Rapid seasonal-like regression of the adult avian song control system

Christopher K. Thompson*†, George E. Bentley‡, and Eliot A. Brenowitz*§

*Graduate Program in Neurobiology and Behavior and §Departments of Psychology and Biology, University of Washington, Box 351525, Seattle, WA 98195-1525; and ‡Department of Integrative Biology, University of California, 3060 Valley Life Sciences Building 3140, Berkeley, CA 94720-3140

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We analyzed how rapidly avian song control nuclei regress after testosterone (T) withdrawal. Regression of neuronal attributes resulting from T withdrawal has been observed in several animal models. The time course over which regression occurs is not known, however. To address this issue, we castrated adult male white-crowned sparrows and rapidly shifted them to short-day photoperiods after being held under breeding conditions (long-day photoperiod and systemic T exposure) for 3 weeks. We found that the volume of one song nucleus, HVC, regressed 22% within 12 h after T withdrawal. Changes in HVC neuron density after T withdrawal were dynamic; density increased at 12 h and then decreased by 4 days. HVC neuron number was reduced by 26% by 4 days. The volumes of Area X and the robust nucleus of the arcopallium (RA) were significantly regressed by 7 and 20 days, respectively. RA somatic area and neuronal spacing were significantly reduced by 2 days. The rapidity of HVC regression is unprecedented among vertebrate models of hormone-sensitive neural circuits. These results reveal that the rapid regression of the song control system provides a model for the important role sex steroid hormones play in mediating adult neural plasticity and in neuroprotection.

birdsong | neurodegeneration | neuroprotection | plasticity | testosterone

Plasticity of structure and function is a fundamental feature of vertebrate brain organization. Steroid hormones play an important role in regulating adult neural plasticity in different brain regions and across taxa (1–6). A question of much interest but that is little investigated is how rapidly brain regions respond to withdrawal of trophic support such as that provided by steroids. Seasonal plasticity of the avian song control system serves as an excellent model for examining the time course of changes in brain structure caused by steroid withdrawal.

Seasonal plasticity of the adult brain has been observed in every vertebrate class (6). Seasonal change of the neural circuits regulating song production, learning, and perception in songbirds, first described by Nottebohm (7) in adult male canaries, has become one of the leading models of adult neural plasticity. The onset of increased day length and elevated levels of circulating testosterone (T) induces rapid and sequential growth of song system nuclei (8, 9). The underlying cellular changes that lead to increases in volume differ between nuclei. Seasonal growth of the volume of one song nucleus, HVC, largely involves increases in neuronal number caused by increased recruitment of new neurons. Increases in the volume of the robust nucleus of the arcopallium (RA) and Area X, both efferent targets of HVC, however, involve increases in neuronal size and spacing, with no change in neuron number (8, 10). These robust changes in brain morphology and circulating levels of hormones are accompanied by changes in song behavior, including song rate, duration, and stereotypy (11).

The seasonal growth of RA and Area X depends on growth of HVC. In captive male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambeli*), a rapid transition from conditions that mimic a nonbreeding state [short days (SD)] to breeding-like conditions [long days + s.c. T implant (LD+T)] leads to rapid and sequential growth of song control nuclei. We refer to neural changes induced under these conditions as "seasonal-like." Under these conditions HVC grows fully within 7 days, whereas the volumes of RA and Area X do not reach full size until 20 days (8). Lesions of HVC prevent the growth or induce the regression of RA and Area X in birds when they are first transferred to LD+T or when they are maintained on LD+T, respectively (12). T acts directly on HVC, which then acts transynaptically to stimulate growth of RA and Area X (13).

At the end of the breeding season, the reproductive system of Gambel's white-crowned sparrows becomes inactive and unresponsive to photostimulation, the testes regress, and T is reduced to basal levels (14). Circulating T decreases from maximal to undetectable levels within days to weeks as males transition from territorial defense to incubating behavior. As T levels decline, the song nuclei regress; the song control system of wild male song sparrows fully regresses by late summer, just after the postbreeding feather molt (11). This and other observations suggest that the song control system regresses rapidly once T levels decline below a certain threshold.

