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Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain

(learning/neurogenesis/neuronal death/glial cells/endothelial cells)

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ABSTRACT **The vocal control nucleus designated HVc (hyperstriatum ventrale, pars caudalis) of adult female canaries expands in response to systemic testosterone administration, which also induces the females to sing in a male-like manner. We became interested in the possibility of neurogenesis as a potential basis for this phenomenon. Intact adult female canaries were injected with [^{3}H]thymidine over a 2-day period. Some birds were given testosterone implants at various times before thymidine. The birds were sacrificed 5 wk after hormone implantation, and their brains were processed for autoradiography. In parallel control experiments, some birds were given implants of cholesterol instead of testosterone. All birds showed considerable numbers of labeled neurons, glia, endothelia, and ventricular zone cells in and around HVc. Ultrastructural analysis confirmed the identity of these labeled neurons. Cholesterol- and testosterone-treated birds had similar neuronal labeling indices, which ranged from 1.8% to 4.0% in HVc. Thus, neurogenesis occurred in these adults independently of exogenous hormone treatment. Conversely, both glial and endothelial proliferation rates were markedly stimulated by exogenous testosterone treatment. We determined the origin of the thymidine-incorporating neurons by sacrificing two thymidine-treated females soon after their thymidine injections, precluding any significant migration of newly labeled cells. Analysis of these brains revealed no cells of neuronal morphology present in HVc but a very heavily labeled ventricular zone overlying HVc. We conclude that neuronal precursors exist in the HVc ventricular zone that incorporate tritiated thymidine during the S phase preceding their mitosis; after division these cells migrate into, and to some extent beyond, HVc. This ventricular zone neurogenesis seems to be a normally occurring phenomenon in intact adult female canaries.**

The primary telencephalic song-control nucleus, HVc (hyperstriatum ventrale, pars caudalis) (1, 2), of adult female canaries doubles in size in response to androgen treatment as these birds develop male-like song (3). We became interested in the possibility that addition of neurons might contribute to this increase in HVc volume. We report here that new neurons are added at relatively high rates to nucleus HVc of adult intact female canaries. This occurs as ventricular zone stem cells proliferate, and then some of the daughter cells migrate into HVc and differentiate into young neurons. This adult neurogenesis occurs independently of exogenous testosterone treatment; however, both glial and endothelial proliferation can be stimulated by testosterone administration.

MATERIALS AND METHODS

On day 0 of this study (June 10, 1981) 18 intact, 1-yr-old female canaries were implanted with Silastic tubes (4) containing either testosterone (14 birds), cholesterol (3 birds), or nothing (1 bird). For successive 2-day periods beginning with day 0, individual birds received intramuscular (M. pectoralis) injections of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 6.7 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; New England Nuclear), a marker of DNA synthesis and inferentially of cellular replication (5–7). Each bird received six injections of 50 μCi each ($\approx$2.5 μCi/g of body weight), spaced 8 hr apart. On days 0 and 1 after testosterone implantation, the first bird was injected; subsequent birds were injected on days 2–3, 4–5, 6–7, 8–9, and 18–19. Duplicates of three of these birds (2–3, 4–5, and 6–7) were also treated with [^{3}H]thymidine and separately used for subsequent ultrastructural analysis. Matched cholesterol-implanted controls were also injected with [^{3}H]thymidine on days 2–3, 4–5, and 6–7. Further controls consisted of one bird given an empty Silastic implant (injected on days 4–5 after implantation) and one bird given [^{3}H]thymidine for a 2-day period ending 14 days before testosterone implantation. All of these birds were sacrificed 37 days after their Silastic implantation (i.e., 3–5 wk after they were injected with [^{3}H]thymidine). Finally, two birds were injected with [^{3}H]thymidine on days 2–3 and 4–5 after testosterone but were killed within 48 hr after the last injection, as opposed to the longer survival times allowed the other 16 birds. All birds were kept on a natural photoperiod for the duration of this study.

