Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries
(neurogenesis/neuronal replacement/song system/gonadal steroids/plasticity)

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ABSTRACT New neurons are added to the high vocal center (HVC) of adult male and female canaries. Exogenous testosterone induces a marked increase in HVC size in adult female canaries, though the mechanisms responsible for this increase remain unknown. To understand the mechanisms, we analyzed the effects of testosterone on neuronal recruitment in the female HVC. Intact adult female canaries received Silastic implants that were empty or filled with testosterone. Birds in the short-survival group received the Silastic implant, followed by a single injection of [3H]thymidine 2 days later, and were killed on the following day. Birds in the long-survival group were injected once a day for 5 days with [3H]thymidine and received the Silastic implant 20 and 40 days later. These birds were killed 60 days after the first injection of [3H]thymidine. The number of [3H]-labeled ventricular zone cells above, rostral, or caudal to HVC was not affected by the hormone treatment in the short-survival birds, suggesting that testosterone did not affect neuronal production. However, the number of [3H]-labeled HVC neurons that projected to robustus nucleus of the archistriatum (RA) in the long-survival birds was three times greater in the hormone-treated than in the control group, though the total number of RA-projecting cells did not change significantly. Testosterone also induced an increase in the size of the HVC cells that project to RA. Thus, these experiments suggest that testosterone affects the recruitment and/or survival of newly generated RA-projecting HVC neurons but does not affect their production.

Canaries learn their song by reference to auditory information (1). The high vocal center (HVC) is an important component of the song system and is used in the acquisition and production of learned song (2). Song is learned for the first time during the months preceding sexual maturity and, thereafter, modified every year (3). Most of these modifications occur at the end of summer and in early fall (4, 5). These, too, are times of peak neuronal recruitment in the HVC of adult males (6, 7). Interestingly, the peak in neuronal recruitment seen in late summer and early fall corresponds in adult males to a time when blood testosterone levels, which fell during the summer, start to rise (5).

Neurons added to the adult avian brain are born in the walls lining the lateral ventricle of the forebrain (8, 9), also referred to as the “ventricular zone” (VZ). HVC is closely apposed to the ventrolateral wall of the lateral ventricle, and it has been suggested that the neurons added to the adult HVC are born on the VZ overlying HVC (8).

Female canaries sing rarely, and the song they produce has fewer syllable types than that of males and is relatively unstable (10). However, treatment of these birds with physiological doses of testosterone induces them to sing more and the song syllables they produce become louder and more stable (11). This work also reported that the size of HVC grows dramatically in these birds under the influence of testosterone over a period of 30 days, although the cellular mechanisms behind this growth were not determined. Thus, these observations on adult male and female canaries suggest that testosterone plays an important role in the recruitment of HVC neurons generated in adulthood.

Here we describe experiments that suggest that testosterone influences the recruitment and/or survival of newly generated neurons in HVC. Testosterone does not seem to influence the number of dividing VZ cells, either above the HVC or elsewhere (see ref. 12). In addition, we will show that testosterone also affects the nuclear and soma size of HVC neurons.

MATERIAL AND METHODS

Animals. One-year-old female waterslager canaries from our own colony were kept under conditions of the natural photoperiod in indoor facilities at the Rockefeller University’s Field Research Center. These birds were divided into two groups, the short-survival (n = 10) and the long-survival (n = 9) groups. The protocol followed with the short-survival birds was meant to quantify the possible effects of testosterone on VZ cell proliferation above, rostral, or caudal to HVC. The protocol followed with the long-survival birds was meant to quantify the effect of testosterone on the recruitment and survival of new HVC neurons. The short-survival birds were killed on October 9 and 10. The long-survival birds were killed on December 18 and 19. Because of the difference in time of year when the birds were killed, our comparisons will be restricted to treatment groups that had the same survival.

Treatment Protocols. Short-survival protocol. Birds in the short-survival group (Fig. 1A) received injections of the retrograde tracer Fluoro-Gold (F-G) (13) bilaterally into nucleus robustus archistriatalis (RA) (14) on day 1. These injections backfill only HVC neurons that project to RA, which is part of the efferent pathway for the production of learned song. RA-projecting cells backfilled with F-G defined the boundaries of HVC. Each of these birds received on day 4 a subcutaneous Silastic implant (i.d., 0.76 mm; o.d., 1.65 mm; length, 5 mm) that was either empty (controls, n = 5) or filled with crystalline testosterone propionate (n = 5). Implants of this size induce in adult female canaries blood testosterone levels comparable to those seen in adult males in the reproductive condition (11). The implants were incubated in physiological saline for 1 day before implantation to ensure immediate release of testosterone upon implantation (15). On day 6 of this protocol, each of the birds received a single 50-µl i.m. injection of [3H]thymidine [6.7 Ci/mmol; New England Nuclear; =2.5 mCi/g (body weight); 1 Ci = 37 GBq]. All birds in the short-survival group were killed on day 7 by an overdose of Nembutal followed by transcardiac