Previous studies have shown that the song control system (15, 16) and hormone-sensitive brain areas in other animal models (17–19) regress in response to T withdrawal, but none has established a time course of regression. To get a better sense of how quickly the song system is able to regress during the transition from breeding to nonbreeding conditions, we rapidly shifted adult male Gambel's white-crowned sparrows from breeding-like (LD+T) conditions to those that mimic a nonbreeding state (SD and T withdrawal) conditions to determine the time course of regression of the song control system. We found that HVC significantly regresses in volume in <12 h after T withdrawal and that RA and Area X regress more slowly. We also found that the rapid regression of HVC volume is accompanied by a dynamic change in HVC neuron density over the first 4 days after T withdrawal and that HVC neuron number significantly decreases by 4 days.

Results

Song system nuclei regressed rapidly after a rapid transition from breeding to nonbreeding conditions (Fig. 1). HVC volume differed significantly across the six time points (Fig. 2*A*). Post hoc tests showed that the average HVC volume of LD+T birds was significantly greater than that of birds killed 12 h after T withdrawal. Twelve-hour birds did not differ significantly from 2-day, 4-day, 7-day, or 20-day birds.

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Abbreviations: BDNF, brain-derived neurotrophic factor; LD, long day; RA, robust nucleus of the arcopallium; SD, short day; T, testosterone.

†To whom correspondence should be addressed. E-mail: ckthomps@u.washington.edu.

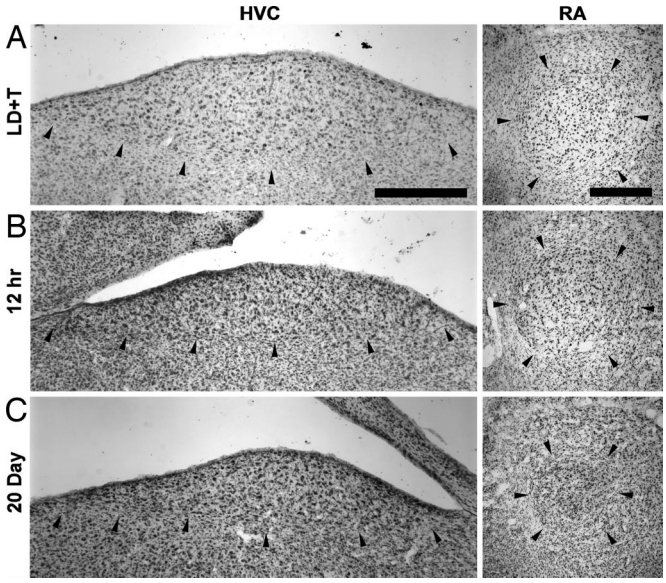

Fig. 1. Coronal sections of HVC and RA of male white-crowned sparrows illustrate the rapid regression of the song control system. (*A*) HVC and RA grow to full size within 3 weeks of exposure to a LD photoperiod and high levels of T. (*B*) Twelve hours after T withdrawal, HVC volume regresses significantly, largely driven by a sharp increase in HVC neuron density. RA volume is unchanged. (*C*) Twenty days after T withdrawal and shift to a SD photoperiod, RA is fully regressed. (Scale bars: 0.5 mm.)

The volumes of RA and Area X regressed more slowly than HVC after the transition from LD+T to SD and T withdrawal (Fig. 2 *B* and *C*). RA volume differed significantly across the six time points, and post hoc tests showed that the average RA volume was not significantly regressed until 20 days after T withdrawal and photoshift. Area X volume differed significantly across the six time points, and post hoc tests showed that the average Area X volume was not significantly regressed until 7 days after T withdrawal and photoshift. The volume of n. rotundus, a thalamic visual nucleus, and the volume of the telencephalon did not significantly vary across the six time points (data not shown).

As with HVC volume, HVC neuronal morphological characteristics regressed rapidly after T withdrawal and photoshift. HVC somatic area significantly regressed by 23% within 2 days after manipulation (Fig. 3*A*). HVC neuronal density was very dynamic after T withdrawal and photoshift; density significantly increased by 47% within 12 h and then significantly decreased back to LD+T levels by 4 days (Fig. 3*B*). The number of HVC neurons varied significantly across time points (Fig. 3*C*). Post hoc tests showed that the mean HVC neuron number was reduced 25% by 4 days after manipulation. The reduction of HVC neuron number coincided with the return to baseline in HVC neuron density, which peaked at 12 h.

