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Targeted Neuronal Death Affects Neuronal Replacement and Vocal Behavior in Adult Songbirds

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Summary

In the high vocal center (HVC) of adult songbirds, increases in spontaneous neuronal replacement correlate with song changes and with cell death. We experimentally induced death of specific HVC neuron types in adult male zebra finches using targeted photolysis. Induced death of a projection neuron type that normally turns over resulted in compensatory replacement of the same type. Induced death of the normally nonreplaced type did not stimulate their replacement. In juveniles, death of the latter type increased recruitment of the replaceable kind. We infer that neuronal death regulates the recruitment of replaceable neurons. Song deteriorated in some birds only after elimination of replaceable neurons. Behavioral deficits were transient and followed by variable degrees of recovery. This raises the possibility that induced neuronal replacement can restore a learned behavior.

Introduction

Neuron production, migration, and differentiation are major developmental events in warm-blooded vertebrates, but it is becoming increasingly clear that these processes—albeit on a smaller scale—can also extend into adulthood (Graziadei and Monti Graziadei, 1978; Bayer et al., 1982; Gould et al., 1992, 1999; Lois and Alvarez-Buylla, 1994; Eriksson et al., 1998). The phenomenon of adult neurogenesis has received particular attention in the high vocal center (HVC) of songbirds, a nucleus that is necessary for the production of learned song (Nottebohm et al., 1976; Simpson and Vicario, 1990; Williams et al., 1992). New neurons are constantly added to HVC, where they replace older neurons that have died (Nottebohm, 1985; Alvarez-Buylla et al., 1990a).

Most of the new HVC neurons added in adulthood extend axons 2–3 mm to the robust nucleus of the archistriatum (RA), thus becoming projection neurons in the motor pathway that controls the production of learned song, referred to subsequently as HVC→RA neurons (Alvarez-Buylla et al., 1988, 1990a; Kirn et al., 1991). A second type of HVC neuron projects to Area X (HVC→X neurons) and is almost exclusively generated in ovo; there is no evidence that the HVC→X neurons are ever produced in adulthood (Alvarez-Buylla et al., 1988, 1992; Gahr, 1990; Kirn et al., 1999) (Figure 1). The projection from HVC to Area X is part of an anterior pathway that is necessary for the acquisition but not for the production of learned song (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). It remains unknown why some neuron types in adult birds are replaced while others are not. Correlative evidence from songbirds (Alvarez-Buylla et al., 1990a; Kirn et al., 1991, 1994; Kirn and Nottebohm, 1993) and other systems (Negishi et al., 1982; Kaplan et al., 1985; Reh and Tully, 1986; Reh, 1987; Gould and McEwen, 1993; Macklis, 1993; Braisted et al., 1994; Sheen and Macklis, 1995; Calof et al., 1996; Hernit-Grant and Macklis, 1996; Snyder et al., 1997; Leavitt et al., 1999) suggests that recruitment of new neurons is regulated, in part at least, by prior neuronal death.

We applied chromophore-targeted neuronal degeneration, a procedure that biophysically induces apoptotic death in specific neuron populations (Macklis, 1993; Madison and Macklis, 1993; Sheen and Macklis, 1994), to test whether death-related events can cause the recruitment of new neurons in a system that shows spontaneous neuronal turnover in adulthood. In adult mouse cortex, where neuronal replacement has not been described, targeted neuronal degeneration induces developmental signals that direct transplanted neural precursors and immature neurons to migrate and differentiate in an appropriate manner (Macklis, 1993; Sheen and Macklis, 1995; Hernit-Grant and Macklis, 1996; Snyder et al., 1997; Wang et al., 1998; Leavitt et al., 1999).

We hypothesized that inducing neuronal death experimentally in the adult HVC would increase the incorporation of new neurons and that the death and replacement of neurons might affect song behavior. Our experiments dealt with HVC's two classes of projection neurons. We tested whether selective death of the HVC→RA neurons that normally turn over at low levels in adulthood was followed by an increased replacement of this neuron type. Previous work had shown that peaks in the recruitment of new HVC neurons were preceded by peaks in HVC neuronal death, but it was not clear that this relationship was causal (Kirn et al., 1994). We also investigated whether the targeted death of HVC→X neurons that normally do not turn over in adulthood induced replacement. In both cases, we explored consequences of the targeted neuronal death on song behavior.

We report here that in adult zebra finches the induced neuronal death of the HVC→RA neurons caused a significant upregulation in the number of new neurons of the same kind. In contrast, induced death of the HVC→X

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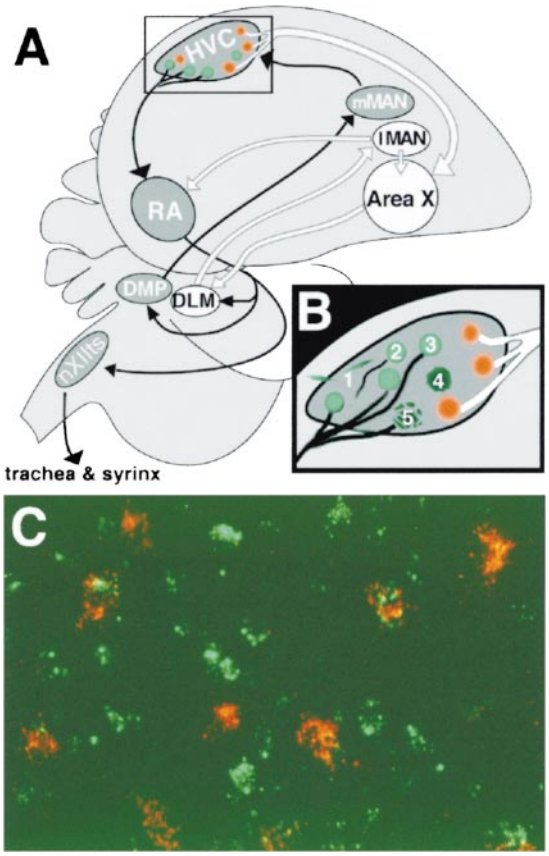

