lateral geniculate nucleus (13). In adult rodents, voluntary exercise and hippocampal-dependent learning enhance neurogenesis in the dentate gyrus (reviewed in ref. 1). In adult songbirds, auditory experience and song production influence neuronal turnover in HVC (14–16). This literature suggests that adult neurogenesis in the vertebrate brain is activity-dependent. In vitro studies show that excitatory stimuli act directly on hippocampal neural progenitor cells and promote survival of new neurons (reviewed in ref. 17). Thus, activity-dependent mechanisms likely influence neuronal recruitment in vivo in both developing and adult brains.

One factor that may influence the recruitment to and survival of new neurons in HVC is the electrical activity of their postsynaptic targets in RA (5). We hypothesized that inhibiting the electrical activity in RA neurons in vivo would reduce neuronal addition to adult HVC. We show that decreasing RA electrical activity does indeed reduce neuronal addition to HVC, indicating that target activity is essential for appropriate neuronal addition to HVC.

Significance

Neural activity in the adult brain plays a key role in mediating experience-dependent neural plasticity. We show that inhibiting electrical activity in the song nucleus, robust nucleus of the arcopallium, in adult bird brain decreases the number of new projection neurons added to the afferent nucleus HVC. Our results are consistent with the general principle of activity-based target selection of newborn neurons during nervous system development and support the idea that developmental and adult plasticity exploit similar mechanisms. Understanding mechanisms influencing the incorporation of new neurons into established neural circuits in adult brains is critical for our basic understanding of neural plasticity and for exploiting the clinical potential of neuronal replacement to repair damage associated with injury and neurodegenerative diseases.

Fig. 1. Inhibition of RA neural activity by muscimol infusion decreases HVC neuronal addition. Birds were injected with BrdU to label adult-born neurons, and muscimol, a GABA_A receptor agonist, was infused unilaterally near RA to inhibit its neural activity. (*A*) A coronal schematic of the song-control system showing major song system projections. Black arrows show projections in the motor output circuit; red arrows show bilateral projections; blue arrows show recursive projections; and green dashed arrow shows a weak projection from RA to HVC. (*B*) Experimental timeline. (*C*) A representative image of BrdU and Hu immunolabeling in HVC. BrdU is shown in red, and Hu, a neuronal marker, is shown in green. Arrows show BrdU-positive neurons. The arrowhead indicates a BrdU-positive cell that does not colabel with Hu. (*D*) The number of BrdU-positive neurons in HVC at time of death. The number of Hu- and BrdU-colabeled neurons in HVC is significantly lower in the hemisphere ipsilateral to muscimol infusion than in the contralateral, uninfused hemisphere and is lower than either hemisphere in vehicle-infused controls. *$P \leq 0.05$. Contralateral hemispheres are shown in light gray, and ipsilateral are shown in black. All data are presented as mean ± SEM.

Results

We tested our hypothesis by inhibiting neural activity within RA in freely behaving adult Gambel's WCS and assaying the addition of new neurons to HVC.

RA contains two populations of neurons: (*i*) neurons that project to motor neurons in the tracheosyringeal portion of the hypoglossal nucleus in the hindbrain and are spontaneously active in vivo; and (*ii*) inhibitory GABAergic interneurons, which are not spontaneously active in vitro (18, 19). To inhibit electrical activity in RA, we infused the GABA_A receptor agonist, muscimol, near RA (20, 21). To label the greatest number of newborn cells, WCS were injected with the thymidine analog BrdU while in nonbreeding conditions [i.e., short days (SDs), when neuronal addition to HVC is highest] (22, 23). Two days after the last BrdU injection, WCS were shifted from SD to breeding conditions [i.e., long days (LDs) with testosterone (T) pellets (LD+T)], which normally increases survival of new neurons in HVC and increases spontaneous activity in RA (5, 23). Simultaneously, birds were implanted unilaterally with a cannula near RA, alternating between the left and right hemispheres in different birds (Fig. 1 *A* and *B*). The cannula was immediately connected to a microosmotic pump containing muscimol, which pumped continuously for the duration of the experiment. In control birds, RA was infused with saline. Vehicle and muscimol cannulae were placed 1,250 ± 100 μm from RA (Fig. S1). WCS were housed on LD+T for 2–4 wk after infusion onset (Fig. 1*B*).