On day 37 after hormone implantation, the birds were sacrificed under pentobarbital anesthesia. Most of the canaries were perfused with 40 ml of phosphate-buffered saline (pH 7.4), followed by 60 ml of buffered 10% (vol/vol) formalin; their brains were removed and postfixed for 2 wk. The brains were embedded into paraffin and cut transversely into 6-μm sections. The sections were mounted onto glass slides, dewaxed, and dipped into Kodak NTB-3 emulsion, which was then exposed at 4°C for 3–7 wk. The slides were then developed (Kodak D-19 at 17°C for 3 min), and the sections were dehydrated and counterstained with cresyl violet. All slides were examined for the presence of labeled cells. For each bird, 5 nonadjacent sections containing the left HVc were chosen randomly for quantification. Each left HVc was photographed in black and white at a magnification of $\times$50 with a Zeiss standard microscope. At this magnification, nucleus HVc fills about half of a 5 $\times$ 7 inch (13 $\times$ 18 cm) print. [^{3}H]Thymidine-labeled cells were marked on these prints by using a colored code to identify different types of labeled cells. The person marking the labeled cells on the photographs did not know what treatment each bird had received.

Meanwhile, the three testosterone-treated birds to be used for electron microscopic analysis were perfused with ice-cold phosphate-buffered saline (40 ml), followed by one-fourth strength phosphate-buffered Karnovsky's fixative (1.25% glutaraldehyde/1% paraformaldehyde/0.1 M phosphate/6% sucrose) (8), and their brains were removed and postfixed for 4

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Abbreviation: HVc, hyperstriatum ventrale, pars caudalis.

hr at 4°C. They were then cut transversely into 200-μm sections on a vibratome, and both right and left HVcs were manually excised from the relevant sections while viewed at ×25 magnification under a Zeiss dissecting microscope. The resultant slides of HVc were osmium-fixed, dehydrated, and embedded into Araldite. Adjacent 1-μm semithin and 100- to 150-nm thin sections were then cut and mounted onto slides or 200-mesh copper grids, respectively (9). The 1-μm sections were dipped into Kodak NTB-3 emulsion, exposed for 6–7 wk at 4°C, and then developed as above and counterstained with methylene blue/azure. These sections were examined for the presence of labeled cells, which were then more precisely identified in the corresponding thin sections. The thin sections were stained with lead citrate and uranyl acetate, and then viewed and photographed at 80 kV in a Philips 300 electron microscope. For both the 6-μm paraffin and the 1-μm Araldite sections, a cell was considered labeled if it had five grains over the nucleus; background labeling was virtually nonexistent.

RESULTS

The birds, both testosterone-treated and controls, showed considerable numbers of labeled cells in and around HVc (Fig. 1). The labeled cells (see Fig. 2) fell into several broad classes when viewed in 6-μm paraffin sections (10, 11): (*i*) large (10- to 18-μm soma diameter) cells with clear nuclei, large basophilic central nucleoli, and scant cytoplasm, which cells we tentatively identified as neurons; (*ii*) smaller cells (6–10 μm) containing light nuclei with stippled heterochromatin, small eccentric nucleoli, and scant cytoplasm, which we considered astrocytes; (*iii*) small cells (5–8 μm) with deeply basophilic cytoplasm, small nuclei, and dark karyoplasm, considered to be oligodendrocytes; (*iv*) thin, fusiform, perivascular cells, clearly endothelial; (*v*) very small (4–6 μm) cells of variable morphology lining the ventricle over HVc (Figs. 1–3), which are presumed to be ependymal and subependymal cells and henceforth referred to as ventricular zone cells (12); and (*vi*) cells of uncertain identity.

In order to substantiate our impression that many of the cells incorporating [^{3}H]thymidine were neurons, we next examined the 1-μm plastic sections, which yielded much better morphologic resolution than the 6-μm sections. The 1-μm sections showed labeling over many nuclei clearly belonging to neurons.

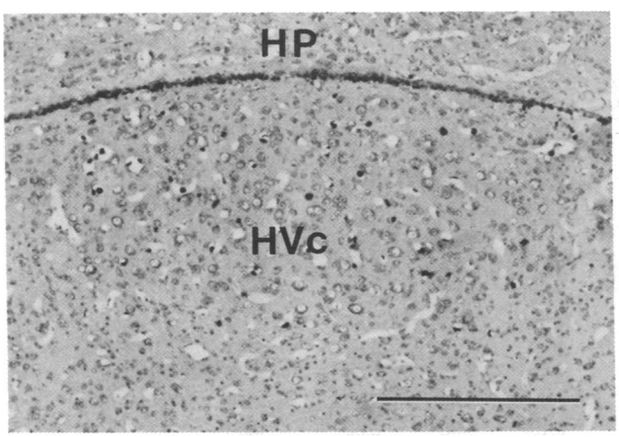

FIG. 1. HVc is the magnocellular region ventral to the collapsed lateral ventricle. The collapsed ventricle is delimited by the two apposed ventricular zones, revealed here as a thin brow of small, darkly staining cells. Notice the presence of labeled cells inside HVc. In this case the labeled cells are all either endothelial or glia. The ventricular zone overlying HVc also shows labeled cells. This section was taken from bird 13 (Table 1), which had been sacrificed two days after the last [^{3}H]thymidine injection. HP, hippocampus. (Bar = 200 μm.)

