Vocal Control Neuron Incorporation Decreases with Age in the Adult Zebra Finch

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In adult male zebra finches, high vocal center (HVC) neurons continuously die and are replaced. Many of these cells are projection neurons that form part of the efferent pathway controlling learned song production. Although it is known that HVC receives new neurons well into adulthood, it is unknown whether this occurs at a constant rate or declines with adult age. We used [^{3}H]thymidine to label new HVC neurons in male zebra finches that were 3–36 months of age. Birds were killed 4 months after ^{3}H injections to measure the long-term incorporation of new HVC neurons. HVC neurons projecting to the robust nucleus of the archistriatum (HVC-RA) were retrogradely labeled with Fluoro-Gold 4 d before death. We found a dramatic age-related decline in the number of ^{3}H-labeled HVC-RA neurons present 4 months after cell birth dating. A similar decline in new HVC neurons was found as soon as 1 month after their formation. These results indicate that the production or early survival of adult-formed neurons decreases with age. HVC volume and total neuron number did not change with bird age, suggesting that the age-related decrease in new neuron addition was balanced by increased survivorship of neurons incorporated previously. Reliance of song structure on auditory feedback also wanes with age. We propose that with aging, fewer new cells are added as the numbers of functionally appropriate cells increase, a process that may be linked to age-related increases in motor program stability.

Key words: adult neurogenesis; birdsong; aging; zebra finch; motor learning; apoptosis

Neurogenesis occurs in the adult brains of several warm-blooded vertebrates, including humans (for review, see Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2002; Gould and Gross, 2002; Kempermann, 2002; Nottebohm, 2002; Rakic, 2002). Understanding the control and functions of this remarkable plasticity may force major revision of existing dogma on normal brain function and may also suggest strategies for brain repair.

Neurogenesis is particularly robust in adult birds because, unlike the case in mammals, new neurons are added to much of the telencephalon. Moreover, of all cases of neurogenesis in adult warm-blooded vertebrates, the songbird is the only one where neurons are added to a motor pathway, and this pathway controls a well characterized behavior. Neurons are continually added to the high vocal center (HVC) (see Fig. 1), and many send an axon 2–3 mm to the robust nucleus of the archistriatum (HVC-RA neurons) to become part of the efferent pathway for song control (Nordeen and Nordeen, 1988; Alvarez-Buylla et al., 1990; Kirn et al., 1991).

HVC neuron addition is accompanied by neuron loss, and neuron replacement may be important for song plasticity. In adult canaries, HVC neuron turnover is highest at times of year when males learn new songs. However, neuronal replacement also occurs in adult canaries when song modification is minimal and in male zebra finches, who normally do not change their songs in adulthood (for review, see Alvarez-Buylla and Kirn, 1997). However, increasing evidence indicates that song stereotypy may normally rely on fine-grained adjustments to motor output that are made based on auditory feedback (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999). These results suggest that even song maintenance requires vocal flexibility, which, in turn, may be promoted by the replacement of old neurons with new ones.

Recent work indicates that the reliance of song stereotypy on auditory feedback wanes with increasing adult age, reflecting an increase in the stability of song motor programs (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). We were interested in seeing whether age-related increases in song stability are paralleled by decreases in new neuron addition. Although it is known that HVC neuron addition persists even in 4-year-old canaries (Alvarez-Buylla et al., 1990), age-related changes in HVC neuron production or long-term survival have never been systematically examined.

It is also not known whether HVC volume or total neuron number change with age. If neuron addition decreases with age, this might lead to a reduction in HVC neuron number. Alternatively, changes in new neuron addition might be compensated by changes in cell loss such that total HVC neuron number does not change (Nottebohm, 1985; Kirn et al., 1991, 1994; Scharff et al., 2000). In the present work, we measured age-related changes in the incorporation and long-term survival of adult-formed HVC neurons, including new HVC-RA neurons, in birds between the ages of 3 and 36 months after hatching. We also measured HVC volume and total neuron number. This age range spans young adulthood and middle age in wild zebra finch populations (Zann, 1996).

MATERIALS AND METHODS

All animal experimentation conformed to National Institutes of Health guidelines and was approved in advance by the Institutional Animal Care and Use Committee at Wesleyan University. Birds were obtained from

Received June 10, 2002; revised Sept. 19, 2002; accepted Oct. 1, 2002.

This work was supported by United States Public Health Service Grants NS29843 and DC04724 and the Scott Family Charitable Trust. We thank Ann Hesla for technical assistance and Anna Dondzillo who provided helpful comments on previous drafts of this manuscript.

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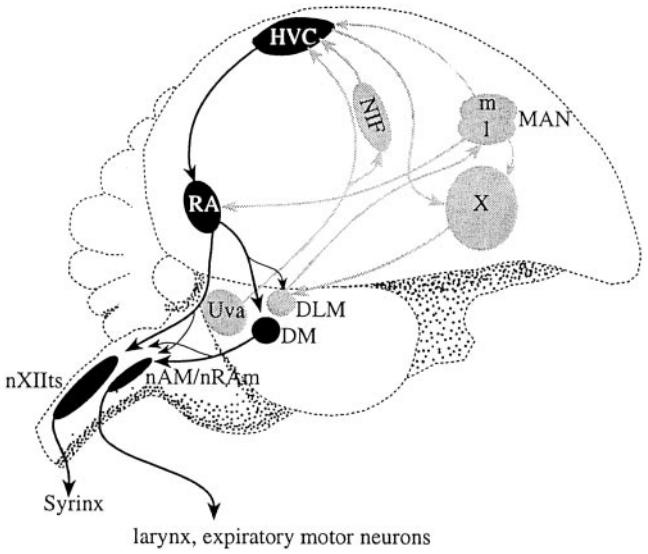

