Afferent Input Is Necessary for Seasonal Growth and Maintenance of Adult Avian Song Control Circuits

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The neural circuits that regulate song behavior in adult songbirds undergo pronounced seasonal changes in morphology, primarily in response to changes in plasma testosterone (T). Most song nuclei have T receptors. We asked whether seasonal growth and maintenance of nuclei within these circuits are direct responses to the effects of T or its metabolites or are mediated indirectly via the effects of T on afferent nuclei. Photosensitive white-crowned sparrows were exposed to one of three treatments. (1) The neostriatal nucleus HVC (also known as the “high vocal center”) was lesioned unilaterally, and the birds were exposed to long-day (LD) photoperiods and breeding levels of T for 30 d. (2) Birds were exposed to LD plus T (LD+T) for 30 d; then HVC was lesioned, and the birds were killed after an additional 30 d exposure to LD+T. (3) HVC was lesioned, and the sparrows were housed on short-day (SD)

photoperiods in the absence of T treatment for 30 d. In both LD+T groups, the direct efferent targets of HVC, the robust nucleus of the archistriatum (RA) and area X, were smaller ipsilateral to the lesion. The lesion did not prevent growth of the hypoglossal motor nucleus, which does not receive direct afferent input from HVC. RA and area X were also smaller ipsilateral to the lesion in the SD birds. These results indicate that afferent input is required both for the growth of adult song circuits in response to typical breeding photoperiod and hormone conditions and for the maintenance of efferent nuclei in either their regressed or enlarged states.

Key words: afferent; bird; birdsong; plasticity; season; songbird; song; song system; testosterone; trophic; white-crowned sparrow

Seasonal changes in the morphology of adult brain nuclei occur in every vertebrate class (Tramontin and Brenowitz, 2000). The nuclei that control song learning and production in birds are easily identified, and their connectivity is well established (Fig. 1) (for review, see Bottjer and Johnson, 1997; Wild, 1997). The avian song control system has emerged as a leading model for studying seasonal plasticity in the brain. There is pronounced seasonal plasticity in the morphology of nuclei in the song control system (for review, see Tramontin and Brenowitz, 2000). These seasonal changes in the song system are primarily regulated by changes in plasma levels of testosterone (T) that are correlated with changes in photoperiod (Gulledge and Deviche, 1997; Smith et al., 1997a; Ball, 2000). Most nuclei within the song control circuits have receptors for androgenic hormones (Fig. 1) (for review, see Bottjer and Johnson, 1997; Schlenger, 1997). We do not yet know, however, whether seasonal growth of different nuclei within these circuits is a direct response to the effects of T or its metabolites or is mediated indirectly via the effects of T on afferent nuclei. This study was designed to test these alternative hypotheses.

Growth of HVC (Fig. 1, *HVC*) in response to breeding season cues precedes that of its direct efferent target nuclei RA and area X in song sparrows (*Melospiza melodia*) and Gambel's white-

crowned sparrows (GWCS; *Zonotrichia leucophrys gambelii*) (Smith et al., 1997c; Tramontin et al., 2000). These observations led to the hypothesis that afferent input from HVC is required for the growth of RA and area X. Afferent input is important in the development and maintenance of diverse neural systems (Levi-Montalcini, 1949; Clarke, 1985; Beyer and Feder, 1987; Furber et al., 1987; Rubel et al., 1990). In the birdsong system, depriving RA and area X of afferent input in juvenile zebra finches (*Taenopygia guttata*) prevents these nuclei from growing to their normal size in males (Akutagawa and Konishi, 1994; Johnson and Bottjer, 1994) and blocks the masculinization of these regions in females that is caused by early treatment with estradiol (Herrmann and Arnold, 1991). If plasticity in adult song circuits exploits mechanisms similar to those of juvenile development (Tramontin and Brenowitz, 2000), then we predict that depriving adult song nuclei of their afferent input would prevent their growth in response to seasonal-like cues.

An alternative mechanism for seasonal plasticity is that T or its metabolites act directly on different song nuclei that contain steroid receptors. Steroid hormones such as T can have direct trophic effects on brain nuclei (Perez and Kelley, 1996, 1997; Kay et al., 1999). Hormones may also act on the efferent targets of neurons that can then provide retrograde trophic support to the neurons (Kelley, 1986; Fishman et al., 1990; Oppenheim, 1991; Fishman and Breedlove, 1992; Lohmann and Gahr, 2000). In either scenario, afferent input may not be required for the growth and maintenance of brain nuclei. A prediction of either model is that we would observe growth of adult song nuclei that contain steroid receptors in response to breeding levels of T, even in the absence of afferent input.

In this study we investigated whether afferent input from HVC

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Figure 1. Simplified schematic sagittal view of the avian song control system showing the distribution of steroid receptors. *Black arrows* connect nuclei in the main descending motor circuit, and *white arrows* connect nuclei in the anterior forebrain circuit. *DLM*, Dorsolateral nucleus of the medial thalamus; *IMAN*, lateral portion of the magnocellular nucleus of the anterior neostriatum; *nXIIIts*, the tracheosyringeal portion of the hypoglossal nucleus; *RA*, the robust nucleus of the archistriatum; *syrinx*, vocal production organ; *V*, lateral ventricle; *X*, area X of the parolfactory lobe.

is necessary for the maintenance and seasonal-like growth of efferent song nuclei in adult male GWCS; this nucleus provides input to both the motor and anterior forebrain circuits (Fig. 1). In white-crowned sparrows there are pronounced changes in the volumes of song nuclei and neuronal attributes of these nuclei in response to seasonal changes in photoperiod and plasma T, both in the wild and in the laboratory (for review, see Tramontin et al., 2000). We found that afferent input is required for both the maintenance and seasonal-like growth of telencephalic song nuclei.

