millivolts) +7.0 ± 2.2, +66.7 ± 6.2, and +76.0 ± 11.6, respectively. Data points for Li^+, however, did not fit well to the GHK equation (the real E_r appeared to be more negative); this and strong rectification upon depolarization may suggest blockage of the channel by Li^+. Channel blockage by permeant ions has been described [D. J. Adams, W. Nonner, T. M. Dwyer, B. Hille, *J. Gen. Physiol.* **78**, 593 (1981); S. Cukierman, G. Yellen, C. Miller, *Biophys. J.* **48**, 477 (1985); G. Eisenman, R. Latorre, C. Miller, *ibid.* **50**, 1025 (1986)].

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17. The channel activity (NPo) was derived from data >15 s either by multi-channel dwell time analyses or by amplitude histogram analyses. For the purpose of describing the activity of an unspecified number of channels, NPo is equal to the product of the number of channels and the probability of channel opening.
18. Wild-type CaM from *Paramecium* was prepared according to the method of T. J. Lukas, M. A. Wallen-Friedman, C. Kung and D. M. Watterson [*Proc. Natl. Acad. Sci. U.S.A.* **86**, 7331 (1989)], with minor modifications. The CaM concentration in *Paramecium* is estimated to be ~1 pg per 200 pl, or 0.3 μM [see the first reference of (7)]. Therefore, the concentration of CaM we used in this report was very close to the physiological concentration.
19. Channel reactivation was observed in ~70% of the trials (>20), including ones under slightly different conditions.
20. Y. Saimi and K.-Y. Ling, unpublished data. The identity of reactivated channels with respect to conductance, and selectivity for Na^+ over K^+, and Ca^{2+} dependence was confirmed in at least three experiments.
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Birth of Projection Neurons in Adult Avian Brain May Be Related to Perceptual or Motor Learning

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Projection neurons that form part of the motor pathway for song control continue to be produced and to replace older projection neurons in adult canaries and zebra finches. This is shown by combining [^{3}H]thymidine, a cell birth marker, and fluorogold, a retrogradely transported tracer of neuronal connectivity. Species and seasonal comparisons suggest that this process is related to the acquisition of perceptual or motor memories. The ability of an adult brain to produce and replace projection neurons should influence our thinking on brain repair.

THE SONG CONTROL SYSTEM OF OSCINE songbirds consists of several discrete nuclei including the high vocal center (HVC) and the robust nucleus of the archistriatum (RA) (*1*). These two brain regions form the backbone of the telencephalic efferent pathway that controls learned song. HVC is remakable in that new neurons continue to be added to it in adulthood (*2, 3*).

HVC includes two types of projection neurons: those that project to area X and those that project to RA (*1, 3*). Area X–projecting HVC neurons are produced during early development (*4*), as is also true for projection neurons in the brains of most warm-blooded vertebrates (*5*). In contrast, the majority of RA-projecting HVC neurons are added after hatching (*4, 6*). According to previous reports, the latter process slows down by the time birds reach sexual maturity (*4, 6*). We now explore the extent to which new RA-projecting neurons continue to be added to the adult HVC.

Twelve adult male canaries received injections of [^{3}H]thymidine (*7*), a marker of DNA synthesis, twice daily for 14 days. One-hundred and twenty-five days later (*8*), we injected fluorogold into the right and left nucleus RA by using stereotactic coordinates (*4*). These birds were killed 4 days later and their brains sectioned at 6-μm intervals; the sections were prepared for autoradiography and counterstained for anatomical detail (*9*). We scored the position and number of (i) neurons, (ii) fluorogold-backfilled neurons, (iii) [^{3}H]thymidine-labeled neurons, and (iv) double-labeled neurons (fluorogold plus [^{3}H]thymidine) within HVC with a computer-yoked microscope (*10*).