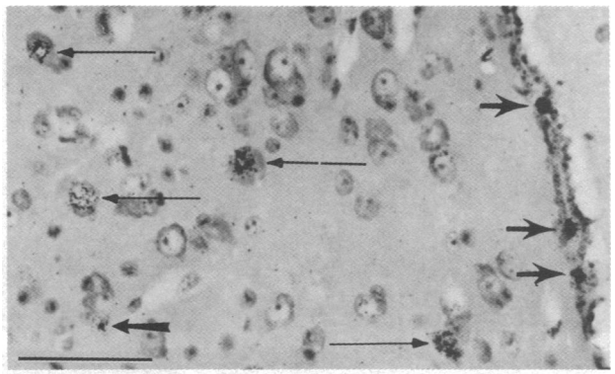

FIG. 2. Paraffin section (6 μm) of HVc (bird 7 in Table 1) showing the ventricular zone on top (to the right). The lateral ventricle normally separating the two ependymal layers is collapsed here. Notice the following labeled cell types: neurons (long arrows), glia (medium arrow), and ventricular zone cells (short arrows). (Bar = 50 μm.)

These cells had the large light nuclei and central nucleoli of neurons and prominent cytoplasmic Nissl substance. Some neurons also had long thin axonal processes and shorter thicker primary dendrites (Fig. 4 *A* and *B*). Electron microscopic examination of the corresponding thin sections of several of these cells confirmed these impressions (Fig. 5); their perikarya contained Nissl bodies composed of extensive rough endoplasmic reticulum and polysomal arrays, the nucleoplasm was homogeneous and devoid of any clumped heterochromatin, and a few cells displayed several long processes filled with arrays of microtubules and polyribosomes, indicative of dendrites. In one case, a labeled cell showed the above traits and an apparent axonal hillock containing filaments and few ribosomes. We failed to see any unequivocal synaptic profiles on these labeled neurons. This is consistent with the paucity of synapses seen on other HVc somata. These characteristics lead us to believe that many of the new cells forming in the adult HVc are, in fact, neurons (13). Also, representatives of several other cell types were found labeled in the 1-μm sections: glia, endothelia (Fig. 4 *C* and *D*), and ventricular zone cells. Interestingly, unlabeled glia cells were often found in satellite association to labeled neurons, and conversely, labeled glia cells were noted to exist in satellite association with unlabeled neurons.

In both the testosterone-treated experimental and choles-

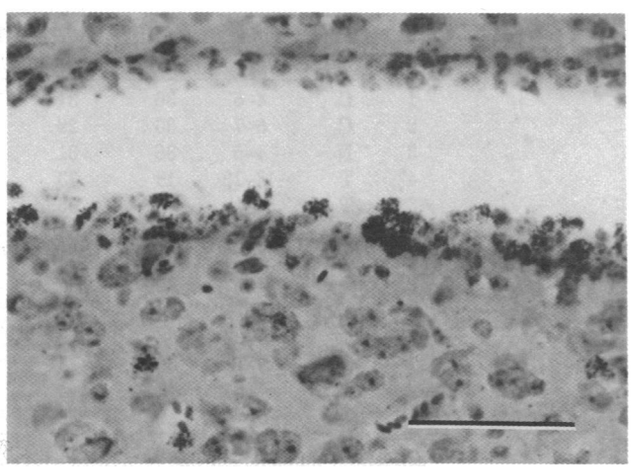

FIG. 3. Ventricular space separates hippocampus (*Upper*) from HVc (*Lower*) in a 15-μm section of HVc taken from a bird injected with [^{3}H]-thymidine and sacrificed 30 days later. The ventricular zone cells overlying HVc are heavily labeled, while the hippocampal ventricular zone is virtually devoid of labeled cells. (Bar = 50 μm.)