RA neuronal morphological characteristics regressed more quickly than RA volume after T withdrawal and photoshift. RA somatic area significantly regressed, and RA neuron density significantly increased within 2 days after manipulation (Fig. 4 *A* and *B*). Both attributes continued to change over the entire time course; by 20 days, RA somatic area regressed by 30% and RA neuron density increased by 47%. The number of RA neurons did not vary significantly across time points (Fig. 4*C*).

We measured circulating levels of T of each bird 7–14 days after the initiation of LD+T and again when they were killed. Circulating T levels for every bird used in the study while under LD conditions were comparable with those seen in breeding Gambel's white-crowned sparrows in the wild (14). Plasma T

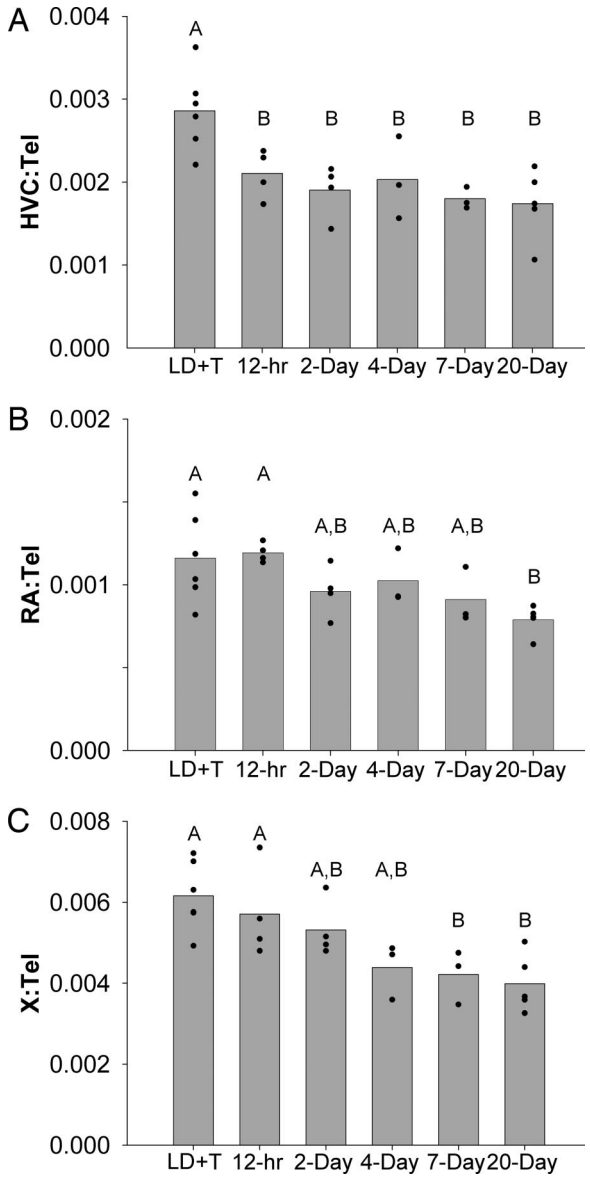

Fig. 2. The volumes of HVC, RA, and Area X regress rapidly after T withdrawal and photoshift. Each dot represents the ratio of song system nucleus to telencephalon volume for an individual bird, and bars indicate mean for each time point. (*A*) HVC was significantly regressed within 12 h after T withdrawal. (*B* and *C*) RA and Area X regressed more slowly, 20 and 7 days, respectively. Letters above bars illustrate significant differences among groups after post hoc test (Holm–Sidak, $P \leq 0.05$).

levels of birds killed under SD/castration conditions were at or below the detection levels of the assay. These data, in addition to determination of absence of the testes when the birds were killed, confirmed successful manipulation of circulating T (LD+T, 11.8 ± 1.22 ng/ml; SD/castration, 0.16 ± 0.03 ng/ml).