Muscimol Effectively Diffused to and Inhibited Neural Activity in RA but Not HVC. To confirm the diffusion of muscimol to RA neurons, but not HVC neurons, we visualized spread of fluorophore-conjugated muscimol [FCM; boron dipyrromethene (BODIPY) muscimol–tetramethylrhodamine (TMR); Sigma-Aldrich] and recorded in vivo spontaneous firing rates in RA and HVC after muscimol infusion for 2 wk. FCM diffused to RA, but not to nearby HVC or VZ (*n* = 4 birds; Fig. 2 *A* and *B*). Muscimol completely inhibited spontaneous activity of RA neurons (*n* = 2 birds; Fig. 2 *C* and *D* and Table S1) (24–26) but did not alter spontaneous activity of HVC neurons (ipsilateral vs. contralateral HVC firing rate; *P* > 0.5; *n* = 28 cells in two birds; Fig. 2 *C* and *D* and Table S1). Together these results demonstrate that muscimol infusion effectively inhibited RA neural activity but did not diffuse to HVC or inhibit its spontaneous neural activity.

Reducing the Activity of RA Neurons Decreased the Addition of New HVC Neurons. The number of newborn neurons (i.e., cells colabeled with antibodies against BrdU and the neuron-specific antigen Hu) and single-labeled BrdU cells (presumably glial or ependymal cells) were counted throughout the full extent of HVC. The number of

BrdU-positive neurons was lower in HVC ipsilateral to muscimol infused in RA [74 ± 19 (mean ± SEM)] compared with HVC contralateral to muscimol infusion (167 ± 19; *n* = 8; *P* = 0.01), HVC ipsilateral to vehicle infusion (170 ± 25; *n* = 5; *P* < 0.05), and HVC contralateral to vehicle infusion (164 ± 24; *n* = 5; *P* < 0.05; Fig. 1*D*). We found no significant difference in new neuron number between HVC contralateral to muscimol infusion and HVC either ipsilateral or contralateral to saline infusion (both *P* > 0.1). We found no effect of hemisphere in birds infused with muscimol on the number of new neurons added to ipsilateral and contralateral
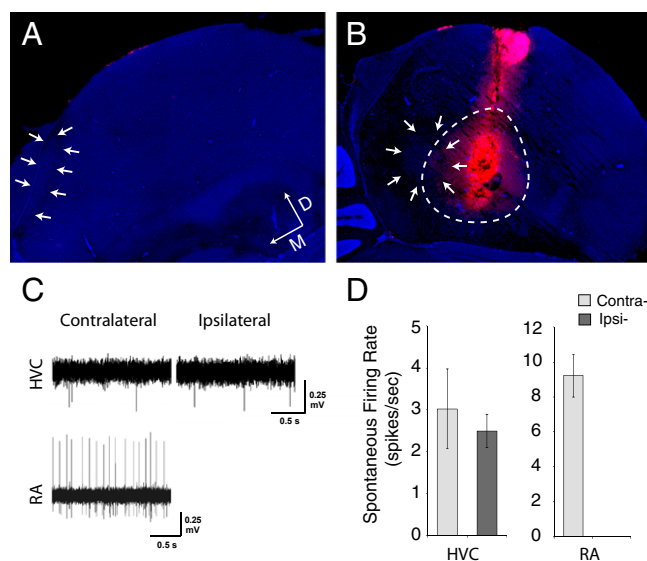
Fig. 2. Reduction in HVC neuronal addition is not an effect of cannula placement or muscimol diffusion. (*A*) FCM diffusion in HVC. D, dorsal; M, medial. White arrows outline HVC. FCM is shown in red; Hoechst 33342, a nuclear marker, is shown in blue. Muscimol did not diffuse to HVC. (*B*) The diffusion of muscimol–TMR in RA. White arrows outline RA. The FCM area of diffusion is outlined with a dotted line. The cannula did not damage RA, and muscimol diffused to most of RA. (*C*) Representative raw electrophysiological trace recordings from ipsilateral and contralateral HVC and RA. Electrical activity was suppressed by muscimol infusion in ipsilateral RA (thus, no spike trace shown), but not ipsilateral HVC. (*D*) The spontaneous firing rates in HVC and RA both ipsilateral and contralateral to muscimol infusion. Spontaneous firing in HVC does not differ between HVC ipsilateral and contralateral to muscimol infusion (see Table S1 for values), further suggesting that muscimol did not diffuse to HVC. Muscimol completely suppressed activity in ipsilateral, but not contralateral, RA (Table S1).