Figure 1. Overview of Song System Components

(A) Diagram of a sagittal view through the songbird brain, highlighting HVC and its connectivity, as well as relationships between other song nuclei. The two identified populations of projections neurons of HVC, those that project to Area X (red) and those that project to RA (green), are schematically indicated. HVC projects toward an anterior pathway (white nuclei, white arrows) and a posterior pathway (gray nuclei, black arrows). The anterior pathway is essential for song learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991); the posterior pathway is essential for production of adult song (Nottebohm et al., 1976; Simpson and Vicario, 1990).

(B) Neuronal replacement occurs in the HVC→RA projecting neurons (green) but not the HVC→X population (red). Newly generated neurons (1) migrate into HVC, where they incorporate, differentiate, and extend axons toward RA (2 and 3). Other HVC→RA neurons die (4) and disappear (5) (Alvarez-Buylla et al., 1990a).

(C) Double-exposed photomicrograph of a 20 μm section showing HVC→X neurons retrogradely labeled red by an injection of fluorescent rhodamine microspheres into Area X, and HVC→RA neurons retrogradely labeled green by an injection with fluorescent fluorescein microspheres into RA. Note that due to section thickness, an Area X projection neuron and a RA projection neuron can superimpose each other, resulting in some overlap of color. Note also that HVC→X neurons are larger but fewer in number than HVC→RA neurons.

neurons in adults or juveniles did not result in the recruitment of new HVC→X neurons. Surprisingly though, targeted death of HVC→X neurons in juveniles increased the number of new HVC→RA neurons. Adult song production was not affected by elimination of HVC→X neurons, but elimination of HVC→RA neurons often induced song deterioration. Subsequent improvement was concurrent with increased recruitment of HVC→RA neurons.

Results

We induced bilateral cell-type specific neuronal degeneration in young and adult zebra finch males by photoactivation of retrogradely labeled HVC projection neurons, using a 674 nm laser. The retrograde label consisted of chlorin e_6–conjugated nanospheres that were injected either into Area X or into RA, thus labeling either HVC→X neurons or HVC→RA neurons. The 674 nm light activates chlorin e_6 to release cytotoxic singlet oxygen, which results in apoptosis of the targeted neurons. The procedure spares all cells that are not labeled as well as those that have taken up the chlorin e_6–conjugated nanospheres but lie outside the region over which the laser is focused (Macklis, 1993; Sheen and Macklis, 1995).

Three control groups were included: (1) a group of birds was injected with chlorin e_6 nanospheres but was not subsequently illuminated with the laser; (2) another group of birds received the laser illumination but no chlorin e_6 nanospheres; (3) a third, intact, group received neither nanospheres nor laser. To monitor cell birth, [^{3}H]thymidine was injected every other day systemically for 10 days, starting on the second day after laser illumination. Three months later, the retrograde tracer Fluoro-Gold (FG) was injected into the same target that had previously been injected with chlorin e_6. This FG injection allowed us to assess how many HVC neurons projected to this target at the time of perfusion, including any neurons that had escaped killing by the targeted photolysis as well as any new neurons that replaced those that were killed by targeted photolysis. The latter cell type could be positively identified by the combined FG and [^{3}H]thymidine label. Song was recorded before and after each experimental manipulation. A summary of the experimental paradigm is schematized in Figure 2. Group sizes, ages of subjects, and treatment times are listed in Table 1.

Enhanced Neuronal Incorporation after Targeted HVC→RA Neuronal Death

There was a 3-fold increase in the number of new HVC→RA neurons in the experimental group compared to the average of the three control groups (Figure 3C). The total number of newly recruited HVC neurons, including interneurons (not labeled with FG), was two times higher in the experimental birds than in the controls (Figure 3D). These increases reflect only those neurons that were born during the first 10 days after induction of neuronal death and survived for an additional 3 months. If the maximal effect of targeted neuronal degeneration on neuronal recruitment did not coincide with the timing of our [^{3}H]thymidine injections, one would expect even larger effects. The new projection neurons appeared to replace those that died; there was no significant difference between the numbers of retrogradely labeled HVC→RA neurons in the experimental birds and in the three control groups at the end of the experiment (Figure 3E). Theoretically, the lack of differences in numbers of retrogradely labeled HVC→RA neurons between experimental and control animals could also mean that the targeted neuronal degeneration was not successful, which would, however, leave unexplained the 3-fold increase in the number of [^{3}H]thymidine-labeled HVC→RA