Discussion

We found that song control nuclei regress extremely rapidly after a transition from LD photoperiods and high levels of circulating T to SD photoperiods and T withdrawal. Notably, HVC volume regressed by 22% within 12 h of T withdrawal. Birds in this group were castrated and killed while housed on LD. Therefore, the initial regression after T withdrawal was not the result of changes in the concentration of melatonin or other factors regulated by photoperiod (20). We also examined attributes of HVC and RA

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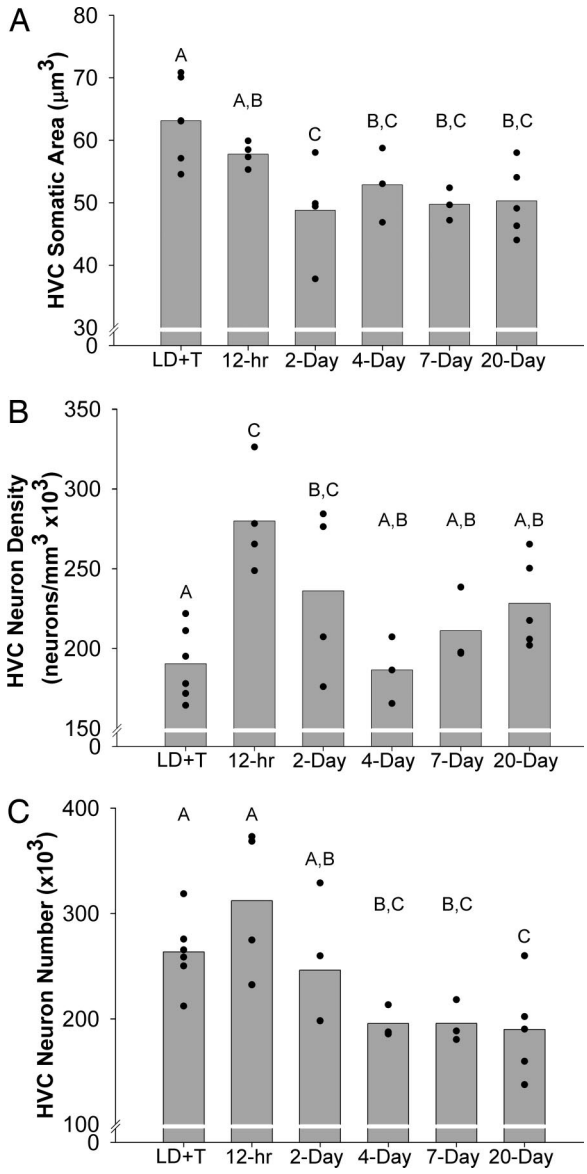

Fig. 3. HVC neuronal attributes regress rapidly after T withdrawal and photoshift. (*A*) Somatic area of HVC neurons decreases within 2 days. (*B*) HVC neuron density changes dynamically after manipulation. Density increases by 47% within 12 h and decreases to LD+T levels within 4 days. (*C*) HVC neuron number significantly decreases within 4 days. Dots indicate individual birds, and bar indicates mean.