MATERIALS AND METHODS

Collection of birds and experimental treatments. We captured 24 adult male GWCS in eastern Washington during their postbreeding season migration in September and October 1998. All birds were housed indoors for 12 weeks on short days (SD; 8 hr light) before the start of the experiment to ensure that their reproductive systems were regressed but sensitive to the stimulating effects of breeding photoperiods (i.e., they were photosensitive). White-crowned sparrows kept on SD indefinitely maintain regressed testes, basal or nondetectable levels of circulating T, and regressed song control nuclei that are typical of the nonbreeding season (Middleton, 1965; Sansum and King, 1976; Smith et al., 1995, 1997b).

The birds were placed in three different treatment groups. (1) SD group. In eight birds, HVC was lesioned unilaterally as described below. On the day after surgery each bird received an empty SILASTIC implant as a control for the T pellets implanted in the other two groups (see below). These birds remained on SD for 30 d after the lesion and were then killed as described below. (2) Long-day (LD) plus T (LD+T) growth ("growth") group. We lesioned HVC unilaterally in nine birds while they were housed on SD and photoshifted them that night to LD (20 hr of light), typical of what they would experience on their Alaskan breeding grounds. On the day after surgery we implanted each bird with a SILASTIC capsule (1.47 mm inner diameter $\times$ 1.96 mm outer diameter) containing 12 mm of crystalline T to elevate rapidly plasma concentrations of this hormone into the range typical of wild breeding birds (4–10 ng/ml) (Wingfield and Farner, 1978). We killed these birds 30 d after their initial exposure to LD+T. This time period is adequate for full seasonal growth of the song nuclei (Smith et al., 1995, 1997a; Tramontin et al., 2000). (3) LD+T maintenance ("maintenance") group. Seven birds were shifted overnight from SD to LD and implanted with the same dose of T that the growth birds received. After 30 d on LD+T we lesioned HVC unilaterally. We replaced each bird's T implant on the

day of surgery to ensure that it maintained high circulating T levels. We killed the birds in this group 30 d after the HVC lesion surgery and 60 d after their initial exposure to LD+T.

Each treatment group was designed to address a different question regarding the role of afferent input and direct trophic effects of T in the song system. The SD group would indicate whether afferent input from HVC is necessary for the maintenance of RA, area X, and other song nuclei even in their regressed, nonbreeding state. The growth birds would show whether afferent input from HVC is necessary for the initial growth of these song regions in response to breeding conditions. The maintenance group would determine whether afferent input from HVC is required for the maintenance of these nuclei after they have grown during the preceding 30 d of LD+T.

The song nuclei of male GWCS will grow in response to spring environmental conditions in the field or to gradual increases in photoperiod in the laboratory, without T implants (Soma et al., 1998; Tramontin et al., 1999). Increases in circulating T are primarily responsible for this growth (Gulledge and Deviche, 1997; Smith et al., 1997a; Ball, 2000). Plasma T levels increase slowly over several days in both the field and laboratory, however, and the timing of this increase varies considerably between individual birds (Wingfield and Farner, 1978; Tramontin et al., 1999). We were concerned that this variability might obscure the effects of the HVC lesions. To control for this variability, we shifted all birds in the growth group to LD overnight after the lesion surgery and implanted them with T pellets that rapidly produced plasma levels of T within or above the physiological range. This treatment should bias against observing an effect of HVC lesions on the growth and maintenance of RA and area X if T does act directly on these nuclei.

Surgical procedure. HVC was lesioned unilaterally with 1% racemic NMA in PBS, pH 7.4. We lesioned both the rostral main corpus and caudomedial extension of HVC at the following stereotaxic coordinates (relative to the intersection of the midsagittal and transverse sinuses and the brain surface): anteroposterior = 0.0 mm (rostral) and -0.3 mm (caudal); mediolateral = 1.8 mm (caudal) and 2.8 mm (rostral); and depth = 0.5 and 0.7 mm (rostral) and 0.65 mm (caudal). We injected 800 nl of NMA at each depth for the rostral site and 800 nl at the single caudal site using a customized pressure delivery system. This system consisted of Tygon tubing connected to the outflow of a Gast vacuum pressure pump, with a T-shaped connector inserted in the pathway to allow us to regulate the flow of air to a calibrated micropipette for the delivery of NMA. We alternated lesions between the left and right HVC in successive birds in each group.

Hormone assay. We collected 300 μ l of whole blood into heparinized microcentrifuge tubes by puncturing the alar vein. Samples were collected from each male the day before he received SILASTIC implants and at 2 and 4 weeks after the implant. The SD and growth birds were killed at 4 weeks. T pellets were replaced in the maintenance birds at 4 weeks, and blood samples were collected 2 weeks later (6 weeks after the start of the experiment) and then again just before death at 7 weeks after the start of the experiment. Each blood sample was immediately centrifuged, and the plasma was removed and stored at -20°C until assay. We measured T in the plasma by radioimmunoassay using the Coat-A-Count Total Testosterone kit (Diagnostic Products). The use of this assay to measure plasma T has been validated for birds (Tramontin et al., 2001). The minimum detectable plasma T concentration was 0.2 ng/ml. Samples with undetectable T levels were treated as having concentrations at this detection limit for statistical analysis.

Histology and brain morphometry. Birds were deeply anesthetized with methoxyflurane and perfused through the heart with heparinized saline followed by 10% neutral-buffered formalin (NBF). Brains were post-fixed in NBF for at least 2 weeks, embedded in gelatin, cryoprotected in a 20% sucrose and NBF solution, and sectioned in the coronal plane at 50 μ m on a freezing microtome. Every other section was mounted and stained with thionin. The Nissl-defined borders of song nuclei coincide with the borders as defined by other labels (for review, see Tramontin and Brenowitz, 2000).