Figure 1. Schematic diagram of the major brain regions involved in song learning and control. The efferent pathway for song control is highlighted in *black*. Our main focus is the HVC and the neurons incorporated into the HVC that project to the RA. *nAM*, Nucleus ambiguus; *DLM*, medial portion of the dorsolateral nucleus of the thalamus; *DM*, dorsomedial nucleus of the intercollicular complex; *lMAN*, lateral subdivision of the magnocellular nucleus of the anterior neostriatum; *mMAN*, medial subdivision of the magnocellular nucleus of the anterior neostriatum; *NIF*, nucleus interface; *nRAm*, nucleus retroambigualis; *Uva*, nucleus uvaeformis; *X*, area X; *nXIIts*, tracheosyringeal part of the hypoglossal nucleus.

our breeding colony and ranged in age from 3.4 to 24 months, with the exception of one bird that was 36 months of age. Birds were kept in a large aviary with their parents and other birds of both sexes until 70–90 d of age, when they were removed and housed together in groups of two to five. Birds were housed on a 14/10 hr light/dark cycle at 22°C. Seed and water were available *ad libitum*, supplemented with a mixture of cooked eggs and baby cereal every 2–3 d.

Cell birth dating. All birds in the 4 month survival study received intramuscular (pectoral muscles) injections of [^{3}H]thymidine (methy-[^{3}H]thymidine, 2.5 μCi/gm; 6.7 Ci/mmol; 1 Ci = 37 GBq; NEN, New Life Products, Boston, MA) every 12 hr (8 A.M. and 8 P.M.) for 6 consecutive days to label dividing cells. Birds in the 1 month survival study received intramuscular injections of bromodeoxyuridine (BrdU; 75 μl of a 10 mg/ml solution in 0.1 M TBS, pH = 7.6; ≈0.06 mg/gm body weight; Boehringer Mannheim, Indianapolis, IN) every 6 hr between 8 A.M. and 8 P.M. for 4 d. The use of different markers and injection schedules for 1 and 4 month survival groups precludes direct comparisons of absolute numbers of labeled cells. However, we were primarily interested in seeing whether similar age-related changes in labeling could be found at 1 and 4 months of survival, not whether the numbers of labeled neurons differed between these two survival times.

Fluoro-gold labeling. Four days before being killed, birds that received [^{3}H]thymidine also received 2-hydroxy-4,4′-diamidinostilbene (Fluoro-Gold; Fluorochrome, Englewood, CO) injections into the RA bilaterally to retrogradely label RA-projecting HVC neurons as described previously. Birds were deeply anesthetized with intramuscular (pectoral) injections of ketamine (Ketalar; Park-Davis, Fort Dodge, IA; 0.3 mg/gm body weight) and xylazine (Rompun; Haver, The Butler Co., Columbus, OH; 0.06 mg/gm body weight). Birds were then placed in a stereotaxic instrument, and Fluoro-Gold [2% (w/v) in 0.9% (w/v) saline] was pressure injected into the RA using glass micropipettes (20–30 μm tip diameter). Injection sites typically encompassed 50–100% of the RA and usually spread to the surrounding archistriatum. In previous work, similar numbers of neurons were labeled despite such targeting variation (Kirn and Nottebohm, 1993), perhaps because HVC neuronal axons ramify extensively within the RA (Vicario and Simpson, 1988). Birds were returned to their home cages after recovery from surgery.

Survival time, perfusion, and fixation. Thirteen birds between the ages of ~4 and 21 months (103–639 d) at the time of BrdU injections were used to measure the initial incorporation of neurons. These birds were killed 28 d after the last BrdU injection. The 28 d survival time was chosen because by this time most if not all labeled neurons would have arrived in the HVC and sent an axon to the RA. The remaining birds (n = 32) were between the ages of 4 and 36 months at the time of [^{3}H]thymidine injection and were killed 120 d after cell birth dating to follow the long-term survival of the [^{3}H]thymidine-labeled neurons. There is a substantial culling of new neurons between 1 and 4 months after birth dating in the zebra finch (Wang et al., 1999), and so this seemed like a promising time interval in which to explore the possibility that differential cell death is involved in age-related changes in new neuron number. For the 4 month survival analysis, hearing intact birds (n = 16) were supplemented with unilaterally deafened birds (n = 16) that are part of a different study. Deafening was accomplished by cochlea removal as described previously (Wang et al., 1999). Otherwise, both hearing-intact and unilaterally deafened groups received identical procedures. Unilateral deafening has no effect on neuronal incorporation (Wang, 2000) or on song acoustic structure (Lombardino and Nottebohm, 2000). Nevertheless, data from these two groups of birds are plotted using different symbols, and statistical analyses were performed with and without data from unilaterally deafened birds. Both analyses yielded similar results, and statistics for both are presented.

At the appropriate survival time, all birds were deeply anesthetized by inhalation of methoxyflurane (Metofane; Mallinckrodt, Mundelgn, IL) and perfused through the left ventricle with 20–30 ml of 0.1 M PBS, pH 7.4, followed by 40–50 ml of 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). The brains of birds injected with [^{3}H]thymidine were postfixed for 3–5 d in the same fixative, washed in PBS, dehydrated in increasing concentrations of ethanol, and embedded in polyethylene glycol (PEG; Polysciences, Warrington, PA) (Smithson et al., 1983). Six micrometer sagittal brain sections containing the HVC were cut on a rotary microtome, and every eighth section was mounted onto chrom-alum-subbed slides and air dried. Sections were then delipidized in increasing concentrations of ethanol and cleared in xylene. The sections were then rehydrated and stored in a dust-free oven overnight. The brains of birds that received BrdU injections were postfixed for 1 hr. Brains were then embedded in PEG, cut and mounted onto glass slides as described for birds that received [^{3}H]thymidine, and then stored at −20°C until immunohistochemical processing.