Abbreviations: HVC, high vocal center; RA, nucleus robustus archistriatalis; VZ, ventricular zone; F-G, Fluoro-Gold.
The length with NTB2 and obtained of each long-survival birds. In applied by labeling (minimum of saline and then with 3% (wt/vol) paraformaldehyde.

**Long-survival protocol.** Birds in the long-survival group (Fig. 1B) received one i.m. injection daily of 50 µl of [3H]thymidine on days 1–5. On day 20, they received a subcortaneous Silastic implant that was either empty (n = 4) or filled with testosterone (n = 5), as described above. These implants were replaced by another implant of the same kind on day 40 to maintain the levels of circulating testosterone. Choice of day 20 was determined by the observation that it takes ~20 days for HVC cells born in adulthood to become identifiable as mature neurons (16). Thus, testosterone treatment started when many of the cells produced during the 5 days of [3H]thymidine injection were already differentiating into mature neurons and more were nearing the end of their migratory phase. Birds in the long-survival group received F-G injections into RA bilaterally on day 55 and were killed on day 60 as described above.

**Histology.** The brains of birds in both groups were removed after perfusion and placed in 3% paraformaldehyde for 3–4 days. Each brain hemisphere was embedded separately in PEG (Mr, 1500) and sectioned sagittally at 6-µm intervals (17). Every tenth section, used for autoradiography, was mounted on chrome alum-coated slides, delipidized, coated with NTB2 (Kodak) nuclear track emulsion, and incubated in the dark at 4°C for 4 weeks. These slides were then developed in D19 (Kodak) for 3 min at 17°C and c overslipped with Krystalon (Harleco, Philadelphia). Sections adjacent to each section above were mounted on separate chrome alum-coated slides, delipidized, and stained with cresyl violet. F-G was visualized in the sections used for autoradiography with UV fluorescence.

**Data Analysis.** All anatomical analysis was done using a computer-yoked microscope (18). The person doing the analysis did not know the treatment group to which each brain belonged. HVC boundaries were mapped in the F-G and cresyl violet series and the area of HVC was calculated for each section. The sum of these areas for each HVC, multiplied by the section thickness and spacing between sections, allowed us to estimate the volume of HVC in short- and long-survival birds. In short-survival birds, the strip of VZ seen in each section was divided into three segments—directly above HVC, rostral to HVC, and caudal to HVC. The length of each segment was measured, and [3H]thymidine-labeled VZ cells were counted.

The position of all F-G-backfilled HVC neurons—with or without [3H]-labeling—was mapped in the long-survival birds and the number of exposed silver grains over the nucleus of each of the labeled cells was recorded. The latter information was obtained only for cells that had 30 times background level of labeling (minimum of seven grains per cell nucleus). Background level was determined with neuropil and was on average 0.019 grain per µm². The minimum number of [3H]-labeled F-G-backfilled cells counted in a bird, both sides combined, was 42; the maximum was 245.

The number of cells per unit of HVC area was obtained by counting all cells of a given category (e.g., all F-G-backfilled cells or F-G cells labeled with [3H]thymidine) that appeared on a particular HVC section. These counts were obtained from four evenly spaced sections per side. In addition, samples of 41–53 F-G-backfilled HVC cells were measured for each bird to obtain cell and nuclear diameters for [3H]-labeled and unlabeled cells. Nuclear size information was used to correct our counts and exclude the possibility that the larger cells were sampled more frequently (19, 20). Counts corrected in this manner were used to estimate total cell numbers.

When possible, we obtained data from both the right and left hemispheres of the brain and compared the two sides. Since there was no systematic difference between the values of the two sides, these birds were represented by the average of the two sides. Where F-G injections on one side were unsatisfactory, the bird was represented by the values of only the successfully backfilled hemisphere.

All statistical comparisons between groups used the Mann-Whitney U test. The paired Student t test was used for comparisons within groups.