We projected a magnified image (46 $\times$) of each mounted section that contained a song nucleus profile (100 μ m sampling interval). We traced onto paper the Nissl-defined borders of unlesioned and remaining lesioned (if any) HVC, as well as RA, area X, IMAN, and nXIIIts ipsilateral and contralateral to the HVC lesion. We used the criteria of DeVogd et al. (1991) to distinguish the tracheosyringeal from the lingual portion of the hypoglossal nucleus (see Smith et al., 1997c). These tracings were scanned into a computer, and the cross-sectional area of each song nucleus profile was calculated using NIH Image software (version 1.57;

Table 1. Plasma T levels (ng/ml) in the three treatment groups before receiving the SILASTIC implant and at different times after the implant

Group	Preimplant	Week 2	Week 4	Week 6	Week 7
SD ($n = 8$)	0.36 ± 0.21^a	0.22 ± 0.19^a	0.07 ± 0.02^a	—	—
Growth ($n = 9$)	0.14 ± 0.05^a	9.76 ± 0.66^b	10.71 ± 1.58^b	—	—
Maintenance ($n = 7$)	0.11 ± 0.04^a	16.69 ± 3.23^c	8.58 ± 1.28^b	$11.70 \pm 0.77^{b,c}$	9.59 ± 0.61^b

Values shown are means $\pm$ SEM. The SD group received a blank implant and remained on short days for 4 weeks, when they were killed. The growth group received a unilateral lesion of HVC and was shifted to LD, received a T implant, and was killed after 4 weeks. The maintenance group was shifted to LD and received a T implant, HVC was lesioned at 4 weeks, and the T implant was replaced then. These birds were killed at 7 weeks.

^{a,b,c} Values with different superscripts differ significantly from each other (SNK *post hoc* comparison, $p < 0.05$, two-tailed).

Wayne Rasband, National Institutes of Health, Bethesda, MD). We estimated the volume of each nucleus using the formula for a cone frustum over each measured profile area (Smith et al., 1995). All brain measurements were made blind to treatment group.

Individual birds may differ in overall brain size and/or histological preparation. To control for such variation, statistical analyses of HVC, RA, area X, and IMAN were conducted on the volumes of each nucleus divided by the volume of the unlesioned telencephalon; a previous study of GWCS reported no size difference between the right and left telencephalon (Tramontin et al., 2000). nXIIIs is located in the brainstem and therefore was only expressed in absolute terms. We estimated the volume of the unlesioned telencephalon by projecting onto paper a magnified image (14 $\times$) of every sixth mounted section through the telencephalon (600 μ m sampling interval). We traced the borders of the telencephalic hemisphere and scanned the tracings into a microcomputer. We defined the borders of the telencephalon as in DeVoogd et al. (1993) and Brenowitz et al. (1998). In sections in which the telencephalon was contiguous with the diencephalon, we used the septomesencephalic tract, anterior commissure, and occipitomesencephalic tract as natural borders. Telencephalon volume was estimated with the formula for a cone frustum.

We also measured neuronal attributes of RA, including neuronal somal area, neuron density, and neuron number, as described below. In wild birds with intact HVC, neuronal size in RA increases and neuronal density decreases when RA grows during the breeding season (Smith et al., 1997c; Tramontin and Brenowitz, 1999, 2000). We wished to determine whether HVC lesions disrupted these cellular changes in RA. In intact birds the number of neurons in RA does not change when this nucleus grows in the breeding season (Brenowitz et al., 1991; Smith et al., 1995, 1997a,c; Tramontin et al., 1998, 1999; Tramontin and Brenowitz, 2000). It is possible, however, that lesions of HVC might lead to the loss of neurons in RA when they are derived of trophic support. We measured neuron number in our birds to test this possibility. Cellular attributes of area X have not been measured in wild birds in different seasons, and we therefore did not measure neuronal traits in this nucleus.

We used a random systematic sampling scheme to measure soma size and neuron density in RA. This procedure was described in detail by Tramontin et al. (1998), who showed that with 50 μ m Nissl-stained sections, this scheme yielded estimates of neuron density and number that did not differ from those obtained using a stereological optical disector procedure. Briefly, we captured a video image of RA at 195 $\times$ magnification using NIH Image. We outlined the borders of RA on-screen and overlaid it with a square grid 42 μ m per side. Using a random-number generator, we selected one of the first six columns and every sixth column thereafter. For each selected column we randomly selected one square. We counted and measured the somal area of all neurons in that square from a second digitized image of that field captured at 1950 $\times$ magnification. In this way we measured at least 100 neurons throughout the RA of each bird, which required sampling 17–23 fields per individual. This sample size is sufficient to encompass all of the variance present in somal area and neuron density in RA (Tramontin et al., 1998).

Statistical analyses. We used separate two-way repeated measures ANOVAs (RMANOVAs) to compare plasma T levels during the first 4 weeks of the study between the SD and growth groups and between the SD and maintenance groups. Sampling time was the repeated measure, and photoperiod or hormone treatment was the unrepeated second factor. A one-way RMANOVA was used to compare plasma T levels across the 7 weeks in the maintenance group. Data for plasma T levels were log-transformed to meet the criteria of normality and equal variance.

We used separate two-way RMANOVAs to compare nuclear and

neuronal measurements between the SD and growth groups and between the SD and maintenance groups. The side of the brain analyzed (ipsilateral vs contralateral to the lesion) was the repeated measure, and the photoperiod or hormone treatment was the unrepeated second factor. The growth and maintenance groups did not differ significantly in any measurements (see Tables 2, 3), and we therefore did not perform a single two-way RMANOVA for all three groups. Because we wanted to compare specific attributes between each LD+T group and the SD group, we did not pool data from the growth and maintenance groups. The RMANOVAs were followed by one-tailed *post hoc* pairwise comparisons using the Student–Newman–Keuls (SNK) test. We used one-tailed tests because we tested the directional hypotheses that lesions of HVC would cause regression or prevent growth of ipsilateral nuclear and neuronal attributes. There is no basis for predicting that lesions of HVC would result in song nuclei or neurons that are larger or more widely spaced than those contralateral to the lesion.

To test the hypothesis that lesions of HVC completely prevented growth or maintenance of the ipsilateral efferent nuclei in the two LD+T groups, we used two-tailed *t* tests to compare measurements contralateral to the lesion in the SD group with measurements ipsilateral to the lesion in the growth and maintenance groups. Two-tailed tests were used for this comparison because there was no *a priori* basis for predicting whether attributes ipsilateral to the lesion would be greater or less than those contralateral to the lesion in the SD group.