Autoradiography and counterstaining. Under a sodium-safe light, slides were dipped in nuclear track emulsion (NTB2; Eastman Kodak, Rochester, NY) in a 37°C water bath, allowed to dry at 37°C in a light-tight oven for 3 hr, and then stored with desiccant for 28 d at 4°C in the dark. Slides were then developed (Kodak D-19 developer) for 3 min at 17°C, rinsed in tap water at 19°C for 1 min, and fixed (Kodak standard fixer) at 19°C for 12 min, followed by running tap water for 10–20 min. Then sections were counterstained through the emulsion with fluorescent cresyl violet. This counterstaining method allows morphological identification of all cells without compromising the Fluoro-Gold signal. Finally, the sections were again dehydrated in ethanol, cleared in xylene, and coverslipped with Krystalon (Harleco; EM Science, Gibbstown, NJ).

Immunohistochemistry. Sections were brought to room temperature and exposed to citrate buffer at 95°C for 10 min, followed by a 5 min wash in phosphate buffer (PB), 3 min in 2.5% pepsin in 0.1N HCl at 37°C, and three 3 min washes in PB. Sections were then blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% Triton X-100 in PB for 1 hr at room temperature, followed by overnight exposure to sheep anti-BrdU (12.5 μg/ml at 4°C; Capralogics, Hardwick, MA). After three 10 min PB washes, sections were processed with an avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA), followed by a 2 hr incubation in biotin-conjugated donkey anti-sheep IgG (1:200; Chemicon International, Temecula, CA). After three 10 min PB washes in the dark, streptavidin conjugated to Alexa 488 (1.25 μg/ml; Molecular Probes, Eugene, OR) was applied for 1 hr in the dark for visualization of BrdU. This was followed by three 10 min washes in PB, 1 hr in blocking solution in the dark, and overnight exposure to mouse anti-Hu primary antibody (10 μg/ml in blocking solution) (Hu MAB16A11; Molecular Probes) at 4°C. After three 10 min PB washes at room temperature in the dark, tissue was exposed to donkey anti-mouse IgG conjugated to Cy-3 (6.25 μg/ml; Jackson ImmunoResearch) for 1 hr in the dark to visualize nuclear and cytoplasmic labeling with anti-Hu (Barami et al., 1995). Sections were then washed, dehydrated in ethanols, immersed briefly in xylene, and coverslipped with Krystalon.

Microscopic analysis. All data were collected without previous knowledge of bird age. Area measurements and cell counting were performed using a computer-yoked fluorescence microscope system. Fluoro-Gold

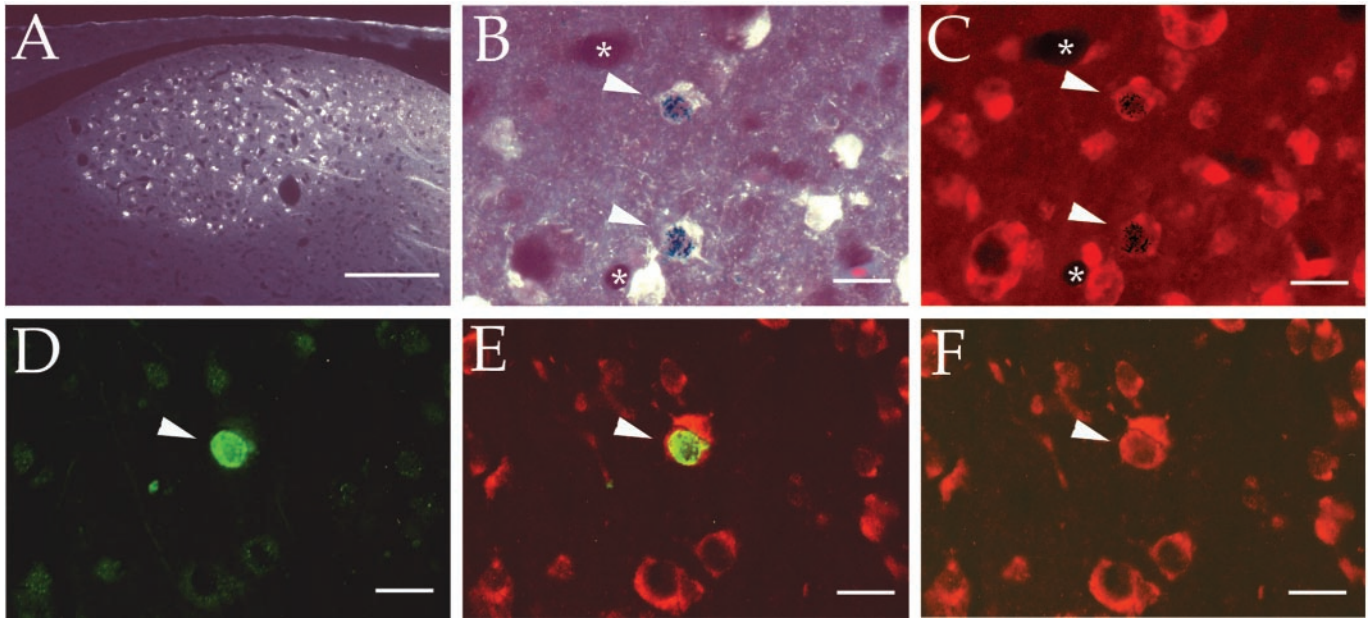

Figure 2. *A*, Low-magnification fluorescence (UV) photomicrograph of the HVC after retrograde labeling with Fluoro-Gold injections into the RA. *B*, Higher-power magnification of two HVC-RA projection neurons formed in adulthood (*arrowheads*) viewed with combined bright-field and UV illumination. These neurons have developed silver grains overlying their nucleus and Fluoro-Gold in their cytoplasm. *C*, Same view as in *B*, showing all cells counterstained with fluorescent cresyl violet and viewed under combined rhodamine fluorescence and bright-field optics. *Arrowheads* point to the same ^{3}H-labeled neurons shown in *B*. *Asterisks* in *B* and *C* label blood vessels cut in cross section. *D–F*, The same field is shown using different fluorescence filters to reveal a cell double labeled with BrdU (*green*) and Hu (*red*; *arrowheads*). *D*, BrdU-labeled cell nucleus viewed under FITC fluorescence. *E*, Same field viewed with dual FITC–rhodamine filter. Cytoplasmic staining with the neuronal marker Hu surrounds the BrdU-labeled cell nucleus. *F*, Same field viewed under rhodamine fluorescence. Scale bars: *A*, 100 μm; *B–F*, 10 μm.