The cell bodies of HVC neurons backfilled with fluorogold stand out unambiguously from surrounding neuropil (Fig. 1A). Fluorogold injections outside, but in the vicinity, of RA failed to label HVC neurons. HVC cells backfilled from RA with fluorogold fall into a rather homogeneous size class [nuclear diameters of 6.18 ± 0.58 (mean ± SD)] and are generally smaller than area X–projecting cells (*3, 4*). The RA-projecting neurons constituted 54% of HVC neurons (*11*).

Many of the fluorogold-backfilled HVC neurons had nuclei labeled with [^{3}H]thymidine (Fig. 1B), indicating that they were born at the time of [^{3}H]thymidine treatment (*12*). Thus, some of the canary HVC neurons generated in adulthood became RA-projecting cells. Previous work in which horseradish peroxidase was used as a retrograde label in adult male canaries revealed very few new RA-projecting HVC neurons, and it was concluded that adult HVC neurogenesis was restricted almost exclusively to the formation of interneurons (*3*). Therefore, in our study we had to rule out the possibility that fluorogold transported retrogradely from RA to HVC passed from projection neurons to interneurons. We injected nucleus RA of an additional five adult male canaries with fluorescent rhodamine beads; these birds were killed 4 days later. Beads are trapped in intracellular organelles and do not leak out of the cell even after long survivals (*13*). The birds had been treated with [^{3}H]thymidine (*7*) and tissue was processed to preserve the fluorescent beads (*14*). Neurons double-labeled with silver grains over their nuclei and beads in their cytoplasm (Fig. 1, C and D) were found in all five canaries. These observations confirm that RA-projecting HVC neurons continue to be generated in adult canary brain.

Adult male canaries learn new songs seasonally (*15, 16*). To address the possible relationship between adult neurogenesis and song plasticity, we injected some canaries with [^{3}H]thymidine in May, when song is stable, and some in October, when song is modified (*7*). The percentage of HVC neurons that were [^{3}H]thymidine labeled was 4.9 times higher in the October group (mean, 3.8%; range, 1.6 to 6.1%) than in the May group (mean, 0.78%; range, 0.23 to 1.5%) (Fig. 2, a and b). Nevertheless, in both the May and October groups, more than half of the new neurons were backfilled with fluorogold (56% and 71%, respectively). Therefore, there were correspondingly more RA-projecting HVC neurons labeled with [^{3}H]thymidine in the October group than in the May group (Fig. 2, a and b). Moreover, in 4-year-old canaries injected with [^{3}H]thymidine in October, the number of new RA-projecting HVC neurons was also considerably higher than in the 1-year-old May group (Fig. 2, a to c) (*17*). Because of the 4-month delay between [^{3}H]thymidine administration and the time when the birds were killed, we do not know whether the lower numbers of new RA-projecting HVC neurons after [^{3}H]thymi-

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dine injections in May compared to October are due to a lower rate of cell production or a higher rate of cell death.

Zebra finches do not modify their songs in adulthood (*18*). As a further test of the relationship between adult neurogenesis and song plasticity, six adult male zebra finches (8 to 11 months old) (*19*) were treated with [^{3}H]thymidine and fluorogold following the same protocol as with the canaries. Forty-nine percent of the zebra finch HVC neurons were backfilled with fluorogold, a proportion very similar to that observed in canaries. Only 1.1% of the neurons in HVC were labeled with [^{3}H]thymidine and of these approximately one-fourth (23.4%) were backfilled with fluorogold (Fig. 2). Assuming similar survivorships of cells, the rate of HVC neuronal recruitment in adult zebra finches would seem comparable to that of adult canaries in May, when song is stable (Fig. 2). However, the proportion of new neurons that project to RA in adult zebra finches is about half that seen in adult canaries.