In some birds the lesions spared part of the HVC. To determine the proportion of HVC damaged by the lesion, we compared the volume of the remaining lesioned HVC (if any) with that of the contralateral unlesioned HVC. Previous studies have not reported a size difference between the left and right HVC in intact GWCS (Smith et al., 1995, 1997a,b; Tramontin et al., 1998, 1999, 2000; Tramontin and Brenowitz, 2000). We calculated Pearson product moment correlations to determine whether the size of ipsilateral RA and area X or neuronal attributes of RA were related to the size of the remaining HVC in all three treatment groups. For this and all other tests, the α level was 0.05.

RESULTS

Plasma T levels

Before receiving SILASTIC implants, birds in all three treatment groups had basal plasma T levels typical of nonbreeding white-crowned sparrows (Table 1). T implants significantly increased circulating T concentrations in the growth and maintenance groups above those in the SD group for all postimplant samples (Table 1; $F \geq 51.78$; $p < 0.001$ for photoperiod or hormone treatment factor; $F \geq 20.44$; $p < 0.001$ for sampling-time factor during the first 4 weeks, two-way RMANOVA). Plasma T levels were within or above the physiological range for breeding birds (4–10 ng/ml) at all postimplant sampling times in both LD+T groups. In the maintenance group, plasma T levels varied significantly across the 7 weeks (Table 1; $F = 4.00$; $p = 0.019$, one-way RMANOVA). This pattern reflects a decrease in circulating T over the 4 weeks after the first implant and then an increase after replacement of the T pellet at 4 weeks.

Song nuclei: HVC

Exposure to the long-day photoperiod and T induced growth of the unlesioned HVC (Table 2). HVC was significantly larger in both the growth [$F_{(1,15)} = 10.39$; $p = 0.006$; SNK $q = 4.138$; $p =$

Table 2. Absolute volumes of brain regions (mm³) ipsilateral and contralateral to the unilateral HVC lesion

Region	Treatment group	Ipsilateral	Contralateral
HVC	SD	0.071 ± 0.019	0.391 ± 0.045
	Growth	0.161 ± 0.065	0.576 ± 0.028
	Maintenance	0.118 ± 0.050	0.637 ± 0.343
RA	SD	0.128 ± 0.019	0.170 ± 0.051
	Growth	0.150 ± 0.035	0.229 ± 0.046
	Maintenance	0.164 ± 0.114	0.216 ± 0.008
Area X	SD	0.879 ± 0.177	1.106 ± 0.304
	Growth	1.405 ± 0.107	1.611 ± 0.289
	Maintenance	1.181 ± 0.062	1.313 ± 0.116
IMAN	SD	0.054 ± .00671	0.059 ± .009
	Growth	0.075 ± .0080	0.071 ± .0066
	Maintenance	0.067 ± .0074	0.066 ± .0061
nXIIIs	SD	0.033 ± .0009	0.034 ± .0119
	Growth	0.044 ± .0002	0.046 ± .0053
	Maintenance	0.039 ± .0048	0.039 ± .0046
Telencephalon	SD		275.15 ± 10.21
	Growth		284.13 ± 5.50
	Maintenance		257.49 ± 9.10

Values shown are means ± SEM. For telencephalon, only the unlesioned contralateral hemisphere was measured. See text for sample sizes.

0.007] and maintenance [$F_{(1,13)} = 7.92$; $p = 0.015$; SNK $q = 4.060$; $p = 0.009$] groups than in the SD group. There was no significant difference in the size of unlesioned HVC between the two LD+T groups [$F_{(1,14)} = 0.567$; $p = 0.464$; SNK $q = 1.628$; $p = 0.260$].

There was individual variation in the amount of HVC eliminated by the lesion. In the SD group, the lesion eliminated 43–100% of HVC, as measured in comparison with the unlesioned contralateral HVC. In the growth group, 28–100% of HVC was damaged by the lesion. In the maintenance group, 36–100% of HVC was lesioned. The three treatment groups did not differ significantly in the percent of HVC lesioned [$F_{(2,23)} = 0.177$; $p = 0.839$, one-way ANOVA].

RA

SD group: volume

Afferent input from HVC was required to maintain RA even at its regressed size in SD birds. RA was significantly smaller ipsilateral to the HVC lesion than contralateral (see Fig. 3; Tables 2, 3). The size of RA was not correlated with the size of the remaining HVC ($r = -0.331$; $p = 0.423$).

SD group: neuronal attributes

In the SD birds, the HVC lesions altered neuron density and number in RA. Neuron density was greater and neuron number was lower ipsilateral to the lesion than contralateral (see Fig. 5; see Table 5). None of these neuronal traits in ipsilateral RA was significantly correlated with the size of HVC remaining after the lesion (Pearson $r \leq -0.529$; $p \geq 0.177$; $n = 8$).

Growth group: volume

Lesions of HVC blocked the LD+T-induced growth of RA in the growth group. Contralateral to the lesion, the volume of RA increased significantly compared with that of the SD birds (see Fig. 3; Tables 2–4). RA was smaller ipsilateral to the lesion than contralateral within the growth birds (Figs. 2, 3; Tables 2, 3). The volume of RA ipsilateral to the lesion was highly correlated with the volume of the remaining HVC ($r = 0.940$; $p = 1.6 \times 10^{-4}$; Fig.

4). The volume of RA ipsilateral to the lesion in the growth group did not differ from the ipsilateral or contralateral volumes in the SD birds (Fig. 3; Tables 2, 3).

Growth group: neuronal attributes

Neuronal attributes in RA were affected by the HVC lesions within the growth birds. In the ipsilateral RA, somal area was significantly smaller than that contralateral to the lesion (Fig. 5; Tables 3, 5). Neuron density was greater in ipsilateral RA, and this difference approached significance ($p = 0.0645$; Fig. 5; Tables 3, 5). Mean neuron number in RA was greater contralateral to the lesion in the growth group (Fig. 5); the side-of-brain factor in the RMANOVA approached significance ($p = 0.063$; Table 3), and the *post hoc* SNK test indicated a significant difference between the ipsilateral and contralateral sides of RA (Table 5). The number of neurons in ipsilateral RA was correlated with the size of HVC remaining after the lesion (Pearson $r = 0.743$; $p = 0.022$; $n = 9$). Ipsilateral to the lesion, the somal area of RA neurons was significantly greater in the growth group than in the SD group (Fig. 5; Tables 3, 5). None of these neuronal traits differed between the ipsilateral RA in growth birds and the contralateral RA in the SD group (Fig. 5; Tables 3, 5).