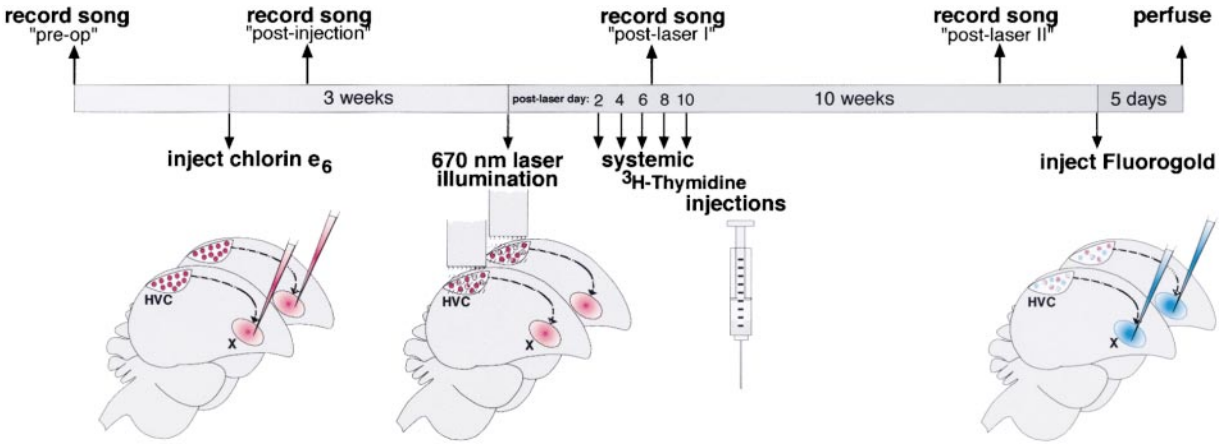

Figure 2. Schematic Timetable of Experimental Design

Song was recorded at the beginning of the experiments involving adult birds. After bilateral injection of the chlorin e_6 nanospheres into either Area X (as depicted) or RA, song was recorded again to assure that no behavioral changes had occurred due to potential tissue damage at the injection site. After allowing sufficient time for retrograde transport of chlorin e_6, HVC was noninvasively illuminated with long wavelength 674 nm laser light, photoactivating neuronal death only in chlorin e_6–labeled neurons. To monitor subsequent neurogenesis, [^{3}H]thymidine was injected five times over a 10 day period, starting with the second day after the laser illumination. During a survival period of 3 months, newly generated neurons were incorporated into HVC. During this time, short-term and long-term effects of the induced neuronal death on song were monitored through song recording. To document new neurons as well as projection neurons that survived, FG was injected 5 days before perfusion into the same target that had previously received chlorin e_6 injection.

neurons. Failure of the procedure to induce neuronal death seems unlikely for several additional reasons: (1) experiments performed in parallel, killing the HVC→X neurons both in juveniles and adults (reported below), directly proved the effectiveness of the method in birds; in the absence of compensatory neuronal replacement, the number of HVC→X neurons was significantly reduced at the end of the experiment; (2) some birds were perfused soon after the laser illumination and numerous apoptotic bodies were observed in HVC (Figure 3A); (3) targeting of RA with chlorin e_6 nanospheres was accurate (Figure 3B); and (4), importantly, song production was often impaired after the procedure intended to induce death of the HVC→RA neurons (described below). These results strongly argue that the initial neuronal death was efficient and that neuronal replacement had in fact compensated for the neuronal loss.

Song Deterioration and Recovery after Induced Death of Adult HVC→RA Neurons

Song deteriorated strikingly in some animals during the first week after the targeted death of HVC→RA neurons. Control birds that received only laser illumination showed no such changes. To control for potential song changes stemming from tissue damage in RA caused by the injection of chlorin e_6, we recorded song from all animals after RA injections and before laser illumination. Only animals in which the RA injections had no or minor effects on song stereotypy were used for comparison between control and experimental birds (compare Figures 4A and 4B; Table 2). Thus, song deficits observed after laser illumination of the animals injected with chlorin e_6 are specific to the induced death of the HVC→RA neurons. In those experimental birds that did show song deterioration (four out of nine), the effect was noticeable by 4 days after induction of targeted neuronal death; the note morphology had degraded and the rendition of songs was more variable (one example shown in Figures 4B and 4C; quantitative group data listed in Table 2). To human observers, deteriorated songs sounded like "mumbled stammering." We subsequently quantified three aspects of song stereotypy: (1) how many different ways notes are ordered into motifs (linearity), (2) how often each particular motif is sung (consistency), and (3) how often each note appears (H value). After induction of neuronal death, the group mean scores of all three measures were lower than before the induced death (Table 2, boldface numbers), indicating that the songs of this group had become less stereotyped. However, the behavioral deficit of this group was very heterogeneous, affecting only morphology (one bird); morphology, linearity, and/or consistency (three birds); or none of the above (five birds) (Figure 5). None of the animals in the two control groups, only injected with chlorin e_6 ($n = 4$) or only laser illuminated ($n = 2$), showed comparable qualitative or quantitative changes in song during the course of the experiment. It is of interest that the four birds that did respond to the induced death with song deterioration and the five birds that did not had, on average, equivalent numbers of newly incorporated HVC→RA neurons (mean of 383/mm^3 for birds with deteriorated song versus mean 392/mm^3 for birds with intact song), as well as equivalent numbers of total HVC→RA neurons (40,308 neurons/mm^3 versus 37,249 neurons/mm^3, $p > 0.05$), suggesting that targeted neuronal death had been effective and had been compensated for by neuronal replacement even in the animals whose song remained stable. However, we could not directly measure the magnitude of the initial reduction of these neurons without terminating the long survival behavioral analysis (see below), and therefore we cannot yet fully characterize how neuronal death interacted with song behavior.