HVC (right hemisphere infusion, 112 ± 21; left, 135 ± 26; P = 0.5). The number of nonneuronal BrdU-positive cells was not significantly different between HVC ipsilateral to muscimol infusion (105 ± 22) and HVC contralateral to muscimol infusion (88 ± 22; n = 8; P > 0.5), HVC ipsilateral to vehicle infusion (120 ± 24; n = 5; P > 0.5), or HVC contralateral to vehicle infusion (137 ± 23; n = 5; P > 0.5). These results demonstrate that inhibiting RA activity unilaterally and selectively reduced the addition of new neurons to the ipsilateral HVC.

Although HVC neuronal addition was reduced with muscimol infusion in RA, neither total HVC neuron number nor HVC volume changed significantly (Table S2). Furthermore, muscimol infusion did not significantly alter RA volume, neuron density, or neuron soma size (Table S2). These results suggest that RA neural activity is required for new HVC neuron addition but may not be necessary for mature HVC neuronal survival or for neuronal growth in RA.

Unilateral Disruption of the Song Production Circuit Transiently Altered Song Behavior. We recorded and analyzed song production before and during muscimol or vehicle infusion. Birds did not differ in the number of songs produced per hour (i.e., rate) or the number of syllables, but did differ in some spectral properties with muscimol infusion (Table S3). Muscimol infused near RA transiently increased the variability and entropy of specific temporal and spectral properties of song during the first 14 d of treatment. Song recovered, however, by 21 d (Fig. 3 and Table S3). Vehicle infusion did not alter song, which demonstrates that cannula placement alone did not cause the transient changes in song attributes found in muscimol-infused birds (Table S4). We found no differences in any song attributes of birds infused with muscimol in the right vs. left hemisphere and no correlation between the degree of song degradation and the number of new neurons or total number of neurons either ipsilateral or contralateral to muscimol infusion. The mechanism underlying song recovery during muscimol infusion and its effect, if any, on neuronal addition remains to be identified.

Discussion

We found that inhibiting electrical activity in RA by infusing muscimol significantly reduced neuronal addition to the ipsilateral, but not contralateral, HVC. These results support the hypothesis that postsynaptic neuronal electrical activity modulates afferent neuronal addition. Our findings are consistent with the general theory that neural activity shapes and refines neural circuits during nervous system development (ref. 13, but see ref. 27) and support the idea that developmental and adult plasticity

exploit similar mechanisms. For example, in rodents, odor deprivation caused by naris closure reduces neurogenesis in the olfactory bulb and survival of newborn olfactory interneurons (28), whereas odor enrichment promotes the survival of newborn neurons and enhances odor memory (29). Similarly, NMDA receptor expression by new neurons in adult mouse dentate gyrus is required for neuronal incorporation into an existing circuit (30). In addition, dysfunctional adult hippocampal neurogenesis has been proposed as a possible biological mechanism of depression in humans. Decreased neural activity in the hippocampus in response to positive social stimuli was found in depressed patients. Electroconvulsive therapy, used to treat severe depression, indiscriminately increases neural activity and enhances hippocampal neurogenesis (reviewed in ref. 31). These studies indicate that neural activity plays an important role in regulating adult neurogenesis. Few studies, however, have tested the role of neural activity in a target nucleus on neuron addition in an afferent nucleus within functional neural circuits. Our study is unique in that it shows in vivo the effects of neural activity in one nucleus on neuron addition in another nucleus of an adult neural circuit that regulates a learned sensorimotor behavior.

Muscimol might influence neuron addition by inhibiting activity of new HVC neurons via direct or indirect projections from RA to HVC (Fig. 1A). If such inhibition were mediated by the weak unilateral direct projection from RA to HVC (24), then we might expect to find lower spontaneous firing of HVC neurons ipsilateral to the muscimol infusion, given the positive relationship between activity and survival of new neurons observed in other models. However, we did not observe a decrease in spontaneous firing rate in ipsilateral HVC in the muscimol-treated birds. The indirect connection between RA and HVC via the dorsomedial thalamic nucleus and the cortical nucleus medial magnocellular nucleus of the anterior nidopallium is bilateral (25, 26). We therefore might expect ipsilateral muscimol infusion in RA to decrease electrical activity and neuronal addition in both sides of HVC. We did not observe differences in the number of new neurons or total number of neurons between contralateral HVC in muscimol-infused birds and either side of HVC in vehicle-infused birds. These observations suggest that there was neither a direct nor indirect effect of muscimol infused in RA on HVC neural activity, although they do not absolutely rule out this possibility.