labeling, used to identify RA-projecting HVC neurons, was visualized with UV fluorescence. ^{3}H labeling was identified with bright-field optics, and the fluorescent cresyl violet counterstain, used to identify neurons not labeled by Fluoro-Gold, was visualized with rhodamine fluorescence (see Fig. 2). Cells of this latter class were recognized based on their size and Nissl-staining characteristics. Cells classified as neurons had a relatively large, clear nucleus and one to two darkly stained nucleoli, criteria that have been validated in ultrastructural work and by retrograde labeling (Goldman and Nottebohm, 1983; Burd and Nottebohm, 1985; Kirn et al., 1991). A neuron was recognized as ^{3}H labeled when the number of exposed silver grains over the nucleus was ≥20 times that of the surrounding neuropil. This threshold typically corresponded to seven or more exposed silver grains.

A combination of three fluorescence filters was used to identify new HVC neurons after immunohistochemistry. BrdU labeling was visualized with an FITC filter, Hu-positive cells were identified under rhodamine fluorescence, and double-labeled cells were confirmed by alternating between these two filters and a dual FITC–rhodamine filter (see Fig. 2).

In all hearing-intact birds in the 1 month survival group (BrdU tissue), all neuronal attributes described were calculated unilaterally, using dark-field optics to define the boundaries of HVC. Previous work has failed to detect any systematic left–right differences in adult neuron addition. In all unilaterally deafened and intact birds used in the 4 month survival group, both hemispheres were analyzed, using Fluoro-Gold to define the boundaries of the HVC. The magnitude of neuron addition in the two hemispheres of these birds was highly correlated, and there were no systematic differences in HVC parameters between hemispheres ipsilateral and contralateral to cochlea removal, nor did these birds show any overall differences from hearing-intact birds on the measures examined (Wang, 2000). Therefore, values obtained for the two hemispheres were averaged.

In each bird, HVC perimeters and cross-sectional areas were determined in 10 sections equally spaced throughout the HVC. HVC volume was estimated using the following formula: sum of the areas measured × sampling interval × section thickness. The HVC in these sections was completely scanned for [^{3}H]thymidine-labeled neurons. Total ^{3}H-labeled neurons represented the sum of [^{3}H]-Fluoro-Gold-positive (double labeled) and ^{3}H-positive but Fluoro-Gold-negative neurons. In animals that received BrdU, all BrdU-positive plus Hu-positive neurons were counted. All HVC neurons and Fluoro-Gold-labeled neurons were counted in four equally spaced sections. The number of cells in each labeling category per volume sampled was multiplied by HVC volume to yield estimates of total cell number. Nuclear diameters were measured for all ^{3}H-labeled neurons encountered and for ≥30 Fluoro-Gold-labeled and 30 non-Fluoro-Gold-labeled neurons in each bird. Cell diameters did not vary systematically as a function of bird age (ANOVA; $p > 0.05$). Therefore, no corrections were made for cell splitting, because we were primarily interested in relative rather than absolute differences in neuronal number (Saper, 1996; Guillery and Herrup, 1997).

Statistical analysis. Statistical comparisons of neuronal attributes were conducted using linear regression and ANOVA (SYSTAT 5.2; Systat, Evanston, IL), with the independent factors being age and survival time. Data are presented as values for individual birds in scatter plots or as means ± SEM in cases in which age groups were combined.

RESULTS

Figure 3 (*left* and *middle*) summarizes the data on the relationship between bird age and the numbers of [^{3}H]thymidine-labeled HVC neurons present 4 months after [^{3}H]thymidine injection. The *left* plot shows age-related changes in total ^{3}H-labeled HVC neuron numbers, and the *middle* plot depicts the relationship between age and the total number of ^{3}H-labeled HVC-RA projection neurons (double labeled with [^{3}H]thymidine and Fluoro-Gold). In both unmanipulated birds (intact hearing) and birds that had been deafened unilaterally, there was a significant decrease in the number of [^{3}H]thymidine-labeled neurons with increasing bird age ($r^2 = 0.51$, $p < 0.002$ for intact-hearing birds alone; $r^2 = 0.58$, $p < 0.0001$ for intact-hearing birds plus unilaterally deafened birds). This was also true for ^{3}H-labeled HVC-RA projection neurons (intact hearing, $r^2 = 0.53$, $p < 0.002$; intact and unilaterally deafened, $r^2 = 0.58$, $p < 0.0001$).