Maintenance group: volume

Afferent input from HVC was necessary to maintain RA at its enlarged size in the maintenance group. Contralateral to the lesion, the volume of RA in this group was larger than that in the SD birds (Fig. 3; Tables 2–4). RA in the maintenance birds was smaller ipsilateral to the lesion than contralateral (Fig. 3; Tables 2–4). The volume of RA ipsilateral to the lesion was correlated with the volume of the remaining HVC ($r = 0.835$; $p = 0.02$; Fig. 4). The volume of RA ipsilateral to the lesion in the maintenance group was significantly larger than the ipsilateral volume in the SD birds but did not differ from the contralateral volume in the SD birds (Fig. 3; Tables 2–4).

Maintenance group: neuronal attributes

Neuron size in RA within the maintenance group was marginally decreased by the HVC lesions (Fig. 5; Tables 3, 5). In this group the lesions increased neuronal density, which was significantly greater in ipsilateral RA (Fig. 5; Tables 3, 5). Within the maintenance group, there was no effect of the lesions on neuron number in RA (Fig. 5; Tables 3, 5). None of these neuronal traits in ipsilateral RA was correlated with the amount of HVC remaining after the lesion (Pearson $r \leq 0.522$; $p \geq 0.229$; $n = 7$). Ipsilateral to the lesion, neither somal area nor neuron density differed between the SD and maintenance groups (Fig. 5; Tables 3, 5). Neuron number was slightly greater in ipsilateral RA in the maintenance than in the SD birds, although this difference did not reach significance ($p = 0.068$; Fig. 5; Tables 3, 5).

Growth versus maintenance groups

The RMANOVA indicated that there was not a significant difference in the volume of RA between the growth and maintenance groups (Table 3). The *post hoc* SNK tests showed, however, that there were some differences in the effects of the HVC lesions on neuronal attributes of RA in these two groups (Table 5). The lesions significantly reduced neuron number in ipsilateral RA in the growth birds but not in the maintenance group. The lesions led to a highly significant increase in neuron density in ipsilateral RA in the maintenance group but a smaller increase that approached but did not reach significance in the growth group ($p =$

Table 3. Statistics for two-way RMANOVAs of volumes of song nuclei and neuronal attributes of RA in SD, growth, and maintenance groups, ipsilateral and contralateral to unilateral lesion of HVC

Attribute	SD × growth		SD × maintenance		Growth × maintenance	
	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
RA volume	6.61 _{1,15} (0.021)	51.01 _{1,15} (<0.001)	18.58 _{1,13} (<0.001)	42.08 _{1,13} (<0.001)	2.31 _{1,14} (0.151)	80.79 _{1,14} (<0.001)
Area X volume	14.23 _{1,13} (0.002)	12.43 _{1,13} (0.004)	12.42 _{1,13} (0.004)	9.14 _{1,13} (0.010)	0.21 _{1,14} (0.656)	15.51 _{1,14} (0.001)
nXIIts volume	8.27 _{1,15} (0.012)	0.52 _{1,15} (0.483)	0.28 _{1,11} (0.607)	0.10 _{1,11} (0.756)	8.49 × 10 ⁻¹⁵ _{1,11} (1.0)	5.48 × 10 ⁻⁴ _{1,11} (1.0)
IMAN volume	2.66 _{1,14} (0.125)	0.01 _{1,14} (0.940)	3.78 _{1,12} (0.076)	0.25 _{1,12} (0.625)	2.49 × 10 ⁻³ _{1,14} (0.961)	0.897 _{1,14} (0.669)
RA somal area	10.15 _{1,15} (0.006)	6.20 _{1,15} (0.025)	1.80 _{1,13} (0.203)	5.27 _{1,13} (0.039)	1.63 _{1,14} (0.223)	8.32 _{1,14} (0.012)
RA neuron density	4.32 _{1,15} (0.055)	4.02 _{1,15} (0.063)	4.65 _{1,13} (0.050)	19.54 _{1,13} (<0.001)	0.11 _{1,14} (0.742)	6.88 _{1,14} (0.020)
RA neuron number	0.94 _{1,15} (0.349)	4.01 _{1,15} (0.063)	1.06 _{1,13} (0.323)	3.15 _{1,13} (0.099)	5.71 × 10 ⁻³ _{1,14} (0.941)	1.82 _{1,14} (0.199)

The volumes for RA, area X, and IMAN were divided by the volume of the telencephalon contralateral to the HVC lesion. The *F* statistic, degrees of freedom (in subscript), and *p* level (in parentheses) for factor 1 (photoperiod+hormone treatment) and factor 2 (side of brain measured) in each RMANOVA are indicated. Significant test results are shown in bold type. See text for explanation of group comparisons.

Table 4. Statistics for pairwise comparisons of volumes of song nuclei in SD, growth, and maintenance groups, ipsilateral and contralateral to unilateral lesion of HVC

Groups compared	RA	Area X	nXIIts
SD contra versus SD ipsi	5.08 (<0.0015)	3.89 (0.0085)	0.20 (0.390)
Growth contra versus growth ipsi	9.34 (<0.0005)	4.81 (0.002)	<10 ⁻⁵ (1.00)
Maintenance contra versus maintenance ipsi	7.34 (<0.0005)	3.07 (0.024)	<10 ⁻⁵ (1.00)
SD contra versus growth contra	4.50 (0.0045)	4.61 (0.002)	3.98 (0.006)
SD ipsi versus growth ipsi	1.64 (0.242)	4.91 (0.0015)	3.64 (0.0095)
SD contra versus maintenance contra	5.92 (<0.0005)	3.68 (0.008)	0.90 (0.269)
SD ipsi versus maintenance ipsi	4.64 (0.002)	4.80 (0.0015)	0.91 (0.265)
Growth contra versus maintenance contra	1.23 (0.20)	0.82 (0.286)	<10 ⁻⁵ (1.00)
Growth ipsi versus maintenance ipsi	2.67 (0.074)	0.452 (0.377)	<10 ⁻⁵ (1.00)
SD contra versus growth ipsi	1.56 (0.07)	-1.56 (0.072)	-2.22 (0.043)
SD contra versus maintenance ipsi	-0.42 (0.342)	-0.75 (0.234)	-0.75 (0.467)