Another mechanism by which reduced activity in RA could affect HVC neuronal addition is through muscimol-induced alteration of song production. This behavioral impairment could indirectly affect neuronal addition in HVC through altered sensory feedback (e.g., proprioceptive or auditory). Proprioceptive
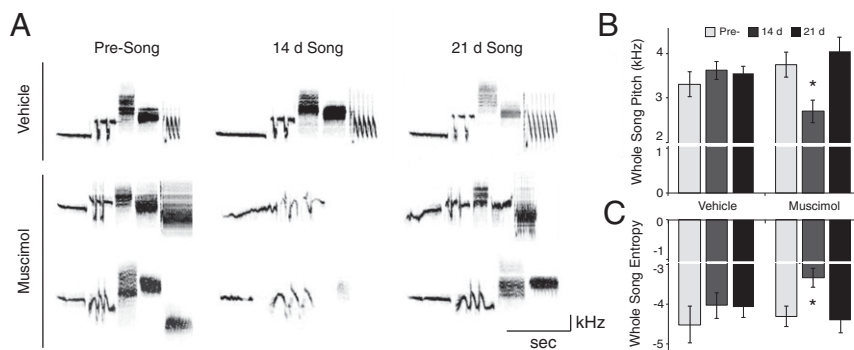
Fig. 3. Muscimol infusion transiently alters song behavior. (*A*) Representative sonograms of songs produced by the same birds before and during unilateral infusion of muscimol or vehicle into RA. (*B*) Whole song pitch of birds infused with vehicle and muscimol. (*C*) Whole song entropy of birds infused with vehicle and muscimol. Pitch and entropy of whole songs produced 14 d after onset of muscimol infusion were significantly different compared with songs produced before and 21 d after infusion of muscimol (all comparisons, n = 7, P < 0.05; see Table S3 for values). Pitch and entropy of songs recorded before experiment and 21 d after infusion onset were not significantly different between vehicle- and muscimol-infused birds (all comparisons, n = 10, P > 0.1; Tables S3 and S4). Preexperiment song is shown in light gray; song recorded 14 d after infusion onset is in dark gray; and 21 d after infusion onset is in black. *P < 0.05.

respiratory and syringeal feedback is conveyed through the rostral ventrolateral medulla, which indirectly projects to HVC via direct bilateral projections to thalamic nucleus uvaeformis (Fig. 1*A*) (32). Wilbrecht et al. (15) reported that in zebra finches, unilaterally denervating the vocal production organ, the syrinx, impaired song production and increased neuronal addition to the contralateral HVC. Deafening birds prevented this compensatory increase in neuronal recruitment. Their results suggest that motor activity and/ or auditory feedback from song can influence neuronal recruitment. One would expect that altered auditory and/or proprioceptive feedback during song impairment would disrupt neuronal addition in both sides of HVC. We found, however, that muscimol infusion only impaired neuronal addition to the ipsilateral HVC. Our results suggest that decreased neuronal addition to HVC in WCS in this experimental context cannot be explained by altered song behavior in a straightforward way. This observation does not rule out the possibility that sensory feedback might modulate neuronal addition.

A possible mechanism through which postsynaptic activity can influence neuronal addition is by activity-induced regulation of genes encoding molecules that promote survival of adult-born HVC neurons, axon path finding, and/or synapse formation (33, 34). Interestingly, activity-induced guidance molecules are seasonally regulated in RA neurons of WCS; microarray analysis of cDNA extracted from RA revealed that the expression of guidance cue genes, including *netrin 4* and *galectin*, is increased in breeding-condition birds (35). Activity in RA may modulate recruitment via activity-induced trophic factors produced by target neurons that are transported retrogradely to influence the survival of new neurons. In canaries, expression levels of brain-derived neurotrophic factor (*BDNF*) mRNA in HVC are higher in singing birds compared with nonsinging birds, and infusion of *BDNF* protein near HVC in nonsinging birds increases neuronal addition in HVC (16, 36). Microarray analysis also revealed that the expression of the proneurogenic genes, including *insulin-like growth factor 1* and *neuromodulin*, is increased in breeding-condition WCS (35). In HVC, the mRNA expression of sex steroid receptors, which facilitate the retrograde transport of trophic factors bound to their receptor toward the neuronal soma, also increases during breeding conditions (37, 38). Once transported back to the soma, trophic factors likely activate signaling cascades that promote the growth and survival of new neurons (39, 40). Muscimol-mediated reduction of neural activity in RA may result in a failure of new HVC neurons to form synapses on RA neurons and/or a decreased production of activity-induced trophic factors in RA. The consequence would be a lack of retrograde transport of the trophic signals and, thus, a decrease in addition of adult-born neurons to HVC.