The decline in new HVC neuron number was much steeper between the ages of 4 and 12 months than it was between the ages of 12 and 24 months at the time of cell birth dating. Indeed, if we

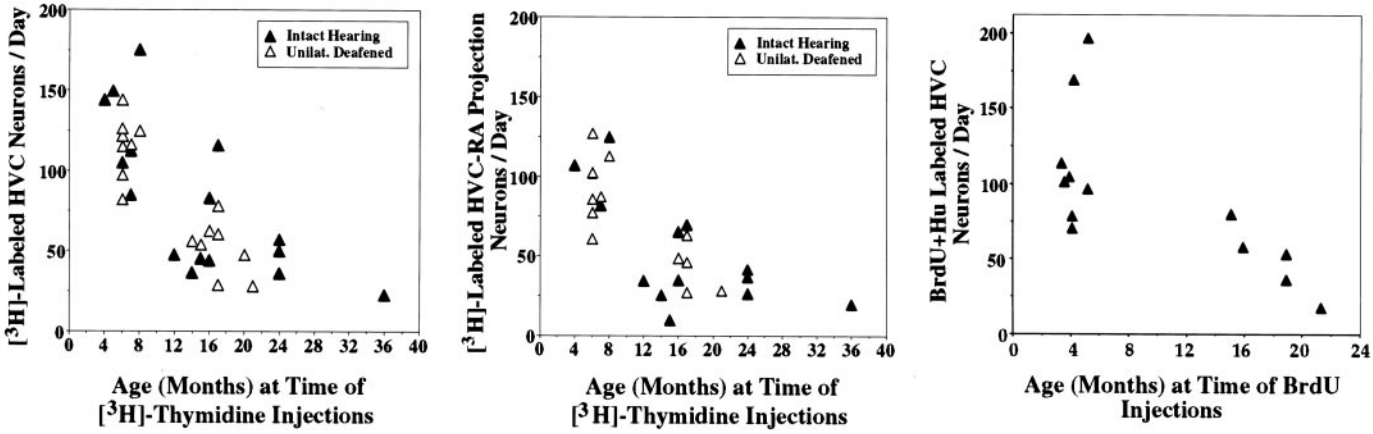

Figure 3. The number of [^{3}H]thymidine-labeled neurons per day of treatment as a function of bird age at the time of injection (*left* and *middle*). Birds were killed 4 months after [^{3}H]thymidine injections. Each *triangle* represents one bird. Both hearing-intact birds and unilaterally deafened birds showed a decline in the total number of new HVC neurons. This was also true for adult-formed HVC-RA projection neurons (*middle*) identified by double labeling with [^{3}H]thymidine and Fluoro-Gold. At 1 month after cell birth dating with BrdU, the number of new neurons double labeled with BrdU and Hu per day of BrdU injections also declined with bird age (*right*). These results indicate that the production or survival of new neurons while migrating or shortly after arrival in the HVC decreases with bird age.

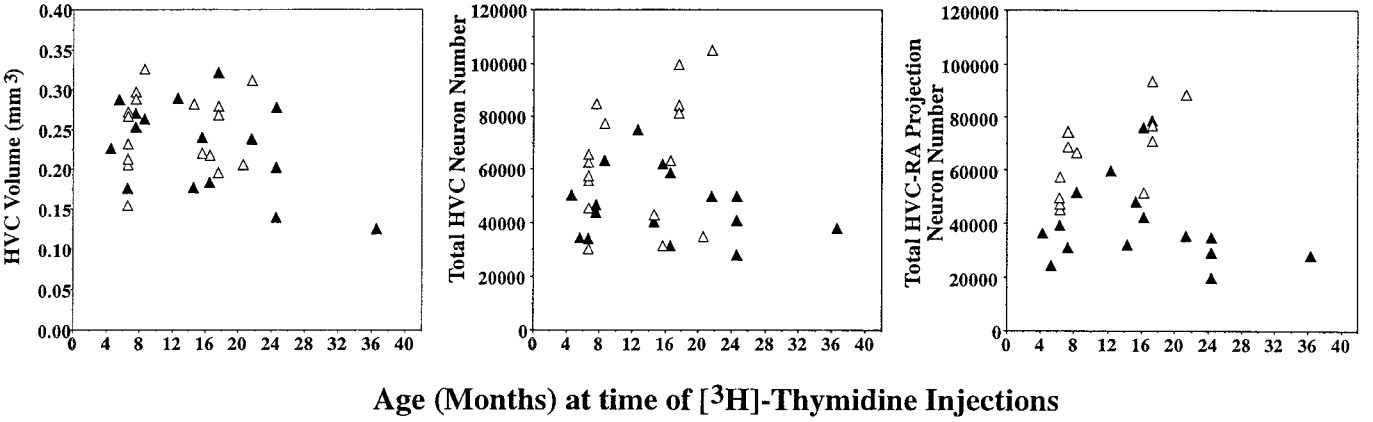

Figure 4. HVC volume (*left*), total neuron number (*middle*), and total HVC-RA projection neuron number (*right*) for all birds used in the 4 month survival study (Fig. 3), plotted as a function of age at the time of [^{3}H]thymidine injections. Despite the dramatic age-related decline in adult-formed neuron number in these birds, HVC volume and the numbers of HVC neurons remained relatively stable with age.

restrict our focus to birds in the latter group, the age effect is lost for total new HVC neurons as well as total new HVC-RA projection neurons ($r^2 < 0.2$; $p > 0.17$).

We wanted to see whether these results might be explained by age-related changes in [^{3}H]thymidine availability. To explore this possibility, we compared the numbers of silver grains overlying the nucleus of labeled neurons in the six youngest and five oldest hearing-intact birds. If the apparent decline in labeled neuron numbers in older birds was caused by decreased availability of [^{3}H]thymidine, we would predict that the average number of silver grains per cell would be lower in older birds. However, old and young birds had very similar grain counts (mean ± SEM: 20.58 ± 0.78 for young birds and 20.22 ± 0.74 for older birds; $p > 0.5$).

To begin to address the potential mechanisms for this age effect, we also killed some birds 1 month after BrdU injections (Fig. 3, *right*). The age-related decline in new neuron number seen at 4 months of survival was already present 1 month after cell birth dating ($r^2 = 0.49$; $p = 0.008$). These results indicate that neuron production, early survival, or both decline with increasing adult age.