**RESULTS**

**Short-Survival Birds.** The VZ rostral to HVC had greater numbers of labeled cells per unit length of ventricular wall than the segments above HVC or caudal to HVC. However, the density of labeled cells in any one of these regions was not significantly affected by the testosterone treatment (Fig. 2). The mean number of silver grains per labeled VZ cell did not differ between testosterone-treated and control groups.

Testosterone treatment did not affect the volume of HVC as determined either in cresyl violet-stained (U = 10; P = 0.602) or F-G-backfilled (U = 12; P = 0.917) sections. Cresyl violet and F-G volumes were very similar to each other (t = 2.045; P = 0.071). However, the nuclear diameters and soma diameters of F-G-backfilled HVC neurons were larger in the testosterone-treated group than in the control group. Mean soma diameter increased from 6.40 µm to 7.07 µm (U = 0; P = 0.009); mean nuclear diameter increased from 5.97 µm to 6.43 µm (U = 3; P = 0.047) (Fig. 3).

**Long Survival Birds.** Testosterone induced a significant increase in the mean volume of HVC, as determined from F-G backfills (Figs. 4 and 5), which went from 0.09 mm³ in the control birds to 0.14 mm³ in the testosterone-treated birds, an increase of 60% (U = 2; P = 0.05). The volume increase in the cresyl violet-stained material (67%) was comparable. There was no significant difference in the volume of HVC estimated by the two methods (r = 0.834; P = 0.428).

Soma diameters were larger in the F-G-backfilled HVC cells of the testosterone-treated than in those of the control birds. Cell diameters increased from 7.36 µm to 8.25 µm (U = 0; P = 0.014). Nuclear diameters were also larger, but not significantly (6.52–6.75 µm; U = 7.5; P = 0.54). When these comparisons are restricted to RA-projecting cells labeled with [3H]thymidine, cell diameters increased from 7.72 µm to 9.13 µm (U = 0; P = 0.014) and the nuclear diameters increased from 6.76 µm to 7.21 µm (U = 1.5; P = 0.04). It is worth noting that the new (i.e., [3H]-labeled) RA-projecting cells are systematically larger than the RA-projecting cells not labeled with [3H]thymidine, regardless of treatment group (for soma diameters, t = −5.242 and P = 0.0008; for nuclear diameters, t = −4.815 and P = 0.001).

In addition, the packing density of F-G-backfilled cells in HVC decreased from a mean of 119,000 cells per mm³ in the
control birds to 84,000 cells per mm$^3$ in the testosterone-treated ones. However, because of the differences in HVC volume, the total number of RA-projecting cells in HVC was comparable between the two groups (10,600 vs. 11,500; $U = 7; P = 0.46$) (Fig. 6).

Testosterone treatment induced a 3-fold increase (Fig. 6) in the mean number of $^3$H-labeled HVC cells that projected to RA—189 in the control group vs. 607 in the testosterone-treated group ($U = 1; P = 0.03$). Labeled cells in both the testosterone-treated and control groups were distributed throughout HVC. Fig. 7 shows that the number of exposed silver grains over the nuclei of RA-projecting neurons was comparable in the testosterone-treated and control birds.

DISCUSSION

Our observations on the short-survival birds showed that testosterone treatment did not influence the number of cells dividing in the VZ above, rostral, or caudal to HVC. This result is similar to one reported earlier (12). However, we cannot conclude, from this alone, that testosterone does not affect the proliferation of HVC neuronal precursors because there is no direct evidence as to where these cells are. Moreover, testosterone might affect proliferation if given over longer periods of time. Testosterone did increase the size of RA-projecting HVC neurons, an effect seen after just 3 days of treatment in the short-survival group, suggesting that testosterone reached the brain. In the long-survival group, the effect of testosterone on nuclear size was more marked in the $^3$H-labeled than in the unlabeled neurons, suggesting that testosterone affects HVC neurons differently depending on their age, a fact that may lead to an understanding of how testosterone acts to rescue dying neurons.

We do not know when all the $^3$H-labeled RA-projecting HVC neurons seen in the long-survival birds were born. Many of them resulted, presumably, from the division of precursor cells that were in S phase when $^3$H-thymidine was administered. Some of those daughter cells could have divided again, and perhaps, testosterone encouraged them to do so. However, had testosterone promoted the repeated division of neuronal precursor cells, then we would have seen relatively fewer numbers of exposed silver grains in the cell nuclei of the testosterone-treated birds than in controls. Yet the histogram of the number of exposed silver grains over the nuclei of HVC neurons backfilled with F-G was comparable in the two treatment groups. Therefore, testosterone exerted its effect by acting on postmitotic cells that did not divide again.