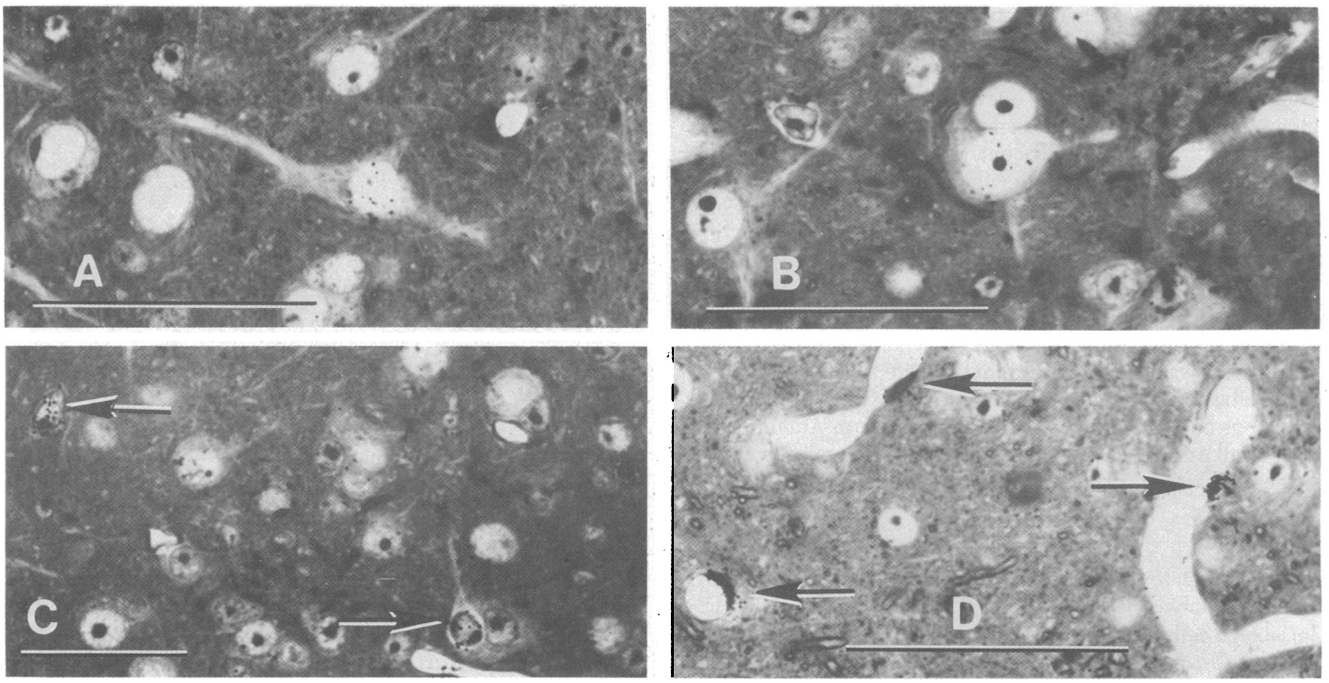

FIG. 4. (*A* and *B*) Two neurons labeled with [^{3}H]thymidine in 1-μm Araldite sections counterstained with methylene blue. Araldite is harder than paraffin; as a result, fewer silver grains are exposed during autoradiography, yielding a seemingly lighter degree of labeling. (*C*) Two labeled astrocytes displaying circumferential nucleoli, one of which shows a probable astrocytic end-foot. (*D*) Several labeled endothelial cells, whose thin, fusiform morphology and pericapillary placement is obvious. These sections were processed for ultrastructural analysis; they were taken from the HVc from a bird sacrificed 32 days after its last [^{3}H]thymidine injection. (Bar = 50 μm.)

terol-treated control birds, sacrificed 37 days after onset of steroid treatment, 1.8–4.0% of the neurons in HVc were labeled by the 2-day [^{3}H]thymidine injection protocol (see Table 1). This represents a neuronal production rate of at least 0.9–2.0% per day in HVc. A labeling index of 1.5% per day was noted in the one bird in which thymidine was given before the testosterone treatment, well within the range observed when steroid treatment preceded the [^{3}H]thymidine injections. However, the two birds that had been sacrificed shortly (24 and 48 hr, respectively) after their last [^{3}H]thymidine injections displayed no labeling of neurons in HVc itself. In these two cases, however, the ventricular zone over HVc was blanketed with labeled cell nuclei of various morphologies, whereas within HVc only endothelial and glia cells were labeled (Fig. 1).