The volumes for RA, area X, and IMAN were divided by the volume of the telencephalon contralateral to the HVC lesion. The top nine comparisons used SNK *post hoc* test; the *q* statistic and *p* level (in parentheses) for each comparison are indicated. The bottom two comparisons used a *t* test; the *t* statistic and *p* level (in parentheses) for each comparison are presented. Significant test results are shown in bold type. See text for sample sizes. contra, Contralateral; ipsi, ipsilateral.

0.0645). Somal area was significantly reduced in ipsilateral RA of both the growth and maintenance groups.

Area X

SD group

This nucleus required afferent input from HVC to remain at its regressed size in SD birds. This nucleus was smaller ipsilateral to the lesion than contralateral (Fig. 3; Tables 2–4). The volume of ipsilateral area X was not correlated with the volume of HVC ($r = -0.281$; $p = 0.50$).

Growth group

The full LD+T-induced growth of area X in the growth group was blocked by the lesion of HVC. The volume of area X contralateral to the lesion in the growth birds was significantly greater than that in the SD birds (Fig. 3; Tables 2–4). Within the growth group, area X was smaller ipsilateral to the lesion than contralateral (Figs. 2, 4; Tables 2–4). The volume of area X ipsilateral to the lesion was marginally correlated with the volume of the remaining HVC ($r = 0.738$, $p = 0.058$; Fig. 4). Area X differed from RA in that the volume ipsilateral to the lesion in the growth group was greater than the ipsilateral volume in the SD birds (Fig. 3; Tables 2–4). The mean volume of area X ipsilateral to the lesion in the growth group was not significantly different from the volume contralateral to the lesion in the SD birds (Fig. 3; Tables 2–4).

Maintenance group

Area X required afferent input from HVC to maintain its enlarged size in the maintenance group. The volume of area X contralateral to the lesion was significantly larger in this group than in the SD birds (Fig. 3; Tables 2–4). Area X within the maintenance group was smaller ipsilateral to the lesion than contralateral (Fig. 3; Tables 2–4). The volume of area X ipsilateral to the lesion was not significantly correlated with the volume of the remaining HVC ($r = -0.687$; $p = 0.088$). The volume of area X ipsilateral to the lesion in the maintenance birds was significantly larger than the ipsilateral volume in the SD birds but did not differ from the contralateral volume in the SD birds (Fig. 3; Tables 2–4).

Growth versus maintenance groups

The effects of HVC lesions on area X were similar whether they were made before or after growth induced by LD+T. There were no significant differences in the volume of area X between the growth and maintenance groups (Fig. 3; Tables 2–4).

IMAN

HVC lesions had no effect on the volume of IMAN in any of the treatment groups. There were no significant differences between the ipsilateral and contralateral volumes of IMAN in the three groups (Tables 2, 3). IMAN did not grow significantly in response

Figure 2. Photographs showing RA (*top*) and area X (*bottom*) ipsilateral (*right*) and contralateral (*left*) to a unilateral lesion of HVC in a male white-crowned sparrow exposed to long days plus testosterone treatment for 30 d after the lesion. The maximum extent of each nucleus is indicated with arrows. Scale bars, 1 mm.

to the LD+T treatment (Tables 2, 3). In the RMANOVAs the photoperiod plus hormone treatment factor was not significant for any of the groups (Table 3).

The volume of IMAN was correlated with the size of other nuclei in some of the treatment groups. Ipsilateral to the lesion in the SD birds, IMAN was correlated with area X ($r = 0.752$; $p = 0.032$). In the growth group, the volume of ipsilateral IMAN was related to the volume of RA ($r = 0.710$; $p = 0.032$).

nXIIIts

The HVC lesions did not affect the size of nXIIIts in any treatment group. There was no difference between the ipsilateral and contralateral volumes of nXIIIts in any treatment group (Tables 2–4). nXIIIts increased in volume significantly in the growth group (Tables 2–4). In the *post hoc* tests, both the ipsilateral and contralateral nXIIIts in the growth group were larger than their counterparts in the SD birds (Tables 2, 4). Ipsilateral nXIIIts in the growth group was also larger than the contralateral nXIIIts in the SD group (Tables 2, 4). The mean volume of nXIIIts on both sides of the brain in the maintenance group was intermediate between those of the other two treatment groups and did not differ significantly from either group (Tables 2–4).

The volume of ipsilateral nXIIIts in the maintenance group was correlated with the size of RA ($r = 0.889$; $p = 0.044$).

DISCUSSION

Afferent input is known to be important for the perinatal development and maintenance of neurons (for review, see Oppenheim, 1991). Furthermore, trans-synaptic effects of gonadal steroids are known to influence the development of neural circuits (for review, see Beyer and Feder, 1987). Our study demonstrates that afferent input is also necessary for the growth of adult brain nuclei in response to seasonal hormonal and photoperiod cues; seasonal growth is a common feature of adult vertebrate brains (for review, see Tramontin and Brenowitz, 2000).

Previous research demonstrated that seasonal growth of the

song circuits is primarily regulated by changes in plasma T levels (Gulledge and Deviche, 1997; Smith et al., 1997a; Ball, 2000). We found that lesions of HVC blocked or decreased the LD+T-induced growth of its efferent targets RA and area X in the growth group. Our study suggests that T acts directly on HVC, which in turn stimulates the growth of RA and area X via trans-synaptic effects. AR are present in both RA-projecting and area X-projecting neurons in HVC (Sohrabji et al., 1989). Additional support for our suggestion comes from the observation that small unilateral implants of T adjacent to HVC induced growth of ipsilateral but not contralateral HVC, RA, and area X in GWCS, whereas T implants near RA did not induce growth of these nuclei (Brenowitz and Lent, 2000).