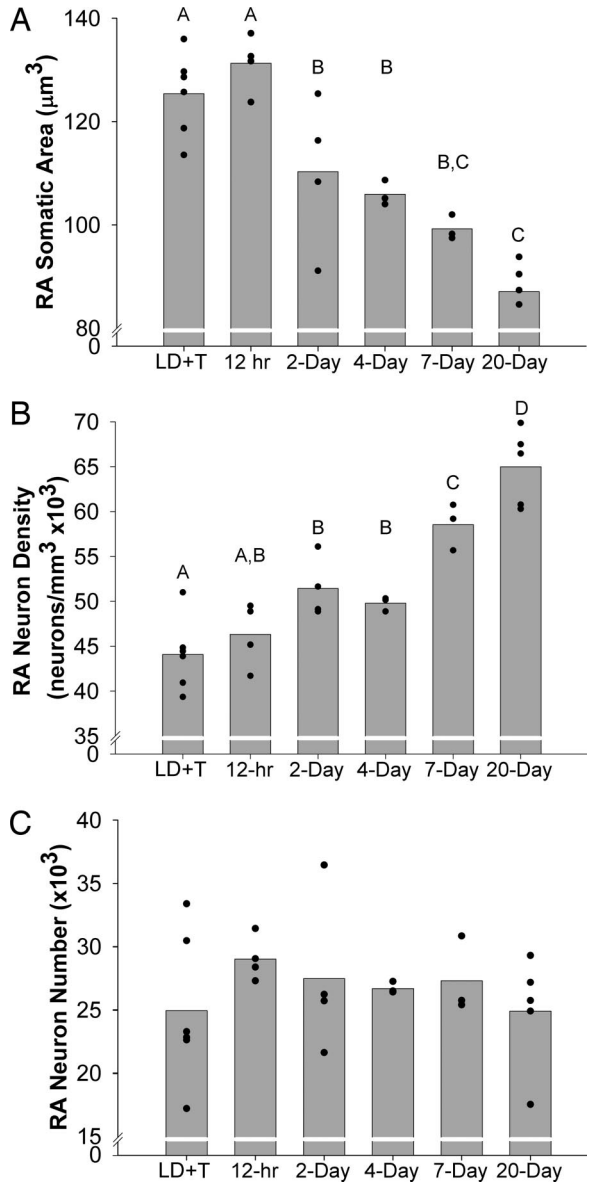

Fig. 4. RA neuronal attributes regress rapidly after T withdrawal and photoshift. (*A*) Somatic area of RA neurons decreases significantly within 2 days and continues to decrease over the time course. (*B*) RA neuron density increases significantly within 2 days and continues to increase over the time course. (*C*) RA neuron number does not decrease significantly across the entire time course. Dots indicate individual birds, and bar indicates mean.

neurons. Average HVC somatic area decreased within 2 days, and HVC neuron density initially increased and then decreased over a 4-day period. HVC neuron number decreased within 4 days, suggesting that there was increased cell death in response to T withdrawal and photoshift. Indeed, there is an increase in the activation of the proapoptotic protease caspase-3 in HVC 24–72 h after T withdrawal and in the incidence of dying neurons (C.K.T., E. W. Rubel, and E.A.B., unpublished results). RA somatic area and neuron density changed significantly within 2 days but continued to change further over the next 18 days after T withdrawal and photoshift.

HVC volume decreased to nonbreeding size by 12 h after T withdrawal; such rapid regression of an entire brain nucleus is unprecedented. Previous studies showed that castration of adult songbirds induced regression of song control system nuclei, but the earliest that these birds were examined was 3 weeks after T withdrawal, by which time their brain nuclei were already significantly regressed (15, 16, 21). Before the current work, we did not know the time course over which brain nuclei regress after hormone withdrawal. Although there are reports of rapid cellular changes such as dendritic retraction after the loss of afferent input (see below), here we report that the volume of an entire telencephalic nucleus regresses over a matter of hours. T, or its metabolites, may either directly or indirectly promote the survival of HVC neurons through enhanced trophic support, which would decrease at the end of the breeding season after the natural reduction of circulating T. One potential mechanism that may explain the loss of HVC neurons is a decrease in the expression of brain-derived neurotrophic factor (BDNF). Rasika *et al.* (22) found that T treatment of adult female canaries increases the synthesis of BDNF protein in HVC, and increased

levels of HVC BDNF increase neuronal recruitment. In addition, the timing of BDNF exposure affects the life expectancy of new neurons (23). T withdrawal may thus decrease BDNF trophic support in HVC and lead to a decrease in HVC neuron number (A. M. Wissman and E.A.B., unpublished data).

We rapidly manipulated both photoperiod and circulating levels of T in the laboratory to mimic seasonal changes in the wild. Changes in photoperiod and plasma T levels occur more gradually in the wild, and we therefore refer to neural changes under these laboratory conditions as seasonal-like. An important advantage of these rapid shifts is that they provide a discrete starting point for experimental manipulations and therefore decrease the individual variance in timing that is characteristic of wild birds' responses to environmental conditions (14).

We believe that regression of the song control system is largely driven by the withdrawal of T for several reasons. First, male white-crowned sparrows held on SD and implanted with s.c. T pellets experience much more growth of the song control system than castrated males that lack measurable circulating levels of T and are exposed to the LD photoperiod (24). Furthermore, increases in song control system nuclei volumes caused solely by exposure to a LD photoperiod are attenuated by exogenous administration of melatonin (25). Second, an intracranial T implant placed near HVC in male birds housed on a SD photoperiod is sufficient to induce growth of ipsilateral song control system nuclei (13). Third, our results show that HVC volume regresses rapidly in castrated birds even when they are kept on a LD photoperiod.