Table 1. Labeling indices after various hormone and thymidine regimens

Bird	Treatment* Steroid type	Treatment* Time, days	Time of sacrifice,† days after treatment: Silastic implant	Time of sacrifice,† days after treatment: Last [^{3}H]dThd injection	Neurons,‡ total no.	Number of labeled cells per 100 neurons§: Neurons	Glia	Endothelia	Ambiguous
1	C	4–5	36	31	1,082	3.70	1.02	1.66	1.20
2	C	6–7	36	29	1,610	2.05	1.42	0.99	0.56
3	N	4–5	36	31	1,862	1.34	1.02	0.32	0.32
4¶	T	−14–13	19	33	1,159	3.02	2.33	1.81	2.42
5	T	0–1	36	35	1,596	3.01	7.46	18.86	1.82
6	T	2–3	36	33	1,122	3.47	3.30	6.86	2.23
7	T	4–5	36	31	1,635	2.20	9.79	21.28	3.79
8	T	4–5	40	35	830	1.92	11.80	26.63	2.05
9	T	6–7	36	29	1,399	2.93	5.36	9.01	1.86
10	T	8–9	36	27	1,281	3.82	8.74	6.01	3.67
11	T	17–18	36	18	2,369	4.01	1.60	0.80	1.35
12	T	4–5	6	1	2,085	0.10	3.98	2.69	0.86
13	T	6–7	9	2	1,093	0.20	5.85	18.57	3.48

* Silastics of the same size had been filled with cholesterol (C), testosterone (T), or nothing (N). Time specifies the days after Silastic implant when [^{3}H]thymidine was given.
† Number of days elapsed since the day of Silastic implant or since the last [^{3}H]thymidine injection.
‡ Counts of all neurons in the five left HVc sections analyzed for each bird.
§ This conversion was done so that the number of labeled cells of each type could be related to a common denominator. Only in the case of labeled neurons does this constitute a proper labeling index.
¶ Notice that bird 4 received its [^{3}H]thymidine starting 14 days before the Silastic implant.