We found that RA neural activity is not required to maintain total HVC neuron number (mature plus new) under breeding conditions. The comparatively small decrease in new HVC neuron number caused by muscimol infused near RA is likely not sufficient to be reflected as a significant decrease in total HVC neuron number. Steroid-induced autocrine signals such as neurotrophic factors produced within HVC itself may be sufficient to maintain mature neurons when RA neural activity is abolished (37, 41).

Local neuronal activity is important in regulating neurogenesis during development (reviewed in ref. 42) and adult neurogenesis (reviewed in ref. 43). Our results show in an in vivo neural circuit that neuronal activity in a target nucleus is important for the addition of new neurons in an afferent nucleus in the adult brain. Understanding the mechanisms underlying the relationship between neuronal activity and adult neurogenesis may provide insight into the etiology of and treatments for many neurological disorders.

Materials and Methods

Animals. We used adult male Gambel's WCS, *Zonotrichia leucophrys gambelii*, captured in eastern Washington during their pre- or postbreeding season migration. Before experiment onset, all birds were housed in indoor

group aviaries exposed to SDs (8-h light, 16-h dark) for 12 wk. All experiments followed National Institutes of Health (NIH) animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee.

Experimental Procedures. We injected birds housed on SD with BrdU (15 mg/ ml in 0.012M NaCl, at a dose of 50 mg/kg; Sigma) intramuscularly once a day for 5 d. Two days after the final BrdU injection, unilateral intracerebral cannulae were implanted stereotaxically adjacent to RA. Briefly, birds were anesthetized with isoflurane (2%) in a non-rebreathing system. We randomly chose a hemisphere and lowered the cannula (Alzet 3–5 mm; Durect) 2.4 mm into the telencephalon caudal to RA (lateral, 3.4 mm; posterior, 1.25 mm, using the intersection of the midsagittal and transverse sinuses as the reference point). We fixed the cannula to the skull with dental cement and attached a microosmotic pump (Alzet Model 1002) to the cannula. Seventeen birds received osmotic pumps containing the inhibitory GABA$_A$ receptor agonist muscimol (2.8 mg/mL in 0.09% NaCl; Sigma), and 7 control birds received only vehicle. Of the 17 birds infused with muscimol, 4 received osmotic pumps filled with BODIPY muscimol–TMR (2.8 mg/mL in 15% DMSO, 60% PEG, and 25% dH$_2$O; Invitrogen). We placed osmotic pumps into sealed microcentrifuge tubes filled with saline and mounted the tubes into custom-made "backpacks" that allowed the birds to fly freely. Two days after surgery, each bird was implanted s.c. with a 12-mm Silastic capsule (1.47-mm inner diameter, 1.96-mm outer diameter) filled with crystalline T. We kept all of the birds on LD+T in individual sound-isolation recording chambers until death 2–4 wk after T implantation to determine whether the effect of inhibiting postsynaptic neurons in RA was most pronounced at a particular stage between cell proliferation and integration of fully differentiated neurons into a functional circuit. Because we found no significant differences in the number of new and preexisting neurons between birds infused with muscimol for 2–4 wk, we pooled these data. To ensure that all birds in the experiment for >14 d received continuous infusion, we exchanged the first osmotic pump with a second pump filled with the same solution at 14 d after surgery.

Tissue Collection and Processing. After we deeply anesthetized birds with isoflurane inhalation, we removed and immediately froze the brains on dry ice. We sectioned the brains in the coronal plane at 40 μm on a cryostat and thaw-mounted each section. We Nissl-stained every third section and stored remaining sections at −80 °C.