Table 1. Number of Birds, Ages, Retrograde Transport Times, and Recordings (in days)

Anatomical Target of Chlorin e_6 Injections	Treatment	Number of Birds	Ages				Recordings				
			At Chlorin e_6 Injection	At Laser	At Last [^{3}H]thymidine Injection	At First Post-laser Recording	After Chlorin e_6 Injection	First after Laser	Second after Laser	Retrograde Transport of Chlorin e_6 before Laser	Survival after Last [^{3}H]thymidine Injection (weeks)
Area X (Adults)	chlorin e_6 only	4	adult			adult	20[2]				
	chlorin e_6 + laser	7	adult	adult	adult	adult	4[1]	9[8]	66[7]	16[5]	10[1]
	laser only	2	adult	adult	adult						9[0]
Area X (Juveniles)	chlorin e_6 only	7	37[6]		59[5]	94[6]					12[0]
	chlorin e_6 + laser	6	34[6]	45[4]	55[4]	90[6]				10[1]	12[0]
	laser only	2		50[2]	68[18]	99[15]					12[0]
	intact	6			60[2]	97[1]					12[0]
RA (Adults)	chlorin e_6 only	10	adult		adult	adult	7[1]				13[0]
	chlorin e_6 + laser	13	adult	adult	adult	adult	10[3]	4[1]	64[5]	23[3]	12[1]
	laser only	2	adult	adult	adult	adult		2[1]	66[0]		15[0]
	intact	5	adult		adult						15[1]

Not all birds qualified for all analyses, i.e., effectiveness of induced neuronal death, song behavior, HVC volumes, and neuron addition. For numbers used per analysis, refer to text.
All birds were recorded at all three recording times; control animals (chlorin e_6 only, laser only, intact) were recorded for each session at equivalent times as the experimental birds.
All adults were older than 120 days at the beginning of the experiments.
Numbers are means; numbers in brackets are standard deviations of the mean.

Surprisingly, three months after induction of neuronal death, the song scores of the experimental group (n = 9) were closer to those of prelaser values, reflecting improved song stability (Table 2). Like the initial song deficits after induction of death, improvements were also heterogeneous (Figure 5): one bird completely recovered his preoperative song motifs (compare Figures 7A and 7D), two birds sang stereotyped motifs that were different from those of their preoperative songs, and one bird improved only the morphology of notes. In this admittedly small group (n = 4), higher levels of neuronal replacement had a tendency to be associated with greater improvements in song linearity ($R^2 = 0.7$, $p = 0.07$; data not shown). Song did not improve over time in animals that had injection-related tissue damage to RA without subsequent laser treatment, suggesting that recovery from RA-related damage does not occur.

Neuronal Recruitment and Song Production Are Unaltered by Induced Death of HVC→X Neurons in Adults

The effect on neuronal recruitment in adults was specific to the induced death of HVC→RA neurons, as the induced death of HVC→X neurons had no such effect; equivalent numbers of ^{3}H-labeled neurons were present in the HVC of experimental and control animals, presumably reflecting the naturally occurring baseline addition of new neurons (Figure 6E). We never encountered any ^{3}H-labeled, FG-filled HVC→X neurons, suggesting that the newly generated neurons following HVC→X neuronal death (Figure 6D) were HVC→RA neurons or interneurons. Since no compensatory neuron incorporation was observed in this set of animals, the efficacy of the induced neuronal death could be directly demonstrated by a mean reduction of 60% in the number of the retrogradely labeled HVC→X neurons compared to controls (Figures 6A–6C). Thus, the death of HVC→X neurons or its sequelae did not induce the replacement of lost neurons of this particular class.

Previous work has shown that electrolytic lesions of Area X in adults have no apparent deleterious effects on song production (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Thus, HVC→X neurons also might not be required for song production. On the other hand, many HVC→X neurons closely appose HVC→RA neurons, forming neuronal clusters (Holzenberger et al., 1997; Kirn et al., 1999), and selective removal of HVC→X neurons from these clusters could have influenced the function of HVC→RA neurons in song production.

Visual comparison of sonograms showed no substantial differences between the songs recorded before and after injection of chlorin e_6 nanospheres into Area X, nor after subsequent induction of HVC→X neuronal death (Figure 6F). Indeed, detailed song analysis for both experimentals and controls established that none of the song parameters measured differed significantly across recordings (Table 3).

Induced Death of HVC→X Neurons in Juveniles Upregulates Recruitment of HVC→RA Neurons

We wondered whether age of the birds was a factor in the failure to bring about incorporation of new HVC→X

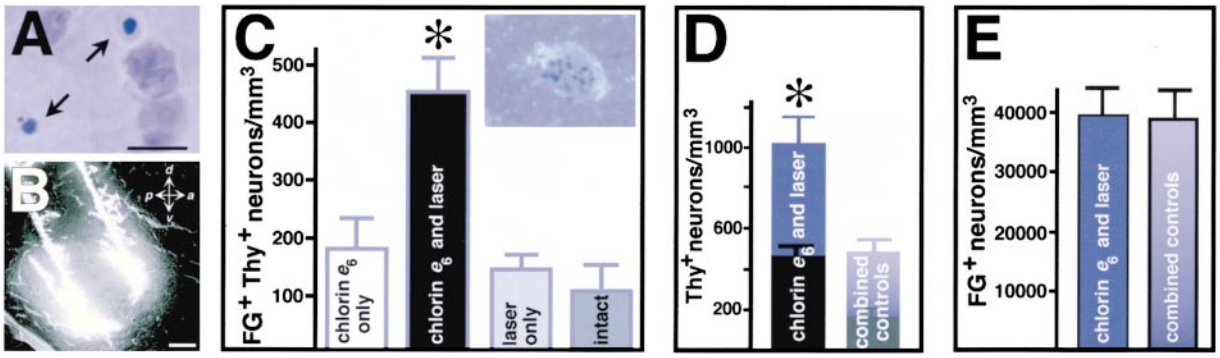

Figure 3. Induced Death of the HVC→RA Neurons in Adults Upregulates Incorporation of New Neurons into HVC