Previous studies have shown that it takes newly formed HVC neurons ~20 days to be born, migrate from their place of origin, and acquire a recognizable neuronal phenotype (9, 16). Our testosterone treatment in the long-survival group started at that point, yet we saw 40 days later a 3-fold increase in the number of labeled RA-projecting neurons. From these results we infer that testosterone influences the recruitment and/or survival of new HVC neurons. An alternative explanation is that testosterone enhanced the transport of F-G toward the soma of $^3$H-labeled RA-projecting HVC neurons, so that more of these cells were counted. There is no precedent for such an effect and it is unlikely that testosterone would have an effect exclusively in the cohort of cells born during the period of $^3$H-thymidine treatment. If the effect was broader than this, then there should have been an increase in the overall number of F-G-labeled HVC neurons, which was not the case.
An earlier study reports that only one-third of the young migratory neurons that move away from the VZ become fully differentiated adult neurons (9). Many of the newly generated cells presumably die before or soon after differentiation. Testosterone could increase the fraction of new HVC neurons that survive during migration and that are drawn into HVC—effects on recruitment. Testosterone may also increase the survival of neurons after differentiation—an effect on survival. Our data cannot distinguish between effects on recruitment or survival of HVC neurons. Testosterone may influence these processes directly or indirectly. Indirect effects would occur if testosterone induced preexisting cells to produce factors that regulated the migration or survival of the new HVC neurons.

The incorporation of new neurons into the HVC of adult male canaries is higher in October than in May. In addition, whereas a majority of the neurons born in May disappear during the next 4 months, there is no such reduction for neurons born in October (21). Testosterone or its metabolites could be responsible for this seasonal effect on survival. The level of testosterone in adult male canaries falls in the summer and starts to rise in the early fall (5). The protocol we followed in females may mimic this seasonal change in testosterone concentration seen in males. Whereas intact adult females have very low serum concentration of testosterone, a Silastic implant of the dimensions we used raises serum testosterone levels to those seen in males in late fall (5, 11). The idea that testosterone promotes the survival of 

![Image A](A) ![Image B](B) ![Image C](C) ![Image D](D)

**Fig. 5.** (A and C) Fluorescent photomicrographs of HVC backfilled with F-G from RA in control (A) and testosterone-treated (C) birds. (B and D) Dark-field photomicrographs of the same fields in A and C, respectively, showing clusters of silver grains from ^3^H autoradiography. Note the increase in HVC size and in the number of ^3^H-labeled F-G neurons (arrows). (Bar = 100 μm.)

**Fig. 6.** Total number of F-G-backfilled HVC neurons and proportion of these cells that are ^3^H-labeled in testosterone-treated (bar T) and control (bar O) birds. Data are the mean ± SEM.

**Fig. 7.** Distribution histograms of number of silver grains for ^3^H-labeled F-G-backfilled HVC neurons in the testosterone-treated (hatched bars) and control (open bars) birds of the long-survival experiment. The means (arrows) of these two distributions are not significantly different.
newly formed HVC cells seems plausible because cells that concentrate testosterone or its metabolites occur in the HVC of adult female canaries (22, 23), and a subset of HVC's androgen-concentrating cells project to RA (24). Still, it is worth noting that the present results were obtained from intact adult females treated with [3H]thymidine and testosterone in the fall. The outcome of our study might have been different if it had been conducted during other times of the year.

The incorporation of new neurons into brain circuits of juveniles and adults has been described in many vertebrates. The function of adult neurogenesis remains speculative; it may play a role in the modification of neural circuits (25–27). In many, if not all animals, seasonal endocrine changes accompany changes in life style, social status, stress, and reproductive state. These changes in behavior might require dramatic modifications of the underlying neural circuitry and in some cases may involve the incorporation of new neurons. Hormones are thus obvious candidates to regulate this form of plasticity. For instance, recent evidence in mammals indicates that adrenal hormones regulate cell division in the dentate gyrus of adult rats (28), a brain region where neurogenesis continues after birth (29, 30).

The present work shows that a gonadal hormone influences the addition of neurons to an adult song control nucleus. Previous work in songbirds indicates that testosterone can also influence neuronal structure and synaptic number (31, 32). Thus, gonadal hormones may have a wide range of trophic effects on the neurons of juvenile and adult brains (33). The HVC adult canaries may serve as a model system in which to study the molecular mechanisms by which testosterone exerts its trophic effects on the recruitment and survival of neurons born in adulthood.

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