Immunohistochemistry. To visualize BrdU-labeled neurons, brain sections were fixed in 4% paraformaldehyde for 15 min, rinsed in PBS with 0.5% DMSO and 0.5% Triton X (PDTX; pH 7.4), incubated with 0.1 mg/mL proteinase K for 5 min, and postfixed for 15 min. Slides were dipped in distilled water, incubated with 2 M HCl at 37 °C for 30 min, and rinsed with PDTX. After blocking for 1 h in 5% goat serum, slides were incubated with rat anti-BrdU (1:200; Accurate) and mouse anti-Hu (1:200; Molecular Probes) antibodies (44). Labeling was visualized with Alexa Fluor 594- or 488-conjugated goat anti-rat and anti-mouse IgG secondary antibodies (1:200; Invitrogen). A confocal laser-scanning microscope (Leica SPE5) was used to verify double labeling. All single- and double-labeled cells in each HVC section were counted. All tissue processing was performed blind to treatment.

Morphometric Measurements in HVC and RA. Volumes were determined by tracing the borders of the nuclei onto paper using a microprojector. These drawings were scanned into a microcomputer, and the area was determined by using NIH ImageJ software. The total volume was calculated from the areas of the tracings by using the formula for a truncated cone (45). We used a random, systematic sampling protocol to measure neuron size and density (46). We measured neuronal density visually by counting all neurons— distinguished by their single, densely staining nucleolus and uniform, non-granular cytoplasm—that fell within an ocular grid (1.9 × 10^{-3} mm^2 at 1,000×) at each sampling site.

Electrophysiological Recordings and Analysis. After a 2-wk continuous infusion of muscimol as discussed above, birds were anesthetized with three intramuscular injections of 25% urethane 30 min apart for a total volume of 6 μL/g. Birds were placed in a stereotaxic apparatus in a sound isolation chamber. Single and multiunit extracellular recordings were made by using glass electrodes filled with 0.9% NaCl. We alternated recordings between the two hemispheres and used the same electrode for multiple passes although each hemisphere. Small, ionophoretic injections of the tracer 10-kDa dextran amine conjugated to Alexa 488 or 568 or 10% fluororuby (10,000 MW tetramethylrhodamine dextran; Invitrogen; loaded into the recording electrode solution before recordings) were made to mark recording locations.

RA was targeted through a separate guide cannula that was implanted with the cannula attached to the osmotic pump. HVC was targeted through a small craniotomy just dorsal to the nucleus. Voltage signals from each structure were amplified 10× with an Axoclamp-2B amplifier (Molecular Devices) in bridge mode and then filtered (0.1-kHz high-pass, 7-kHz low-pass) and amplified a further 100× with a Model 440 amplifier (Brownlee Precision). Signals were digitized at 25 kHz with a Micro 1401 data acquisition unit and Spike2 software (Cambridge Electronic Design). We measured spontaneous rates of single units in RA and both single and spike-sorted units (Spike 2 software) in HVC that had a stable firing rate for at least 2 min.

Each bird was transcardially perfused with 4% paraformaldehyde. The brains were removed and postfixed in 20% sucrose/4% paraformaldehyde solution before slicing 40-μm thick on freezing microtome. Sections were counterstained and examined to verify that recording locations were within RA or HVC.

Song Recording and Analysis. We individually recorded song of all birds housed in sound-isolation chambers using Syrinx (www.syrinxpc.com). Baseline song was obtained just as experimental song in a prior exposure to LD+T conditions. We measured song rate and the variability of 16 different song attributes using the protocol described in ref. 6. To determine the stereotypy of the song attributes, we compared coefficients of variance (SD/mean). We also calculated song completeness from the number of syllables produced in 20 randomly selected songs and divided by the maximum number of different syllables produced by the bird—typically five syllables in Gambel's WCS. We analyzed the spectral properties of individual syllables using Sound Analysis Pro (SAP) software (http://ofer.sci.ccny.cuny.edu/sound_analysis_pro) (47). For each syllable, we used SAP to automate measurement of four spectral properties: pitch (indicates the period of oscillation, or mean frequency, of the sound), frequency modulation (the mean slope of frequency contours), entropy (a measure of randomness), and pitch goodness (a measure of periodicity) (47). Using the same 20 digitized songs, we calculated each property's mean value per syllable and averaged syllable values for each bird before statistical analysis (6). We measured song rate as the average number of songs produced during a 1-h period beginning 2 h after lights on for each bird between 13 and 22 d. This period is the time of maximal daily singing in WCS (6).