The withdrawal of circulating T leads to neural changes in other well characterized model systems, albeit less pronounced than those we report here. In the telencephalon, castration of adult male rats led to regression of volume and neuronal somatic area of the posterodorsal medial amygdala (26). In adult male gerbils, rats, and Japanese quail, castration induced regression of the volume of sexually dimorphic nuclei within the preoptic area (27–29) and, in the case of Japanese quail, a decrease in somatic area and axosomatic synapses of preoptic area neurons (30, 31). In the spinal cord, castration of adult male rats, mice, and gerbils led to decreased motoneuron size in the spinal nucleus of the bulbocavernosus, a sexually dimorphic nucleus (32–34). Castration had no effect on neuron number in any of these cases, however. A major difference between our analysis and those reported for hormone-sensitive brain areas in other animal models is that the earliest any of these animals was examined after castration was ≈4 weeks, after these areas were significantly regressed. A time course of regression for these systems is not known. Other model systems in which brain nuclei are sensitive to steroid hormone levels may also demonstrate rapid changes on the order of hours or days upon the withdrawal of hormones similar to those seen in the song control system.

Tramontin *et al.* (8) previously showed that the song control system rapidly grows when male white-crowned sparrows are exposed to LD photoperiod and increased circulating T. The rate of song control system growth also differs between nuclei; HVC is fully grown by 7 days after manipulation, whereas RA and Area X are fully grown between 7 and 20 days. These results suggest that growth of RA and Area X depends on the prior growth of HVC. Further evidence supports this hypothesis; unilateral lesions of HVC prevent the growth of ipsilateral RA and Area X in birds exposed to LD and elevated T (12). In addition, an intracerebral T implant placed unilaterally near HVC in birds housed on SD induces the growth of the ipsilateral RA and Area X, but a T implant near RA has no effect (13). These results suggest that the growth of RA and Area X depends on trophic support from HVC and that the sequential growth of the song control system at least partially depends on the action of T and its metabolites in HVC.

The results of the current work are consistent with the hypothesis that regression of RA and Area X are also dependent on changes in HVC, given that the regression of HVC volume and neuron number precedes changes in efferent nuclei and that HVC lesions abolish growth and maintenance of RA and Area X (8, 12). The sequential regression of song control nuclei follows the same pattern seen when these nuclei grow. Regression, however, occurs more rapidly than the growth; preliminary results in our laboratory suggest that HVC takes 4 days to increase in volume significantly, whereas it regresses in 12 h or less. This discrepancy may be explained by the fact that growth and regression involve different cellular mechanisms. HVC increases in volume largely by the addition of new neurons, yet these new neurons do not arrive in HVC for at least 3 days after being born in the ventricular zone (35). In addition, newly recruited HVC projection neurons probably do not make synaptic connections with RA neurons for at least 8 days after birth (based on results from male canaries) (35).

The results from the current work clearly indicate that the underlying neuronal changes that mediate the regression of the song control system nuclei follow a different course from that of growth. HVC volume regresses within 12 h, which is driven by an initial sharp increase in neuron density. The increase in density is reversed over the next few days, perhaps as neurons progressively die. The rapid changes in HVC neuron density suggest that T withdrawal results in significant retraction of axonal and/or dendritic branches within HVC within 12 h (see below). Although it needs to be experimentally confirmed, our results suggest that hormone withdrawal alone can result in rapid retraction of neuronal processes.

The rapid changes in HVC after T withdrawal ultimately have effects on trophic support to its efferent targets, RA and Area X. Given that RA and Area X regress more rapidly than they grow, the presumed loss of trophic support from HVC neurons to its efferent targets is likely a more immediate process than the integration of newly recruited HVC neurons and establishment of synaptic connections with efferent neurons that is essential for growth of the song circuits during the breeding season. The rapid retraction and loss of HVC neurons, potentially mediated by apoptotic mechanisms, with an associated decrease in transsynaptic release of a trophic signal, most likely explains the more rapid changes seen in RA and Area X in the current work compared with the slower changes observed in growth of RA and Area X (8). Unilateral infusion of caspase inhibitors near HVC reduces the incidence of caspase-3 activity in the ipsilateral HVC and the regression of ipsilateral RA neuron size up to 7 days after T withdrawal (C.K.T., E. W. Rubel, and E.A.B., unpublished results), providing further evidence that HVC neurons transynaptically promote the growth of efferent nuclei.