Circuits for song learning are put together at the very time song models are acquired and imitated (*16, 20*); during this period, neurogenesis is restricted to only some song control nuclei and some neuronal types (*4, 6, 21*). In adult male canaries, higher numbers of RA-projecting neurons with a survivorship of at least 4 months are recruited in fall, when song is being modified, than in spring, when song is stable. The greater number of these RA-projecting neurons added in the fall is probably related to an increase in song learning. However, neurons that survive 4 months are also added (albeit at lower rates) in adult canaries and zebra finches at times when song appears to change little or not at all. If the rate observed in adult zebra finches were to hold for the entire year, then the number of HVC neurons would increase by 30% over a 12-month period and that of RA-projecting neurons would increase by 15%. The purpose of neurogenesis at times when song is stable remains unknown.

HVC and RA may be involved not only with the production and modification of learned song, but also with its perception (*22*). Acquisition of new perceptual memories for song may require new RA-projecting neurons. Alternatively (or in addition), new RA-projecting neurons could influence other vocalizations such as calls (*2, 23*) that may be subject to subtle changes in adulthood.

Earlier work showed that new neurons are generated, migrate, and become incorporated into existing circuits of the adult canary brain (*2, 24*). We have now shown that some of these new neurons can project an axon over roughly 3 mm, the distance between HVC and RA, and link separate brain regions, for example, neostriatum and archistriatum. This process continues even in 4-year-old canaries. Since the ratio of RA-projecting HVC neurons to other HVC neurons does not change between September and February, and the size of HVC does not change from year to year (*25*), we infer that over the time of our study some RA-projecting neurons were discarded and replaced by new ones, indicating a significant degree of plasticity in a major motor pathway. These results should influence our view of the extent to which self-repair is possible in the adult central nervous system.

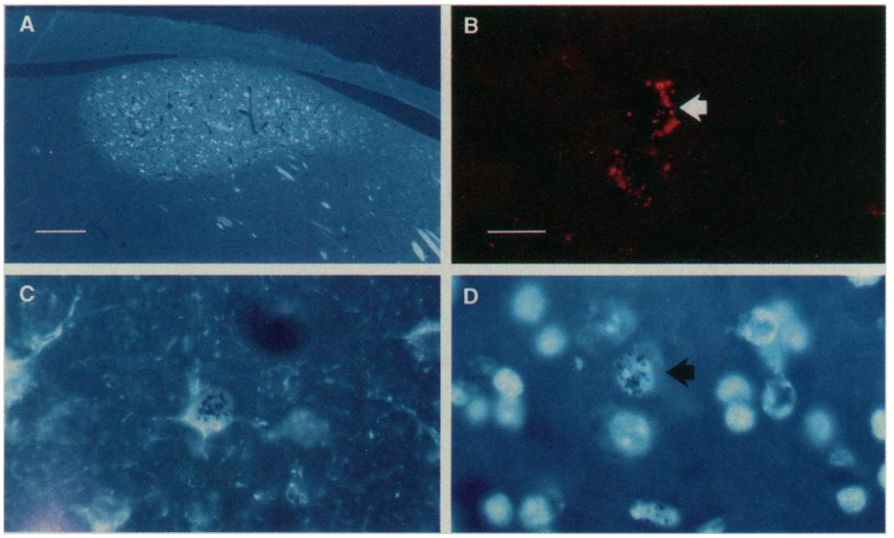

Fig. 1. [^{3}H]Thymidine-labeled RA-projecting HVC neurons. (**A**) HVC backfilled from RA with retrograde tracer fluorogold; there are discrete boundaries of HVC and fascicles of fluorogold-labeled axons that connect HVC and RA (ventro-lateral to HVC, lower right outside of this photomicrograph). (**B**) A fluorogold-backfilled neuron in HVC also labeled with silver grains (black spots) on its nucleus. (**C**) Two HVC neurons retrogradely labeled with fluorescent rhodamine beads injected into nucleus RA. (**D**) The same field as in (C), but viewed under different filter combination to reveal Hoechst 33258 nuclear staining. One of the neurons filled with fluorescent beads in (C) (arrow) is labeled with [^{3}H]thymidine (arrow) in (D). Scale bars: (A), 200 μm; (B) and (D) are as in (C), 10 μm.