Given the age-related decline in new neuron addition, we were interested in the potential impact this would have on HVC volume, the total number of HVC neurons, and the number of HVC-RA projection neurons. Figure 4 summarizes these data. Interestingly, there was no relationship between age and any of these HVC attributes (intact birds alone: $r^2 = 0.026$, $p > 0.3$ for HVC volume; $r^2 = 0.002$, $p > 0.80$ for total neuron number; $r^2 = 0.001$, $p > 0.90$ for total HVC-RA projection neurons; intact or intact and unilaterally deafened birds combined: $r^2 < 0.03$, $p > 0.30$). We infer from this that age-related reductions in neuron addition are balanced by increased survival of neurons generated previously. However, it is also possible that age-related differences in neuron addition occur in the absence of changes in the survival of neurons added previously. The kinetics of cell loss beyond our 4 month sampling time are unknown, and this information is necessary before we can estimate the extent to which age-related differences in the numbers of new neurons added per day of ^{3}H treatment are amplified over time. The difference between young and older birds in terms of the absolute number of neurons added and that persist beyond 4 months of survival may be sufficiently small as to be masked by total HVC neuron counts.

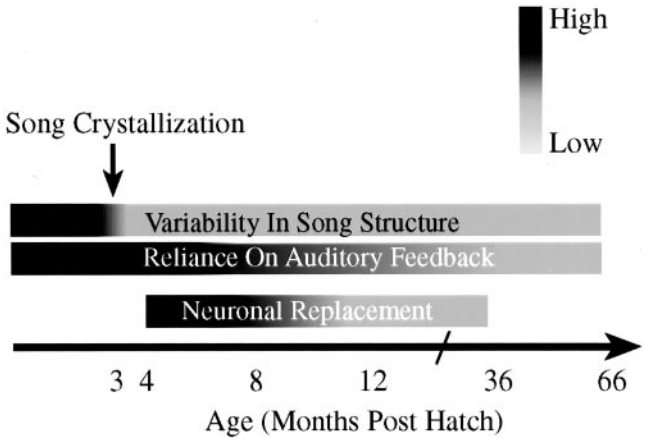

Figure 5. A model integrating previous behavioral work with the present results. Song crystallization occurs at ~90 d after hatching. Before this age, song structure and amplitude are highly variable. After crystallization, song structure remains highly stereotyped throughout adulthood. However, even after song structure becomes stable, song stereotypy relies on the bird's ability to compare vocal output with song memories and make motor adjustments accordingly. Therefore, song stereotypy requires sensorimotor flexibility. With increasing adult age, the motor program for song becomes increasingly stable and independent of auditory feedback. Our results indicate that over the same interval, neuronal incorporation decreases, and the population of replaceable HVC neurons becomes more stable.

A better understanding of the longer-term survivorship of adult-formed neurons would help resolve this issue.

DISCUSSION

We demonstrate that new HVC neurons, including HVC-RA projection neurons, are produced in male zebra finches at all ages examined. However, the total number of adult-formed HVC neurons that persists for 4 months decreases with increasing adult age. Age-related changes in new neuron number could result from changes in cell proliferation or survival. Most adult-formed neurons arrive in the HVC and have begun to differentiate by 4 weeks after their formation (Burek et al., 1994; Kirn et al., 1999), and then many die when they are between 1 and 4 months of age (Nottebohm et al., 1994; Wang et al., 1999). We wanted to determine whether the age effect emerged between these two times because of differential cell death. However, we found that the number of new neurons present even 1 month after their formation declined with age. These results raise the possibility that the production of HVC neurons decreases with age. Alternatively, many adult-formed neurons die before our 1 month survival time, while migrating (Alvarez-Buylla and Nottebohm, 1988) or shortly after their arrival in the HVC (Kirn et al., 1999), and so we cannot rule out cell death as a possible contributor to the age-related changes in new neuron number. Regardless, our results indicate that events occurring before 1 month after cell birth dating are the likely locus for age-related change.

Age-related decreases in new granule cell numbers in the dentate gyrus have been described in adult rodents, and preliminary indications suggest that this is also true in primates, including humans (Kuhn et al., 1996; Eriksson et al., 1998; Cameron and McKay, 1999). Neuronal incorporation in the telencephalon of the ring dove, a non-songbird, also decreases with age (Ling et al., 1997). Our results extend these observations to HVC neurons that are part of the efferent pathway for song control. These results raise the possibility that cell proliferation and/or the survival of new neurons decreases with age in most if not all systems that undergo continued postnatal neuron addition.

Several trophic factors are potential candidates for mediating age-related changes in adult HVC neuron turnover. Changes in glucocorticoid levels have been implicated in the reduced production of hippocampal granule cells in senescent rats (Cameron and McKay, 1999). In adult songbirds, gonadal steroids influence neuronal replacement, and this effect is mediated by BDNF (Brown and Bottjer, 1993; Rasika et al., 1994, 1999; Hidalgo et al., 1995). Thyroid hormones may also play a role in controlling HVC neuronal survival (Tekumalla et al., 2002). However, it is presently not known whether any of these trophic factors change in adult zebra finches over the age range studied, although decreases in steroid levels with advancing age have been found in other avian species (Ottinger, 2001).

One of the most interesting findings of the present study was that despite age-related decreases in the addition of new HVC neurons, HVC volume and the total number of HVC neurons were not affected by age in the same birds. This suggests that the addition and loss of HVC neurons are maintained in dynamic equilibrium. We cannot rule out the possibility that estimates of total HVC neuron number mask age-related differences in neuron addition that are not compensated for by changes in the survival of neurons generated previously. Nevertheless, we favor the former interpretation based on previous work. Experimentally induced increases in HVC neuron death are followed by augmented neuronal replacement (Scharff et al., 2000). In addition, seasonal increases in HVC cell death precede increases in neuronal replacement (Kirn et al., 1994) in a manner that maintains stable HVC volume and total neuron number throughout much of the year (Kirn et al., 1991). Additional exploration of the age effects is necessary before this issue is resolved. However, regardless of which interpretation is correct, progressive decreases in neuron incorporation indicate that the HVC becomes more stable with age.