(A) Numerous apoptotic bodies (arrows) were seen in HVC 1 week after initiation of death of the HVC→RA neurons.

(B) Injection site of chlorin e_6 into RA in a sagittal section. This injection did not cause song impairments. RA can be seen as a faint egg-shaped region slightly brighter in fluorescence than the surrounding tissue. One injection is located in the anterior (a) pole of RA, the other is located in the central/ventral (v) part of RA. Other abbreviations: d, dorsal; p, posterior.

Scale bars: 10 μm (A) and 100 μm (B).

(C) The number of newly recruited HVC→RA projection neurons (double-labeled with [^{3}H]thymidine and FG; inset) was significantly higher after targeted neuronal death of the HVC→RA neurons (black bar) than in the three control groups ($n = 6$ hemispheres for white, 18 for black, 4 for light gray, and 4 for dark gray; ANOVA, $p = 0.012$, F4.3). The control groups were not significantly different from each other ($p > 0.05$). There was no significant difference in HVC volumes among groups (not shown).

(D) In addition to the increase of projection neurons shown in (C), there were also significantly more total [^{3}H]thymidine-labeled HVC neurons in the experimental group (black bar, $n = 15$) than in the combined control group (gray bar, $n = 14$), suggesting that more interneurons were also added to HVC after the induction of HVC→RA neuronal death (unpaired, two-tailed t test, $p = 0.004$). The bars outlined in purple indicate the fractions of the newly recruited [^{3}H]thymidine neurons that were also labeled with FG, i.e., HVC→RA neurons (same data as in [C]).

(E) The total number of FG-labeled HVC→RA neurons did not differ between the experimental and control groups at the end of the experiment, suggesting that the newly generated neurons (C and D) had in fact compensated for the selective neuronal death. Control groups (chlorin e_6 only, laser only, intact) were not significantly different from each other and are shown as a combined group.

neurons after induced neuronal death. Favorable conditions for axonal outgrowth toward Area X might cease to exist after HVC→X development is completed, between ~40 and 54 days of age (Burek et al., 1993, Soc. Neurosci., abstract; Nordeen and Nordeen, 1997). Molecules transiently expressed or upregulated during development (reviewed by Clayton, 1997; Holzenberger et al., 1997; Akutagawa and Konishi, 1998) could contribute to those favorable conditions.

We tested whether targeted death of HVC→X neurons during the time of ongoing HVC→X axon outgrowth would provide a favorable context for replacement of this neuron type, even though the normal generation of HVC→X neuronal precursors is largely completed before hatching (Alvarez-Buylla et al., 1988). Indeed, in juveniles, there was a significant increase in the number of new HVC neurons (Figure 7C, second bar) after the selective reduction of HVC→X neurons (Figure 7A). However, when we analyzed whether the new neurons had projected axons to Area X (retrogradely filled with FG) or to RA (retrogradely filled with green fluorescein microspheres; inset in Figure 7C), no new neurons were found projecting to Area X. Instead, many new neurons projected to RA, and significantly more so in the group that had undergone targeted neuronal death (Figure 7C, green bars). In this group, there were also significantly more new neurons that were not retrogradely labeled, suggesting that they were interneurons (Figure 7C, black bar). Further evidence that the increased addition of new neurons to HVC resulted from targeted neuronal death is the observation that the magnitude of the neuronal addition was inversely related to the number of HVC→X neurons still present; the more HVC→X neurons underwent induced death in the experiment, the more new neurons were encountered in HVC (Figure 7D). This relationship was significant (see Figure 7 legend).

Discussion

Our first goal was to address the role of neuronal death in the regulation of neuronal replacement. We tested directly whether neuronal death causes an increase in the recruitment of new neurons and whether the effects of death on replacement depend on age and cell type. We found that targeted death of the HVC→RA neurons upregulated the recruitment (which includes survival) of new neurons of this kind. In contrast, we observed no cell type–specific replacement after HVC→X neurons were killed either in juveniles or in adults. Apparently, the absence of recruitment of new HVC→X neurons in intact birds after hatching is not determined by a lack of death of HVC→X neurons.