T has neuroprotective effects *in vitro* (36, 37) and in some (38, 39) but not all (40, 41) *in vivo* animal models of neuronal injury. T regulates the expression of antiapoptotic genes such as *bcl-2* in the spinal nucleus of the bulbocavernosus in male rats (42). 17β-Estradiol (E_2), a metabolite of T, promotes the expression of several antiapoptotic genes (43–48). In zebra finches, neuronal injury is accompanied by an increase in expression of aromatase, an enzyme that converts T to E_2 (49), and inhibition of aromatase increases apoptosis near the injury (50). These results suggest that T and/or its metabolites may reduce programmed cell death in HVC of songbirds. The seasonal increase in plasma T levels in male songbirds may contribute to the survival of HVC neurons through the up-regulation of antiapoptotic genes such as *bcl-2*.

Although the rapidity with which forebrain circuits regress because of hormone withdrawal reported here is unprecedented, the time course of song control system regression is comparable with degeneration associated with acute neuronal injury. For example, the morphological effects of global ischemia (51, 52)

and status epilepticus (53) first appear hours to days after injury. The sharp increase in HVC neuronal density within 12 h of T withdrawal suggests that there is substantial degeneration of dendritic and/or axonal processes. In patients afflicted with schizophrenia, neuropil degeneration is correlated with an increase in neuronal density and decrease in somatic area (54–56) and is regulated by apoptotic mechanisms (57). Neuropil degeneration can occur quickly; dendrites of dentate gyrus neurons show signs of degeneration within hours of status epilepticus (58), and deafferentation leads to rapid atrophy of dendritic arbors within hours (59–62). In hibernating ground squirrels, the dendritic structure and somatic area of neurons in multiple brain areas rapidly regress in response to temperature-induced torpor on the order of hours to days, but there is no evidence that torpor of this form is driven by changes in circulating hormone levels (63). The speed of the regression that we report here illustrates the pronounced sensitivity of the song control system of seasonally breeding songbirds to fluctuations in circulating steroids. Neuropil degeneration may also occur on the order of hours in other model systems in which hormone withdrawal leads to regression of dendrites (1, 64).

In summary, we report that the volumes of three song control nuclei regressed rapidly after a transition from breeding to nonbreeding conditions. The number of HVC neurons also decreased significantly within 4 days, suggesting that there is substantial cell death after the withdrawal of T. These results also suggest that T may provide essential trophic support to HVC neurons. Like growth, regression is sequential; the efferent targets of HVC do not regress until after HVC has undergone substantial regression. This report demonstrates the rapid regression of adult brain nuclei caused by hormone withdrawal and photoshift in adult animals.

Experimental Procedures

All procedures followed National Institutes of health animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee. We captured 25 male Gambel's white-crowned sparrows in eastern Washington during their postbreeding season migration. We housed the birds in indoor-group aviaries for at least 12 weeks on SD (8 h of light) before the start of the experiment to ensure that the song system and reproductive system were sufficiently regressed and sensitive to the stimulatory effects of T and LD photoperiods.

The song control nuclei in wild male white-crowned sparrows increase in size in response to a gradual increase in circulating T levels as day length increases and the testes grow. The timing of the increase in circulating T levels varies across individuals, however. To reduce individual variability, we exposed all birds to the same LD photoperiod and administered T s.c. to elevate plasma T concentrations to those seen in breeding birds (4–12 ng/ml; ref. 14). Even though such a transition occurs more gradually in the wild, this laboratory manipulation recreates the two most important seasonal influences on white-crowned sparrows: elevated T levels and a LD photoperiod typical of their Alaskan breeding grounds (20 h of light per day).

At the beginning of the experiment, we transferred the birds from L:D = 8:16 (SD) to L:D = 20:4 (LD) overnight. The next day we implanted each bird s.c. with a 12-mm Silastic capsule (1.47-mm inner diameter × 1.96-mm outer diameter) filled with crystalline T. Each bird was housed individually in visual and auditory contact with the other birds used in this experiment. We kept all of the birds under LD+T for 20 days. This time period is sufficient to induce full growth of the song control system under these conditions (8).