The functional significance of adult neurogenesis in species in which brain and body size do not continue to increase remains unclear. If adult neurogenesis serves adaptive functions, then perhaps equally perplexing is why neurogenesis and/or new neuron survival should decrease with increasing age. Although in some cases it could be argued that decreases in cell addition are attributable to extreme senescence and generalized brain deterioration, this seems unlikely in the zebra finch. Much of the observed age effect in the present study occurred over the first year of adulthood, and zebra finches in the wild are known to live and breed for as long as 5 years (Zann, 1996). Therefore, another explanation is needed.

The important role that the HVC plays in song motor control provides an especially promising context for understanding the functional significance of neuronal replacement. Song development in zebra finches involves the matching of vocal output with an internalized song model based on auditory feedback and becomes stereotyped by the age of 90–100 d after hatching (Fig. 5). Thereafter, song does not change in acoustic structure (Immelmann, 1969; Arnold, 1975; Price, 1979). However, song maintenance in adulthood also requires auditory feedback (Nordeen and Nordeen, 1992; Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997; Leonardo and Konishi, 1999; Wang et al., 1999). Recent work has shown that this reliance on auditory feedback wanes with adult age in terms of the onset and magnitude of song deterioration after deafening, suggesting that the song motor program becomes increasingly stable with age, vocal practice, or

both (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). The decline in new HVC neuron addition reported here occurs over the same age range associated with a decline in the effects of auditory deprivation on song structure (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). Our neurobiological results further correlate with these behavioral studies on a more fine-grained level in that age-related changes do not appear to be linear. The decline in new HVC neuron number was much steeper between the ages of 4 and 12 months than it was between the ages of 12 and 24 months at the time of cell birth dating. Similarly, the greatest age-related declines in song deterioration after deafening occur between ~4 and 7 months of age (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001) (Figs. 3, *left* and *middle*, 5). Collectively, these results point to a need for more investigation of what happens to song and the underlying neural circuits as adults grow older with special attention focused on the first few months after song crystallization.

It has long been hypothesized that HVC neuronal replacement contributes to the cellular basis for song plasticity (Nottebohm, 1989; Kirn et al., 1994; Scott et al., 2000). We do not know whether the age effect we report is specific to HVC or generalizable to much of the forebrain, a question currently under study. If age-related changes in neuron addition are causally linked to changes in motor program stability, the directionality of causation remains an intriguing question. Increasing stability of HVC neuron populations might constrain vocal plasticity and stabilize learned motor commands. Alternatively, the relative stability of song motor programs may influence cell turnover rates. These two scenarios need not be mutually exclusive, and both would predict progressive loss of susceptibility to auditory deprivation with age (Lombardino and Nottebohm, 2000). The proposed relationship between age-related changes in neuron turnover and song motor program stability would be strengthened if it could be shown that the effects of experience on neuron turnover (Wang et al., 1999; Li et al., 2000) also diminish with age.

In the classical description of song sensorimotor development (Thorpe, 1958), song crystallization has been viewed as the hallmark change from plastic to fully stereotyped song. It is now clear from several studies that this stereotypy belies a dynamic process whereby birds rely on auditory feedback to make slight adjustments to motor output to maintain stereotypy. Furthermore, song stereotypy undergoes a progressive emancipation from auditory feedback between young adulthood and more advanced ages. Our results indicate that behavioral stability also masks age-related changes in the underlying neural circuitry, in this case, neuronal turnover in the motor pathway for song control. Our results reveal an intriguing parallel between age-related increases in song motor program stability and the stability of the HVC. Adult-formed neurons have variable life spans ranging from days to ≥8 months (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1991, 1999; Nottebohm et al., 1994). Perhaps new neurons that fit well with their environment live for relatively longer periods of time. These latter neurons may become increasingly abundant as a function of age. The protracted survival of earlier formed neurons could reduce the amount of available space for incorporation of subsequent neurons. As a result, incoming neurons might die in increasing numbers as birds age. Alternatively, or in addition, signals generated by an increasingly stable cell population might suppress proliferation (Scharff et al., 2000). In the zebra finch, cellular turnover may thus be a lifelong cell-sorting process whose magnitude depends on age-related changes in behavioral and neural network demand.

REFERENCES

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629–634.

Alvarez-Buylla A, Kirn JR (1997) Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. J Neurobiol 33:585–601.

Alvarez-Buylla A, Nottebohm F (1988) Migration of young neurons in adult avian brain. Nature 335:353–354.

Alvarez-Buylla A, Kirn JR, Nottebohm F (1990) Birth of projection neurons in adult avian brain may be related to perceptual or motor learning. Science 249:1444–1446.

Arnold AP (1975) Effects of castration on song development in zebra finches (*Poephila guttata*). J Exp Zool 191:261–277.

Barami K, Iversen K, Furneaux H, Goldman SA (1995) Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. J Neurobiol 28:82–101.

Brainard MS, Doupe AJ (2001) Postlearning consolidation of birdsong: stabilizing effects of age and anterior forebrain lesions. J Neurosci 21:2501–2517.

Brown SD, Bottjer SW (1993) Testosterone-induced changes in adult canary brain are reversible. J Neurobiol 24:627–640.

Burd GD, Nottebohm F (1985) Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol 240:143–152.

Burek MJ, Nordeen KW, Nordeen EJ (1994) Ontogeny of sex differences among newly-generated neurons of the juvenile avian brain. Brain Res Dev Brain Res 78:57–64.

Cameron HA, McKay RD (1999) Restoring production of hippocampal neurons in old age. Nat Neurosci 2:894–897.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317.

Gage FH (2002) Neurogenesis in the adult brain. J Neurosci 22:612–613.

Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci USA 80:2390–2394.