Our second goal was to dissect the contribution that different sets of HVC projection neurons make to adult song behavior. We show that even though these two neuron types are intermingled in HVC and often apposed in tight clusters (Burd and Nottebohm, 1985; Holzenberger et al., 1997; Kirn et al., 1999), the induced death of more than half of all HVC→X neurons had no apparent deleterious effect on song production. This suggests an unexpected functional independence of the HVC→RA neurons from a majority of their neighboring HVC→X neurons. In contrast, induction of HVC→RA neuronal death resulted in highly deteriorated song in a subset of the birds. Surprisingly, the song impairment recovered

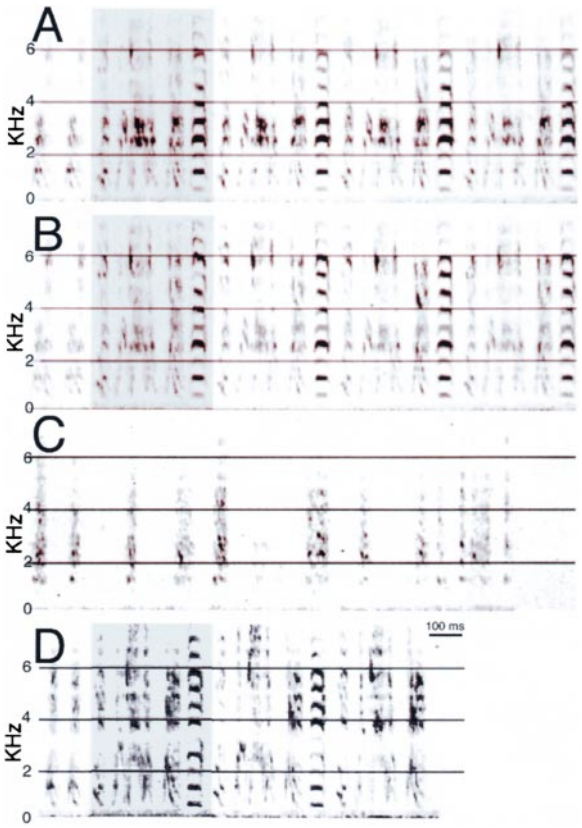

Figure 4. Induced Death of the HVC→RA Neurons in Adults Can Result in Reversible Song Degradation

Examples of song from one bird before (A) and 7 days after (B) chlorin e_6 injection, 3 days after induced neuronal death (C), and 9 weeks after initiation of neuronal death (D). Motif (highlighted) structure and sequence of notes are essentially identical before and after chlorin e_6 nanosphere injection, demonstrating that the injection itself has not impaired RA function. After subsequent laser illumination and initiation of neuronal degeneration, song production is grossly aberrant. Surprisingly, recovery of song that was identical in all characteristics was observed 9 weeks later. Other birds showed partial recovery of songs.

partially or completely after 2 months. We do not know whether this recovery was causally related to the coincident upregulation of neuronal replacement that was observed in this group.

The variability in the behavioral consequences of targeted death of HVC→RA neurons is puzzling. This variability could have been due to differences in the exact placement of the chlorin e_6 injections into RA or due to properties of the birds' learned song. Some songs may have been more vulnerable to partial loss of HVC→RA neurons than others, depending on the complexity of their sounds and how difficult they were to produce. Moreover, characteristics of injection placement and of the song may have interacted to determine a particular behavioral outcome. Our analysis of the data revealed no simple correlation between site of injection or song properties and the vulnerability of the song to targeted neuronal death; this is a topic much in need of future attention.

Table 2. Song Parameters Quantified after Induced Death of HVC→RA Neurons in Adults

Chlorin e_6 Only (n = 6)					Chlorin e_6 + Laser (n = 9)				Laser Only (n = 2)		
	Recording 1 Preinjection	Recording 2 Postinjection	Recording 3 (n = 5) Post Injection 2	Recording 4 (n = 5) Post Injection 3	Recording 1 Preinjection	Recording 2 Postinjection	Recording 3 Post Laser 1	Recording 4 Post Laser 2	Recording 1 Prelaser	Recording 3 Post Laser 1	Recording 4 Post Laser 2
Linearity	0.56 ± 0.08	0.56 ± 0.07	0.51 ± 0.06	0.51 ± 0.09	0.70 ± 0.11	0.64 ± 0.10	**0.57 ± 0.17**	0.66 ± 0.11	0.50 ± 0.00	0.60 ± 0.41	0.62 ± 0.16
Consistency	0.83 ± 0.08	0.88 ± 0.04	0.82 ± 0.10	0.86 ± 0.07	0.92 ± 0.02	0.90 ± 0.04	**0.78 ± 0.03**	0.87 ± 0.07	0.87 ± 0.04	0.81 ± 0.20	0.82 ± 0.16
H Value	2.31 ± 0.50	2.35 ± 0.53	2.28 ± 0.54	2.29 ± 0.54	2.39 ± 0.45	2.38 ± 0.493	**2.19 ± 0.63**	2.40 ± 0.41	2.16 ± 0.95	2.27 ± 1.03	2.27 ± 1.03

Numbers are group means, errors are standard deviation, and an average of 48 ± 19 motifs per bird were analyzed from each recording session.
Recordings 1,2,3, and 4 were made at equivalent times (relative to the first recording) in all groups.
Scores at the four recording times were compared for each group with repeated measures ANOVA; chlorin e_6 + laser group: linearity $p = 0.02$, consistency $p = 0.01$, H values $p = 0.07$; scores in other groups were not significantly different.
Number of notes per motif and number of introductory notes were also quantified but did not differ significantly and are not listed.