Afferent input from HVC was also necessary to maintain RA and area X after they had grown in response to LD+T treatment. RA and area X contralateral to the lesion in the maintenance group were larger than these nuclei in the SD group and did not differ in size from the contralateral nuclei in the growth birds. These observations show that RA and area X grew on both sides of the brain before the HVC lesion in the maintenance birds. The smaller volume of the efferent nuclei ipsilateral to the lesion in this group can therefore be interpreted as indicating a reduction in size as a result of the lesion, rather than a failure to initially grow in response to LD+T.

The volume of the ipsilateral RA was strongly correlated with the size of the remaining HVC, if any, in both the growth and maintenance groups. These correlations indicate that the extent to which LD+T induced or maintained growth of RA was directly related to the amount of trophic support that HVC continued to provide after the lesion.

The size of area X, in contrast to RA, was only marginally correlated with the amount of HVC that remained after the lesion in the growth group and was not correlated in the maintenance group. Furthermore, ipsilateral area X was significantly larger in these groups than in the SD birds. Two factors might have

Figure 3. The volumes (mean $\pm$ SEM) of RA (*top*) and area X (*bottom*) ipsilateral and contralateral to the lesioned HVC in the SD, growth, and maintenance (*maint.*) treatment groups. The volume of each nucleus was divided by the volume of the entire telencephalon contralateral to the lesioned HVC. Different letters above the vertical bars indicate significant differences in volume between sides of the brain and between treatment groups.

contributed to this pattern. First, there are area X-projecting (but not RA-projecting) neurons in para-HVC, a region immediately caudal to HVC (Nordeen et al., 1987; Johnson and Bottjer, 1995). Some of these neurons were likely spared by our HVC lesions and may have provided adequate trophic support to prevent area X from regressing to the size found in the SD birds, regardless of how much HVC remained. These area X-projecting neurons contain receptors for estradiol, which is available by aromatization of T (Nordeen et al., 1987; Johnson and Bottjer, 1995; Schlenger, 1997). Second, there is evidence that area X in GWCS grows to a small extent in response to effects of photoperiod independent of gonadal steroids. In a previous study, area X in castrated GWCS exposed to LD was intermediate in size between castrates exposed to SD and castrates exposed to SD+T (Smith et al., 1997a). A similar effect may have played a role in preventing area X in the LD+T birds from regressing fully down to the size observed in the SD birds.

Neurons in RA of intact birds have high levels of AR (Smith et al., 1996). In the absence of afferent input from HVC, however, high plasma T concentrations were not sufficient to induce or maintain growth of RA. Potentially, the loss of afferent input from HVC downregulated AR expression in RA neurons. A

Figure 4. The relationship between the volume of HVC remaining after the lesion, if any, and the volumes of RA (*top*) and area X (*bottom*) ipsilateral to the lesion in the growth and maintenance groups.

decrease in AR levels would have made RA less sensitive to the stimulatory effects of T. Alternatively, the growth and maintenance of RA may require an interaction between trans-synaptic influences from HVC and the direct action of T on RA.

Systemic T treatment clearly influences area X (present report) (Konishi and Akutagawa, 1981; Smith et al., 1997a), but it is unlikely that T acts directly on this nucleus. Steroid accumulation, immunocytochemical, and *in situ* hybridization studies show that area X neurons have few or no AR (Arnold et al., 1976; Balthazart et al., 1992; Smith et al., 1996; Bernard et al., 1999; Metzendorf et al., 1999).

The SD group demonstrated that afferent input from HVC is also required for RA and area X to maintain their normal regressed, nonbreeding season size. Apparently, HVC continues to provide trophic support at a basal level that keeps the efferent nuclei somewhat larger than they would be in the absence of this support. It seems unlikely that support of the song nuclei in SD birds is based on gonadal androgens because the testes are regressed and plasma T levels are unmeasurably low in GWCS outside the breeding season (Wingfield and Farnar, 1978). It may be that there is a constitutive level of electrical activity across the synapses between HVC axon terminals and their postsynaptic targets. Alternatively, there may be an ongoing release of neuro-

Figure 5. Neuronal attributes (mean $\pm$ SEM) of RA ipsilateral and contralateral to the lesioned HVC in the SD, growth, and maintenance groups. Different letters above the vertical bars indicate significant differences between sides of the brain and between treatment groups.

trophic factors by HVC axon terminals that maintain RA and area X neurons. The potential roles of synaptic activity and neurotrophins in providing trophic support are discussed in more detail below.

The neuronal measurements in RA provide insights into the cellular basis of the HVC lesion effects in the different treatment groups. Previous reports showed that the seasonal growth of RA

is primarily caused by increases in soma size, dendritic arborizations, and neuronal spacing and that neuron number in RA does not change seasonally (for review, see Tramontin and Brenowitz, 2000).

The HVC lesions significantly decreased neuron number in both the SD and growth groups but not in the maintenance birds. This comparison suggests that withdrawing afferent input from RA when birds had low plasma T and RA was regressed led to neuronal loss. The strong correlation ($r = 0.74$) between RA neuron number and the amount of HVC remaining after the lesion in the growth birds supports this suggestion. High plasma T and a fully grown RA in the maintenance birds appear to have protected RA neurons from dying after HVC was lesioned. Neuron number in RA of the maintenance group was not correlated with the size of HVC after the lesion. It is possible that T acted directly on RA neurons to prevent them from dying in these birds, although high T was not adequate to maintain the size or spacing of these neurons when afferent input was removed. If this was the case, then neuronal loss must have occurred relatively rapidly in the growth birds because they received a T implant 1 d after HVC was lesioned.

Neuron density in RA was increased by the HVC lesions in all three groups, although the decrease in the growth group did not reach significance ($p = 0.0645$). Decreased spacing may reflect a decrease in dendritic arborizations (DeVoogd and Nottebohm, 1981; Hill and DeVoogd, 1991). The change in density was most pronounced for the maintenance birds, as shown in the p levels of the *post hoc* tests. In the SD and growth groups, increased neuron density may have been partially offset by the decreases in neuron number.