Gould E, Gross CG (2002) Neurogenesis in adult mammals: some progress and problems. J Neurosci 22:619–623.

Guillery RW, Herrup K (1997) Quantification without pontification: choosing a method for counting objects in sectioned tissues. J Comp Neurol 386:2–7.

Hidalgo A, Barami K, Iverson K, Goldman SA (1995) Estrogens and non-estrogenic ovarian influences combine to promote the recruitment and decrease the turnover of new neurons in the adult female canary brain. J Neurobiol 27:470–487.

Immelmann K (1969) Song development in the zebra finch and other estrildid finches. In: Bird vocalizations (Hind RA, ed), pp 64–74. Cambridge, UK: Cambridge UP.

Kempermann G (2002) Why new neurons? Possible functions for adult hippocampal neurogenesis. J Neurosci 22:635–638.

Kirn JR, Nottebohm F (1993) Direct evidence for loss and replacement of projection neurons in adult canary brain. J Neurosci 13:1654–1663.

Kirn JR, Alvarez-Buylla A, Nottebohm F (1991) Production and survival of projection neurons in a forebrain vocal center of adult male canaries. J Neurosci 11:1756–1762.

Kirn JR, O'Loughlin B, Kasparian S, Nottebohm F (1994) Cell death and neuronal recruitment in the high vocal center of adult male canaries are temporally related to changes in song. Proc Natl Acad Sci USA 91:7844–7848.

Kirn JR, Fishman Y, Sasportas K, Alvarez-Buylla A, Nottebohm F (1999) Fate of new neurons in adult canary high vocal center during the first 30 days after their formation. J Comp Neurol 411:487–494.

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033.

Leonardo A, Konishi M (1999) Decrystallization of adult birdsong by perturbation of auditory feedback. Nature 399:466–470.

Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F (2000) A relationship between behavior, neurotrophin expression, and new neuron survival. Proc Natl Acad Sci USA 97:8584–8589.

Ling C, Zuo M, Alvarez-Buylla A, Cheng MF (1997) Neurogenesis in juvenile and adult ring doves. J Comp Neurol 379:300–312.

Lombardino AJ, Nottebohm F (2000) Age at deafening affects the stability of learned song in adult male zebra finches. J Neurosci 20:5054–5064.

Nordeen KW, Nordeen EJ (1988) Projection neurons within a vocal motor pathway are born during song learning in zebra finches. Nature 334:149–151.

Nordeen KW, Nordeen EJ (1992) Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. Behav Neural Biol 57:58–66.

Nottebohm F (1985) Neuronal replacement in adulthood. In: Hope for a

new neurology (Nottebohm F, ed), pp 143–161. New York: New York Academy of Sciences.
Nottebohm F (1989) From bird song to neurogenesis. Sci Am 260:74–79.
Nottebohm F (2002) Why are some neurons replaced in adult brain? J Neurosci 22:624–628.
Nottebohm F, O'Loughlin B, Gould K, Yohay K, Alvarez-Buylla A (1994) The life span of new neurons in a song control nucleus of the adult canary brain depends on time of year when these cells are born. Proc Natl Acad Sci USA 91:7849–7853.
Okanoya K, Yamaguchi A (1997) Adult Bengalese finches (*Lonchura striata var. domestica*) require real-time auditory feedback to produce normal song syntax. J Neurobiol 4:343–356.
Ottinger MA (2001) Quail and other short-lived birds. Exp Gerontol 36:859–868.
Price PH (1979) Developmental determinants of structure in zebra finch song. J Comp Physiol Psychol 93:260–277.
Rakic P (2002) Adult neurogenesis in mammals: an identity crisis. J Neurosci 22:614–618.
Rasika S, Nottebohm F, Alvarez-Buylla A (1994) Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. Proc Natl Acad Sci USA 91:7854–7858.
Rasika S, Alvarez-Buylla A, Nottebohm F (1999) BDNF mediates the effects of testosterone on the survival of new neurons in an adult brain. Neuron 22:53–62.
Saper CB (1996) Any way you cut it: a new journal policy for the use of unbiased counting methods. J Comp Neurol 364:5.
Scharff C, Kirn JR, Grossman M, Macklis JD, Nottebohm F (2000) Targeted neuronal death affects neuronal replacement and vocal behavior in adult songbirds. Neuron 25:481–492.
Scott LL, Nordeen EJ, Nordeen KW (2000) The relationship between rates of HVc neuron addition and vocal plasticity in adult songbirds. J Neurobiol 43:79–88.
Smithson K, MacVicar B, Hatton G (1983) Polyethylene glycol embedding: a technique compatible with immunocytochemistry, enzyme histochemistry, histofluorescence and intracellular staining. J Neurosci Methods 7:27–41.
Tekumalla P, Tontonoz M, Hesla M, Kirn JR (2002) Effects of thyroxine on cell death and cell proliferation in the adult zebra finch neostriatum. J Neurobiol 51:323–341.
Thorpe WH (1958) The learning of song pattern by birds, with especial reference to the song of the chaffinch, *Fringilla coelebs*. Ibis 100:535–570.
Vicario DS, Simpson HB (1988) Control of syringeal muscles in nucleus RA of zebra finches. Soc Neurosci Abstr 14:89.
Wang N (2000) The effects of cochlea removal and age on neuronal turnover in the high vocal center of adult male zebra finches. PhD dissertation, Wesleyan University.
Wang N, Aviram R, Kirn JR (1999) Deafening alters neuron turnover within the telencephalic motor pathway for song control in adult zebra finches. J Neurosci 19:10554–10561.
Woolley SM, Rubel EW (1997) Bengalese finches *Lonchura striata* domestica depend on auditory feedback for the maintenance of adult song. J Neurosci 17:6380–6390.
Zann RA (1996) The zebra finch: a synthesis of field and laboratory studies (Zann RA, ed), pp 139–156. New York: Oxford UP.