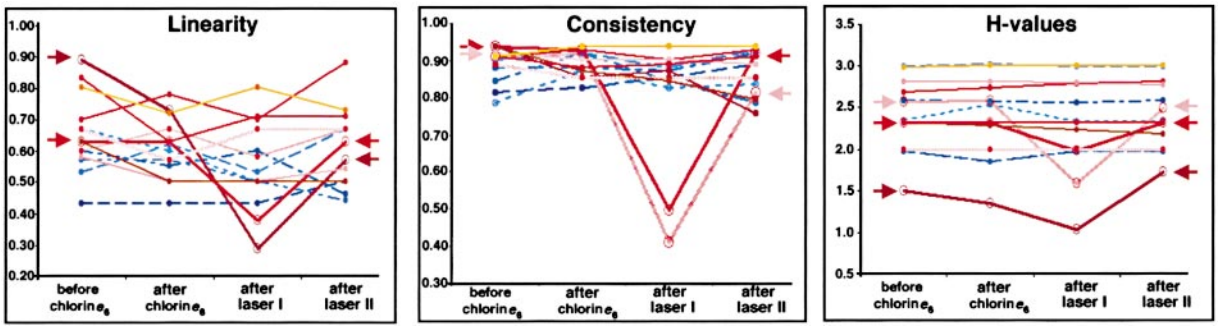

Figure 5. Behavioral Effects of Induced Death of the HVC→RA Neurons in Adults Are Heterogeneous

(A) Song linearity, consistency, and H values are plotted for individual control birds that were only injected with chlorin e_6 nanospheres (broken lines in shades of blue) and the experimental animals that were also illuminated with laser (solid lines in shades of red). Each line represents the values for one bird across the four different recording times, spanning a total of 5 months. Linearity, consistency, and H values of control birds (and most experimental birds) varied little, indicated by flat lines (average standard deviation of the control birds was 0.6, 0.5, and 0.5 for the three scores, respectively). Those experimental birds whose standard deviation was more than twice the average standard deviation of the control birds are indicated with arrows and open symbols at each recording point. Note that one bird (red line and arrows) had a major drop in linearity and consistency scores after the induced neural death, whereas two other birds (pink and maroon) show impairments in only one of those measures. All three birds had a drop in H value scores after the induced neuronal death, indicating that some notes appeared less frequently than before. Also note that those experimental birds that did show a dramatic decrease in the quantified parameters improved by the second recording after the induced neuronal death.

The Role of Neuronal Death in the Regulation of Neuronal Replacement

It has long been intriguing why of the two types of projection neurons present in the adult HVC, HVC→RA neurons undergo adult turnover, whereas HVC→X neurons do not. Similarly, in the few other systems where spontaneous adult neurogenesis has been described, only a subset of neuronal phenotypes seem to be generated (Hinds, 1968; Kaplan et al., 1985; Cameron et al., 1993). This limited diversity in new neuron types could be due to lineage restriction (intrinsic cues) of adult neuronal precursors, or to limits imposed by the local microenvironment into which new neurons incorporate (extrinsic cues). Lineage restriction of neuronal precursors is well documented during the development of the invertebrate and vertebrate central and peripheral nervous system (McConnell, 1995; Morrison et al., 1997; Doe et al., 1998; Shen et al., 1998; Cepko, 1999). In many cases, developmental age seems to be critical in determining the relative contribution of intrinsic versus extrinsic cues toward final neuronal fate. Among the environmental factors determining what types of neurons can be generated in adulthood could be a restriction of the type of neurons that die. There is good correlative data (Alvarez-Buylla et al., 1990a; Kirn et al., 1991, 1994; Kirn and Nottebohm, 1993; Calof et al., 1996; Gould and Cameron, 1996) and some direct evidence (Bengzon et al., 1997; Gould and Tanapat, 1997; Snyder et al., 1997) that cell death triggers compensatory neuron addition. In those cases, the specificity of replacement could be induced by the nature of signals directly produced by the dying cells or by the type of vacancy left behind by the dying cells. The notion of a vacancy may be simplistic, but is meant to stand for the possibility that when a cell dies it leaves behind anatomical and chemical consequences that are characteristic of the type of cell lost, thereby influencing its replacement. Such consequences could include both permissive and/or instructive signals. Whether those influences act on the generation of neurons in the ventricular zone or whether they act on the migration, incorporation, or survival of neurons in HVC remains to be determined.

The absence of replacement of HVC→X neurons in our experiment indicates that apoptotic death of the HVC→X neurons is apparently not a sufficient signal to induce cell-specific replacement. Of course, we can only assert this for the neurons that were birth-dated during the first 10 days after the induction of death and that survived the ensuing 3 months. Whether manipulation of the molecular environment in HVC could induce newly arriving neurons to differentiate into neurons of the type that is not usually replaced awaits future experimentation. Since the HVC→X neurons grow their axons toward Area X during the very time when we did our experiments in juveniles (Burek et al., 1993, Soc. Neurosci., abstract; Nordeen and Nordeen, 1997), it seems unlikely, though, that the failure to produce new HVC→X neurons was solely determined by conditions that precluded axonal growth toward Area X.