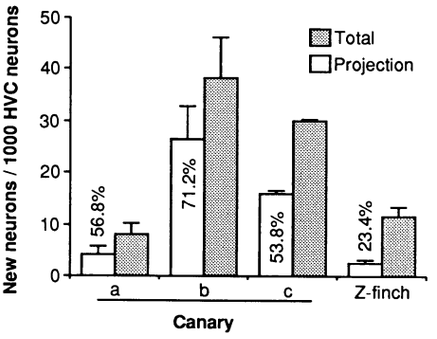

Fig. 2. Proportion of all HVC and RA-projecting HVC neurons labeled with silver grains on their nuclei (7). The numbers indicate the percentage of all labeled neurons that projected to RA in each group. (a) One-year-old canaries injected with [^{3}H]thymidine in May and killed in September (n = 4). (b) Canaries injected with [^{3}H]thymidine in October (16 to 18 months old) and killed in February (n = 6). (c) Four-year-old canaries injected with [^{3}H]thymidine in October (52 months old) and killed in February (n = 2). Zebra finches (Z-finch; n = 6) were 8 to 13 months old at the time of [^{3}H]thymidine treatment. Means ± SEM.

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7. Waterschlager canaries were kept under a New York State photoperiod. Under these conditions canaries became sexually mature approximately 8 months after hatching. [^{3}H]Thymidine (6.7 Ci/mM, New England Nuclear; 2.5 μCi per gram of body weight) was injected into the pectoral muscle. The experimental canary groups were injected with [^{3}H]thymidine in May (n = 4) at age 12 months, killed in September; injected in October (n = 6) at age 17 months, killed in February; and injected in October (n = 2) at age 53 months, killed in February at 57 months of age.
8. It takes 20 to 40 days for cells born in the ventricular zone of the adult canary forebrain to become recognizable neurons (*24*). Because axonal growth might