Neuron size in RA was decreased by the HVC lesions in the growth and maintenance groups but not in the SD birds. Afferent input from HVC is necessary both to grow and to maintain large RA neurons in birds exposed to LD+T. There may have been a “floor” effect in the SD birds, in which neurons in the regressed RA were already at a minimum size and not able to regress further after the lesions.

The specific nature of the trophic support provided by HVC to its efferent targets remains to be determined. Chemical neurotransmission associated with electrical activity across synapses may have neurotrophic effects on postsynaptic neurons (Balazs et al., 1989; Brenneman et al., 1990a,b; Rubel et al., 1990; Oppenheim, 1991; Galli-Resta et al., 1993). The release of neurotransmitter molecules from HVC axon terminals in the current study may have had trophic effects on neurons in RA and area X. Alternatively, or in addition, presynaptic terminals from HVC may have released neurotrophins that stimulated growth of postsynaptic neurons. Neurotrophins can be transported anterogradely and taken up by postsynaptic neurons (von Bartheld et al., 1996). Neurotrophins influence the juvenile development of the song circuits (Johnson et al., 1997; Akutagawa and Konishi, 1998; Ditttrich et al., 1999). Also, Rasika et al. (1999) showed that T-induced growth of HVC in adult female canaries (*Serinus canaria*) is mediated by the action of brain-derived neurotrophic factor (BDNF) on HVC neurons. BDNF mRNA is expressed in RA-projecting and, to a lesser extent, area X-projecting neurons of HVC in singing adult male canaries (Li et al., 2000). The effects of LD+T on HVC and the efferent nuclei in our study may have also been mediated by the action of neurotrophins. It is possible that the specific nature of trophic activity provided by HVC to its efferents (i.e., chemical neurotransmission vs neurotrophins), or

Table 5. Statistics for pairwise comparisons of neuronal attributes of RA in SD, growth, and maintenance groups, ipsilateral and contralateral to unilateral lesion of HVC

Groups compared	Somal area	Neuron density	Neuron number
SD contra versus SD ipsi	1.46 (0.158)	3.12 (0.023)	3.22 (0.02)
Growth contra versus growth ipsi	3.92 (0.008)	2.27 (0.0645)	2.70 (0.038)
Maintenance contra versus maintenance ipsi	2.53 (0.048)	3.03 (0.025)	0.42 (0.384)
SD contra versus growth contra	4.45 (0.002)	2.23 (0.063)	1.56 (0.139)
SD ipsi versus growth ipsi	2.79 (0.030)	1.82 (0.104)	0.28 (0.4225)
SD contra versus maintenance contra	1.92 (0.0955)	3.63 (0.009)	0.33 (0.408)
SD ipsi versus maintenance ipsi	1.58 (0.1395)	1.62 (0.133)	2.20 (0.068)
Growth contra versus maintenance contra	2.117 (0.074)	0.96 (0.2525)	1.32 (0.180)
Growth ipsi versus maintenance ipsi	0.90 (0.266)	0.49 (0.367)	1.18 (0.206)
SD contra versus growth ipsi	−0.92 (0.374)	−0.02 (0.981)	1.24 (0.235)
SD contra versus maintenance ipsi	−0.32 (0.752)	−0.46 (0.653)	−0.02 (0.985)

The top nine comparisons used SNK *post hoc* test, and the *q* statistic and *p* level (in parentheses) for each comparison are indicated. The bottom two comparisons used a *t* test, and the *t* statistic and *p* level (in parentheses) for each comparison are presented. Significant test results are shown in bold type. See text for sample sizes.

the relative contributions of these factors, differed between the SD and the LD+T conditions.

RA and area X each receive afferent input from IMAN as well as HVC, but the IMAN input was not sufficient for the maintenance or for full LD+T-induced growth of these regions after HVC lesions. IMAN neurons contain AR, as do HVC neurons, but IMAN does not change in size seasonally in white-crowned sparrows (present report) (Smith et al., 1995, 1997a; Brenowitz et al., 1998; Tramontin and Brenowitz, 2000). Taken together, these observations indicate that the presence of AR in a given nucleus is not sufficient to induce or maintain growth either of that nucleus or of its efferent targets in response to high plasma T levels.

The HVC lesions had no effect on the volumes of IMAN or nXIIIs, which are both separated from HVC by multiple synapses. These results suggest that the effects of the HVC lesions were limited to nuclei that receive direct afferent input from HVC (i.e., RA and area X).

nXIIIs did grow in response to LD+T (see also Smith et al., 1997a). There are several possible explanations for this growth in the absence of HVC input to the motor circuit. (1) Neurons in nXIIIs have AR (Arnold et al., 1976) and may have responded directly to the T treatment. (2) T may have directly stimulated the syringeal muscles, which have AR (Lieberburg and Nottebohm, 1979; Luine et al., 1980, 1983; Smith et al., 1996). The motor neurons in nXIIIs innervate these muscles and might have derived retrograde trophic support from them, as observed in the spinal nucleus of the bulbocavernosus of rats (Fishman et al., 1990; Fishman and Breedlove, 1992) and the laryngeal motor nucleus of *Xenopus* (Kelley, 1986). (3) Neurons in nXIIIs receive afferent input from the dorsomedial portion of the intercollicular nucleus (ICo), which has AR (Soma et al., 1998). T may have acted on ICo neurons that could then have had trophic effects on postsynaptic nXIIIs neurons. (4) RA may have provided adequate trophic support to nXIIIs neurons even in the absence of input from HVC.

In conclusion, seasonal change in brain morphology is a common feature of vertebrate brain organization. We have shown that afferent input from the avian song nucleus HVC is necessary for both the seasonal growth and maintenance of its efferent targets. Exploring the specific mechanisms underlying the trophic support provided by HVC represents a logical extension of our study. It will be productive to address this issue in a broad array

of vertebrate neural circuits that demonstrate adult seasonal plasticity.

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