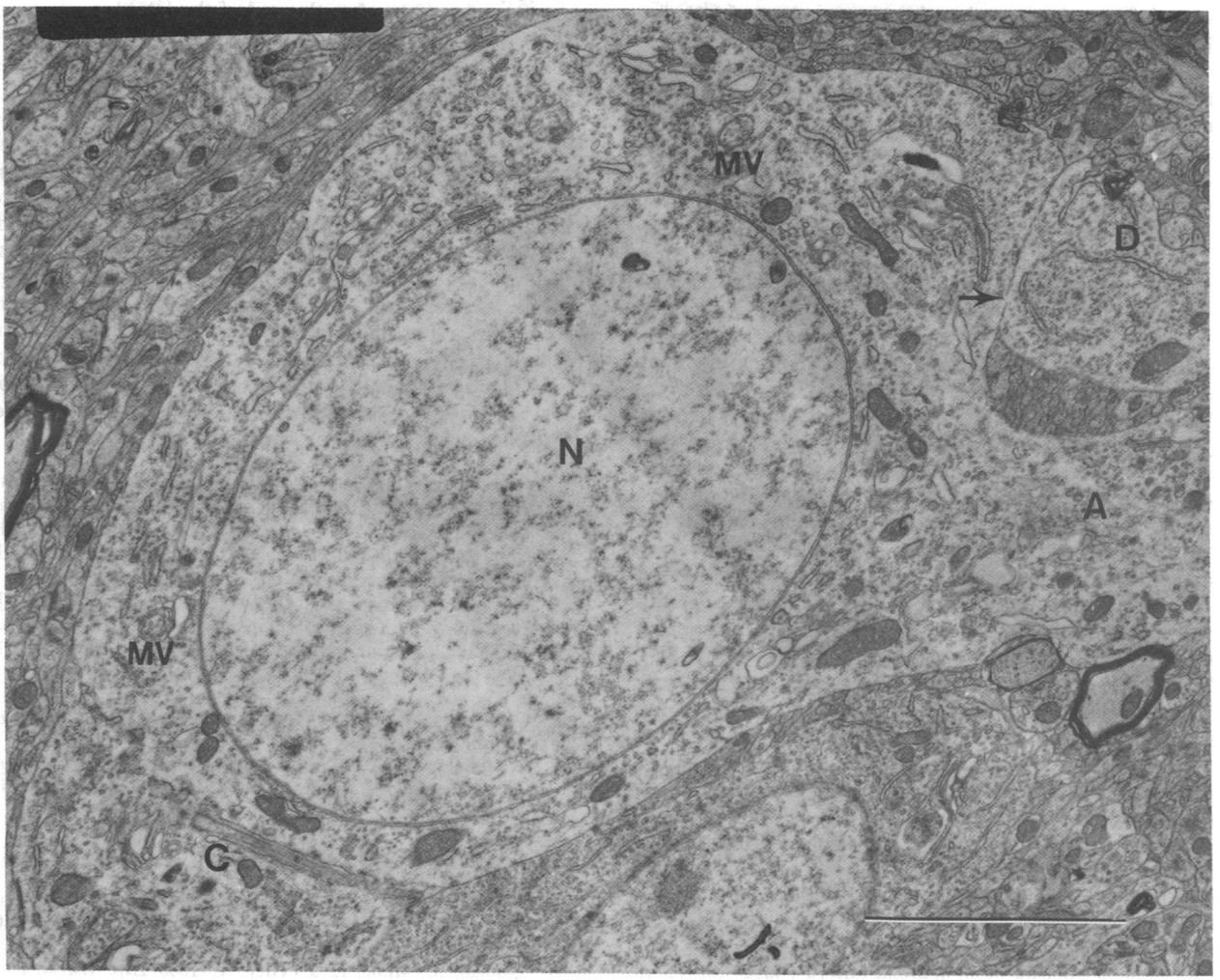

FIG. 5. Electron micrograph of a thin (100–150 μm) section adjacent to that shown as a 1-μm section in Fig. 4B. This cell has a large nucleus (N) with dispersed chromatin, prominent Golgi apparatus, an abundant cytoplasm with extensive rough endoplasmic reticulum, axonal hillock (A), and in this section a primary dendrite (D) cut transversely, whose connection to the cell body is indicated by the arrow. All of these characteristics are indicative of this cell's neuronal identity. This cell also contains several multivesicular bodies (MV), which are common in HVc neurons, and what appears to be an invaginated cilium, perhaps related to this cell's ventricular zone origin. (Bar = 4.4 μm.)

Labeled neurons also occurred in neostriatum adjacent to HVc. None were found in nucleus robustus archistriatalis, a forebrain vocal control nucleus to which HVc projects (1). Label in ventricular zone cells, as found over HVc, was restricted to a few parts of the brain (Fig. 3). The occurrence of labeled cells in non-HVc parts of the brain will be reported elsewhere.

The total number of labeled glia and endothelial cells in HVc was larger in the testosterone-treated birds than in the cholesterol controls (Table 2). This effect retains its sign and magnitude when the data are expressed either as total counts or in terms of number of labeled glia or endothelial cells per 100 neurons. It was noticeable in birds sacrificed 24–48 hr or 17–35 days after the end of the [^{3}H]thymidine treatment (Table 1).

Table 2. Effect of testosterone on labeling indices of neurons, glia, and endothelial cells (from Table 1)

Treatment	Birds	Number of labeled cells per 100 neurons		
		Neurons*	Glia*	Endothelia*
No testosterone†	1–3	2.36 ± 1.21	1.15 ± 0.23	0.99 ± 0.67
Testosterone‡	5–10	2.89 ± 0.73	7.74 ± 3.07	14.77 ± 8.63
		$P > 0.20$	$P < 0.01$	$P < 0.05$

* [^{3}H]Thymidine-labeled cells; values shown correspond to the mean ± SD. P values were obtained by using a two-tailed t test.
† Birds with cholesterol-filled and empty Silastic tubes, pooled; $N = 3$.
‡ [^{3}H]Thymidine injections given sometime during the 10 days after testosterone implant, with a survival of 27–35 days after the last injection; $N = 6$.

DISCUSSION

Neurons in nucleus HVc of adult female canaries incorporate systemically administered [^{3}H]thymidine. We have attempted to determine the origin of labeled HVc neurons, by noting where this label first appears. As noted above, two of the birds in this study were sacrificed within 48 hr after their [^{3}H]thymidine injections to reduce the significant migration of any cells that had incorporated label. Whereas long (3–5 wk) survival times after [^{3}H]thymidine administration resulted in many labeled neurons and glia cells in HVc, the shorter survival times yielded HVcs that were devoid of labeled neurons. Instead, in these early-sacrificed birds, the HVc manifested a heavily labeled ventricular zone, along with many labeled endothelial and glia cells. Therefore, we conclude that a population of ventricular zone precursor cells normally proliferates in the adult female canary brain, with subsequent migration of these cells into HVc and their differentiation therein into neurons and perhaps additional glia.