also take time, we chose a survival of 4 months to ensure that a stable population of neurons was in place.
9. Serial polyethylene glycol (PEG) sections were cut in the sagittal plane at 6-μm intervals (*4*). Sections were delipidized in ethanol-xylene-ethanol and coated with NTB2 (Kodak) nuclear tract emulsion. After 30 days of incubation at 4°C, the emulsion was developed in D19 (Kodak) for 3 min at 17°C. Sections were counterstained with fluorescent cresyl-violet (A. Alvarez-Buylla, C.-Y. Ling, J. R. Kirn, *J. Neurosci. Methods*, in press) and cover slips were mounted with Krystalon (EM Diagnostic Systems).
10. A. Alvarez-Buylla and D. S. Vicario, *J. Neurosci. Methods* **25**, 165 (1988). A cell was considered positive when seven or more grains were seen overlying the cell nucleus (>×20 background). ^{3}H-Labeled neurons and ^{3}H-labeled fluorogold–backfilled neurons were counted and mapped in ten noncontiguous HVC sections under a ×63 objective. The area sampled ranged from 3 to 5 mm^2 and 40 to 500 [^{3}H]thymidine-labeled neurons were counted per brain. In addition, all fluorogold-backfilled cells and unfilled neurons were counted in three HVC sections (sample 2000 to 3000 neurons per brain). Averages from left and right HVCs were treated as individual samples; only one HVC was used when the fluorogold injection on the other hemisphere was outside RA.
11. The proportion of RA-projecting neurons to all neurons was similar in all groups studied. Number of fluorogold-backfilled neurons divided by total neurons was: 0.51, for the canaries in the May group; 0.53, for the October 17-month-old group; 0.59, for the October 54-month-old group; 0.49 for the zebra finch group.
12. Cell labeling with [^{3}H]thymidine occurs during the first 60 min after intramuscular injection; no dividing cells become labeled after 90 min [A. Alvarez-Buylla, M. Theelen, F. Nottebohm, *Neuron* **5**, 101 (1990)].
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14. Brains that received fluorescent rhodamine beads were fixed with 3% paraformaldehyde, washed in phosphate-buffered saline, equilibrated in 30% sucrose, and frozen in Tissue-Tek (Miles Inc.). Sagittal, 6-μm frozen sections were thaw-mounted onto gelatinized slides and dried overnight at 4°C under vacuum. They were then rinsed in water and dried again, treated for 1 min in xylene, dried, rinsed in water, dried, and dipped in NTB2 (Kodak) emulsion. They were incubated and developed as were fluorogold sections (*9*), counterstained with Hoechst 33258 nuclear stain (Aldrich), and the cover slips were mounted with immersion oil.
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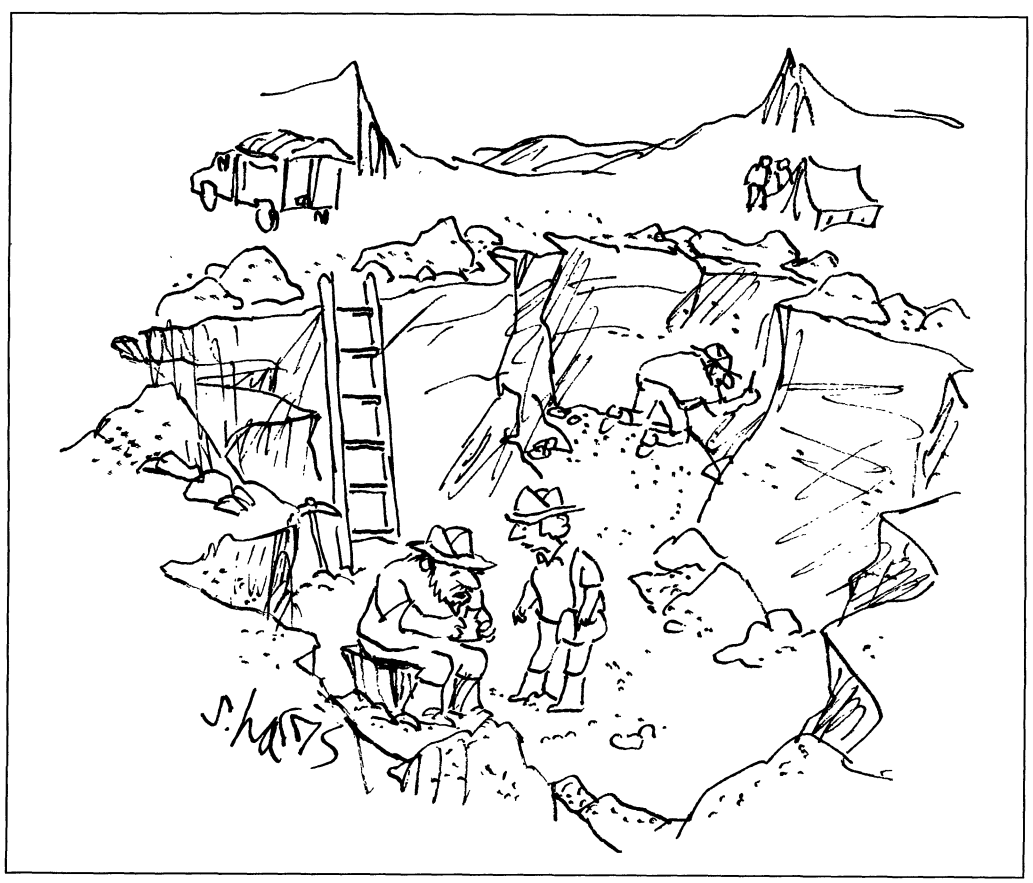

" From the variety of objects found here – cooking utensils, furniture, tools, games – and the multi-levels on which they are found, I'd say we have come across man's earliest shopping mall."