Statistical Analysis. Spike rates between ipsilateral and contralateral HVC and RA were analyzed with nonparametric t tests. All other comparisons were made with a two- or three-way mixed-model ANOVA and post hoc Tukey tests by using JMP 8 (SAS).

1. Gage FH, Kempermann G, Song H (2008) *Adult Neurogenesis* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), p 673.
2. Reiner A, Perkel DJ, Mello CV, Jarvis ED (2004) Songbirds and the revised avian brain nomenclature. *Ann N Y Acad Sci* 1016:77–108.
3. Brenowitz EA (2008) Plasticity of the song control system in adult birds. *Neuroscience of Birdsong*, eds Zeigler HP, Marler P (Cambridge Univ Press, Cambridge, UK), pp 332–349.
4. Meitzen J, Thompson CK (2008) Seasonal-like growth and regression of the avian song control system: Neural and behavioral plasticity in adult male Gambel's white-crowned sparrows. *Gen Comp Endocrinol* 157(3):259–265.
5. Meitzen J, Perkel DJ, Brenowitz EA (2007) Seasonal changes in intrinsic electrophysiological activity of song control neurons in wild sparrows. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 193(6):677–683.
6. Meitzen J, Thompson CK, Choi H, Perkel DJ, Brenowitz EA (2009) Time course of changes in Gambel's white-crowned sparrow song behavior following transitions in breeding condition. *Horm Behav* 55(1):217–227.
7. Nottebohm F, Nottebohm ME, Crane L (1986) Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. *Behav Neural Biol* 46(3):445–471.
8. Scotto-Lomassese S, Rochefort C, Nshdejan A, Scharff C (2007) HVC interneurons are not renewed in adult male zebra finches. *Eur J Neurosci* 25(6):1663–1668.
9. Scott BB, Lois C (2007) Developmental origin and identity of song system neurons born during vocal learning in songbirds. *J Comp Neurol* 502(2):202–214.
10. Alvarez-Buylla A, Theelen M, Nottebohm F (1990) Proliferation "hot spots" in adult avian ventricular zone reveal radial cell division. *Neuron* 5(1):101–109.
11. Kirn JR, Fishman Y, Sasportas K, Alvarez-Buylla A, Nottebohm F (1999) Fate of new neurons in adult canary high vocal center during the first 30 days after their formation. *J Comp Neurol* 411(3):487–494.
12. Paton JA, Nottebohm FN (1984) Neurons generated in the adult brain are recruited into functional circuits. *Science* 225(4666):1046–1048.
13. Catalano SM, Shatz CJ (1998) Activity-dependent cortical target selection by thalamic axons. *Science* 281(5376):559–562.
14. Scharff C, Kirn JR, Grossman M, Macklis JD, Nottebohm F (2000) Targeted neuronal death affects neuronal replacement and vocal behavior in adult songbirds. *Neuron* 25(2):481–492.
15. Wilbrecht L, Crionas A, Nottebohm F (2002) Experience affects recruitment of new neurons but not adult neuron number. *J Neurosci* 22(3):825–831.
16. Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F (2000) A relationship between behavior, neurotrophin expression, and new neuron survival. *Proc Natl Acad Sci USA* 97(15):8584–8589.
17. Ma DK, Kim WR, Ming GL, Song H (2009) Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. *Ann N Y Acad Sci* 1170:664–673.
18. Mooney R (1992) Synaptic basis for developmental plasticity in a birdsong nucleus. *J Neurosci* 12(7):2464–2477.
19. Spiro JE, Dalva MB, Mooney R (1999) Long-range inhibition within the zebra finch song nucleus RA can coordinate the firing of multiple projection neurons. *J Neurophysiol* 81(6):3007–3020.
20. Pinaud R, Mello CV (2007) GABA immunoreactivity in auditory and song control brain areas of zebra finches. *J Chem Neuroanat* 34(1-2):1–21.
21. Mooney R, Konishi M (1991) Two distinct inputs to an avian song nucleus activate different glutamate receptor subtypes on individual neurons. *Proc Natl Acad Sci USA* 88(10):4075–4079.
22. Alvarez-Buylla A, Kirn JR (1997) Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J Neurobiol* 33(5):585–601.