Several factors suggest that the incorporation of [^{3}H]thymidine by the labeled neurons actually represents proliferation *per se* of ventricular zone precursor cells. The turnover rate of neuronal DNA is typically extremely low and is not sufficient to account for the heavy labeling seen over HVc neurons (14, 15). Polyploidization of extant HVc neurons is a similarly untenable alternative; earlier claims of magnocellular neuronal polyploidy are now considered in doubt (16, 17), and besides, the labeled neurons described here originate in the ventricular zone, not *in situ*. Any scenario of DNA repair, increased turnover, or polyploidization in ventricular zone cells, with subsequent migration and differentiation of these cells into neurons, seems dubious. If ventricular zone neuronal precursors underwent migration and differentiation without mitosis, one might expect the ventricular zone over HVc to become depleted of cells with age; this situation seems unlikely because the HVc ventricular zone of neonatal and adult birds appears similar. We are left with the conclusion that the [^{3}H]thymidine-labeled neurons found in nucleus HVc are in fact the result of the proliferation and subsequent migration and neuronal differentiation of ventricular zone precursor cells. Presumably there continues to be in adulthood a source of neuronal stem cells in the ventricular zone overlying HVc. The mechanism for this may involve precursor cells that give rise to both stem and neuroblastic cells.

Neuronal production and differentiation does not appear to be testosterone-induced in intact adult females, who show this phenomenon both in the presence and absence of exogenous testosterone. However, because all of these females had intact ovaries, we have not excluded some gonadal influence on neuronal recruitment, S phase, or survivability. In one pilot experiment (unpublished data), four ovariectomized adult female canaries treated with [^{3}H]thymidine showed neuronal labeling indices that were depressed with respect to those of normal females.

We have determined here that neurons are added to the HVc of adult female canaries at an average rate of 1.46% per day of the total HVc neuronal pool. From earlier work (18) we know that the size of the HVc does not differ between females sacrificed at ages 1 and 2 yr. If the HVc neuronal number were to remain constant over time, then one might expect an HVc neuronal death rate roughly balancing the neuronal production rate. Thus, the 1.46% daily neuronal labeling index may either represent the overall turnover rate of HVc neurons or be indicative of an even higher turnover rate of a given subpopulation of neurons within HVc. We do not yet know if these recruitment and turnover rates remain constant throughout the year, nor do we know whether the neuronal recruitment rate varies with increasing age of the birds.

Earlier workers have shown that new neurons occur in areas of the adult rodent brain, including rat hippocampus (19–25), olfactory bulb (24), and olfactory epithelium (26). Despite these precedents for neurogenesis in adulthood, the production and directed migration of central neurons from the neuroepithelium of higher vertebrates has generally been thought to be confined to embryonic and perinatal brain (27). Our observations on HVc show that new neurons can arise from ventricular zone stem cells and migrate to other parts of the adult brain. Furthermore, the HVc pattern of neurogenesis is reminiscent of the constant replacement of neurons in the adult olfactory epithelium (26). It contrasts sharply with the constant neuronal addition without loss that has been postulated for the adult rodent hippocampus (22–24).

An increase in the number of labeled glia and endothelial cells in HVc occurred in testosterone-treated birds relative to the cholesterol controls. This increase was apparent even in birds sacrificed 24–48 hr after the end of the [^{3}H]thymidine treatment. This suggests that, unlike neurons, glia and endothelial cells proliferate inside HVc and that this process is markedly stimulated by testosterone.

The aspect of our results which surprises us most is that such a high level of neuronal recruitment should occur in the HVc of untreated adult female canaries. In the absence of exogenous testosterone, such birds do not normally sing. It seems likely, though, that as in other cardueline songbirds studied to date (28), adult female canaries alter some of their social calls to match the calls of other canaries. It is tempting to speculate that ventricular zone neurogenesis in the adult female canary's HVc may contribute to the acquisition or maintenance of such learned calls. Regardless of its behavioral significance, the ventricular zone neurogenesis we have observed in the adult brain is both provocative and reassuring of the plasticity that may reside in adult nervous systems.

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