We killed six birds 2–4 h after their subjective dawn after 20 days of exposure LD+T conditions; these birds are referred to as the LD+T group. We removed the s.c. T capsules from the remaining birds and castrated them at the same time of day that the LD+T group was killed, to induce regression of the song control system. To castrate birds we anesthetized them with isoflurane through a nonrebreathing system. We made a small incision on the left side anterior to the caudal-most rib and dorsal to the uncinate process and aspirated the testes. This procedure is completed within 10 min, and the animals recover within minutes from anesthesia and are returned to their cage. We killed five birds 12 h after T withdrawal; these birds are referred to as 12-h birds, and they did not experience a change in photoperiod. We shifted the remaining birds overnight to L:D = 14:10 the same day as the T withdrawal and the next day shifted them overnight to SD. The intermediate photoperiod helped birds adjust to the reduction in available feeding time. These birds were killed at 2, 4, 7, or 20 days after photoshift and T withdrawal (n = 3–5 per group).

Blood Draw and Hormone Analysis. We took blood samples from the birds at various time points throughout the experiment to measure circulating T levels. We drew 250 μl of blood from the alar vein in the wing into heparinized collection tubes. We immediately centrifuged the tubes to separate the plasma, which was stored at −20°C until assay. We measured plasma T concentration by using the Coat-A-Count total testosterone RIA kit (Diagnostic Products, Los Angeles, CA). The minimum detectable plasma T concentration was 0.09 ng/ml. Samples with undetectable levels of steroid were treated as having concentrations at this detection limit for statistical analysis.

Killing, Perfusion, Tissue Processing. We deeply anesthetized the birds with methoxyflurane inhalation and perfused them through the heart with heparinized saline followed by 4% phosphate-buffered paraformaldehyde. We postfixed the brains in 4% paraformaldehyde for 24 h, embedded the brains in gelatin, and postfixed the gelatin-embedded brains in a 20% sucrose/neutral buffered formalin solution for 48 h. We sectioned the brains in the coronal plane at 40 μm on a freezing microtome, mounted every third section, and stained them with thionin.

Measurement of Nuclei and Telencephalon Volume. Using an overhead projector, we traced the borders in both hemispheres throughout the full rostral–caudal extent of HVC, RA, Area X, and n. rotundus. We traced either the right or left hemisphere of the full extent of the telencephalon, chosen at random, of every sixth mounted section. We scanned these tracings into a microcomputer and measured the surface area of each cross-section by using a customized module of Image software (National Institutes of Health, Bethesda, MD). We determined the volumes of nuclei and telencephala by summing the estimated volume between sections calculated with the formula for the volume of a cone frustum (65). We doubled the value of the unilateral telencephalon hemispheric volume to calculate total telencephalon volume.

Measurement of Neuronal Attributes in HVC and RA. We sampled neuron size by measuring the cross-sectional area of the soma in every mounted section throughout the rostral–caudal axis of HVC and RA. We distinguished neurons from glia by their single nucleolus and uniform, nongranular cytoplasm. We used a random, systematic sampling protocol that has been previously described and validated (65), which yields estimates of neuronal density and size that do not differ from those obtained using the stereological optical dissector method. We measured the somatic area of at least 150 HVC neurons and 100 RA neurons per bird in fields chosen randomly by computer in each section. In thin sections there is a likelihood of splitting of neuronal nuclei between sections, which could overestimate cell counts (66, 67). To estimate neuron density, we therefore counted neuronal

nucleoli in every field and used Konigsmark's (68) formula no. 4 to correct for nucleus splitting:

$$N/n = t/(t + 2(r^2 - (k/2)^2)) \quad [1]$$

where N is the number of nucleoli present, n is the number of nucleoli counted, t is the section thickness in micrometers, r is the nucleolus radius, and k is the diameter of the uncounted fragments of nucleoli. We set k equal to 1 μm, which equaled the smallest nucleolus fragment encountered in this work. Konigsmark-corrected neuron counts were divided by the volume of the tissue sampled to obtain neuronal density.

We estimated neuron number by multiplying neuron density by total nucleus volume. We sampled at least 150 HVC neurons and 100 RA neurons throughout the rostral–caudal extent of each nucleus in each brain. This sample size is sufficient to encompass the entire range of variability in neuron density and somatic area in these nuclei, based on Tramontin *et al.* (65). All measurements were made blind to the time of killing for each bird.

Statistics. We used an ANOVA to make comparisons across the various time points. We used a post hoc Holm–Sidak pairwise multiple comparison procedure to compare the time points. The α level for all tests was 0.05 (two-tailed).

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