23. Tramontin AD, Brenowitz EA (1999) A field study of seasonal neuronal incorporation into the song control system of a songbird that lacks adult song learning. *J Neurobiol* 40(3):316–326.
24. Roberts TF, Klein ME, Kubke MF, Wild JM, Mooney R (2008) Telencephalic neurons monosynaptically link brainstem and forebrain premotor networks necessary for song. *J Neurosci* 28(13):3479–3489.
25. Foster EF, Mehta RP, Bottjer SW (1997) Axonal connections of the medial magnocellular nucleus of the anterior neostriatum in zebra finches. *J Comp Neurol* 382(3):364–381.
26. Coleman MJ, Vu ET (2005) Recovery of impaired songs following unilateral but not bilateral lesions of nucleus uvaeformis of adult zebra finches. *J Neurobiol* 63(1):70–89.
27. Oppenheim RW, et al. (2003) Rescue of developing spinal motoneurons from programmed cell death by the GABA(A) agonist muscimol acts by blockade of neuromuscular activity and increased intramuscular nerve branching. *Mol Cell Neurosci* 22(3):331–343.
28. Corotto FS, Henegar JR, Maruniak JA (1994) Odor deprivation leads to reduced neurogenesis and reduced neuronal survival in the olfactory bulb of the adult mouse. *Neuroscience* 61(4):739–744.
29. Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *J Neurosci* 22(7):2679–2689.
30. Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH (2006) NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. *Nature* 442(7105):929–933.
31. Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. *Nat Neurosci* 10(9):1110–1115.
32. Striedter GF, Vu ET (1998) Bilateral feedback projections to the forebrain in the premotor network for singing in zebra finches. *J Neurobiol* 34(1):27–40.
33. Zhang LI, Poo MM (2001) Electrical activity and development of neural circuits. *Nat Neurosci* 4(Suppl):1207–1214.
34. Kay L, Humphreys L, Eickholt BJ, Burrone J (2011) Neuronal activity drives matching of pre- and postsynaptic function during synapse maturation. *Nat Neurosci* 14(6):688–690.
35. Thompson CK, et al. (2012) Seasonal changes in patterns of gene expression in avian song control brain regions. *PLoS ONE* 7(4):e35119.
36. Rasika S, Alvarez-Buylla A, Nottebohm F (1999) BDNF mediates the effects of testosterone on the survival of new neurons in an adult brain. *Neuron* 22(1):53–62.
37. Jezierski MK, Sohrabji F (2003) Estrogen enhances retrograde transport of brain-derived neurotrophic factor in the rodent forebrain. *Endocrinology* 144(11):5022–5029.
38. Fusani L, Van't Hof T, Hutchison JB, Gahr M (2000) Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. *J Neurobiol* 43(3):254–268.
39. Gottschalk WA, et al. (1999) Signaling mechanisms mediating BDNF modulation of synaptic plasticity in the hippocampus. *Learn Mem* 6(3):243–256.
40. Yoshii A, Constantine-Paton M (2007) BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nat Neurosci* 10(6):702–711.
41. Wissman AM, Brenowitz EA (2009) The role of neurotrophins in the seasonal-like growth of the avian song control system. *J Neurosci* 29(20):6461–6471.
42. Linden R (1994) The survival of developing neurons: A review of afferent control. *Neuroscience* 58(4):671–682.
43. Ge S, Pradhan DA, Ming GL, Song H (2007) GABA sets the tempo for activity-dependent adult neurogenesis. *Trends Neurosci* 30(1):1–8.
44. Louissaint A, Jr., Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. *Neuron* 34(6):945–960.
45. Smith GT, Brenowitz EA, Beecher MD, Wingfield JC (1997) Seasonal changes in testosterone, neural attributes of song control nuclei, and song structure in wild songbirds. *J Neurosci* 17(15):6001–6010.
46. Tramontin AD, Smith GT, Breuner CW, Brenowitz EA (1998) Seasonal plasticity and sexual dimorphism in the avian song control system: Stereological measurement of neuron density and number. *J Comp Neurol* 396(2):186–192.
47. Tchernichovski O, Nottebohm F, Ho CE, Pesaran B, Mitra PP (2000) A procedure for an automated measurement of song similarity. *Anim Behav* 59(6):1167–1176.