The outcome of our experiments with juveniles was surprising; as in adults, we failed to induce the recruitment of new HVC→X neurons, but instead the loss of HVC→X neurons was followed by an increased incorporation of new HVC→RA neurons and interneurons. This suggests that in juveniles, in contrast to adults, vacancies created by death of HVC→X neurons can be colonized by HVC→RA neurons. One reason why this might be so is that in juveniles the newly generated HVC→RA neurons may still find unoccupied target space in RA, while in adults this space is presumably fully occupied. Alternatively, the HVC→RA neurons of juveniles might be more dependent for their survival on trophic support provided by the HVC→X neurons—e.g., IGF-II (Holzenberger et al., 1997) and retinoic acid (Denisenko et al., 1997, Soc. Neurosci., abstract). Targeted death of

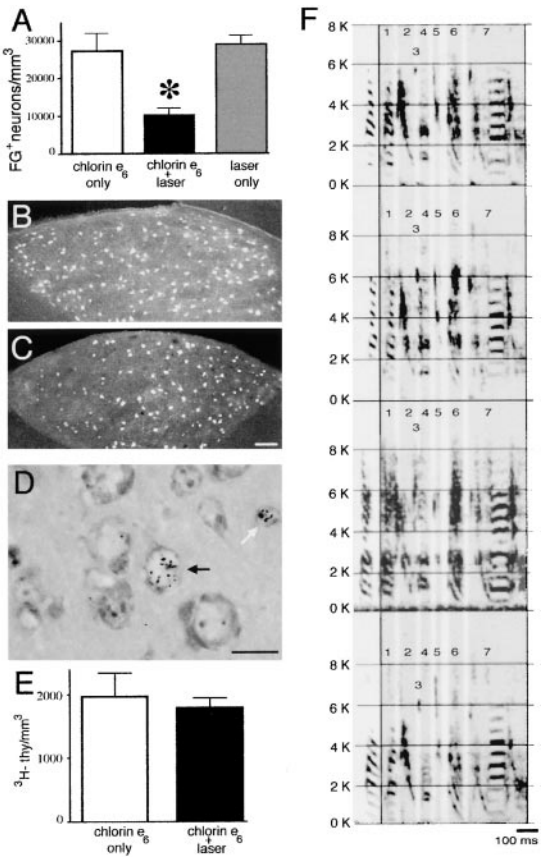

Figure 6. Induced Death of the HVC→X Neurons in Adults Affects Neither Song Production Nor Incorporation of New Neurons into HVC

(A) Significantly fewer FG-labeled HVC→X neurons were counted in the group that was injected with chlorin e_6 nanospheres and illuminated with laser (black bar, n = 7) than in the chlorin e_6–only group (white bar, n = 4) or the laser-only group (light gray bar, n = 2). Error bars are SEM (Kruskal-Wallis, H = 9.2, p = 0.01). HVC volumes did not differ significantly between experimental and control birds (data not shown; p = 0.6, Mann-Whitney U test, Z = −0.463, n = 2 and 4, respectively).

(B) Representative 14 μm section through HVC of a control bird that was injected with chlorin e_6 only and photographed under UV illumination, showing 153 FG-labeled (white) HVC→X neurons.

(C) HVC section of an experimental bird that was injected with chlorin e_6 nanospheres and later illuminated with laser shows only 70 retrogradely labeled neurons.

(D) Cresyl violet–counterstained autoradiograph of a 14 μm HVC section, showing a typical neuron (large, pale cytoplasm and prominent nucleoli) labeled with [^{3}H]thymidine (black grains, black arrow) and a smaller, heterochromatin-rich labeled glial cell (white arrow).

(E) Neuron addition to HVC after laser illumination was equivalent in the experimental group that received chlorin e_6 injections before the laser (black bar, n = 3) and the control group that received laser illumination only (gray bar, n = 2; p = 0.8, Mann-Whitney test, Z adjusted for ties = −0.286).

(F) Representative sonograms of a song motif consisting of seven notes sung by a 2.8-year-old male before injection of chlorin e_6 (first panel), 5 days after injection of chlorine e_6 (second panel), 6 days after induced neuronal death (third panel), and 61 days after induced neuronal death (fourth panel). Frequency (kHz) is plotted on the y axis; time (ms) is plotted on the x axis. The order, timing, and appearance of notes is essentially unchanged by either injection of tracer (second panel) or death of neurons (third and fourth panels).

Scale bars: 50 μm (B and C), 10 μm (D), and 100 μm (F).

HVC→X neurons may reduce, perhaps temporarily, the supply of such trophic substances, resulting in the death of some of the HVC→RA neurons and thus secondarily fostering their replacement. Such a mechanism could provide a common explanation for why HVC→RA neuron addition was upregulated by the induced death of HVC→X neurons in juveniles and by the death of HVC→RA neurons in adults. Future experiments could distinguish between these possibilities by combining targeted neuronal death with manipulation of growth factors in HVC or RA.

In the mammalian hippocampus, where only granule neurons are added during adulthood, excitotoxic cell death of granule neurons upregulates proliferation of neuronal precursors in the subjacent subgranular and the hilar zone (Gould and Tanapat, 1997). The new granule cells are inserted at the bottom of the granule layer and gradually move up as new cells are inserted from below (Gould and Cameron, 1996). However, when other hippocampal cell types that are not normally produced in adulthood are killed by the excitotoxic lesion, they are not replaced, a situation that parallels our findings. It is important to notice, though, that these two apparently similar experiments are not equivalent because of the difference in geography; in HVC, newly immigrating neurons had equal access to the vacancies created by induced death of HVC→RA neurons or of HVC→X neurons. Because of the layered organization of the hippocampus, newly immigrating neurons there did not have equal access to the different layers where exitotoxic death was induced.

Two other experimental paradigms, however, suggest that targeted neuronal death can result in replacement of neurons that do not normally die. In the tadpole retina, selective ablation of either of two neuron types that do not normally die results in differentiation of multipotent retinal precursors that specifically replace the ablated cell types (Reh and Tully, 1986; Reh, 1987). In the adult mouse neocortex, induced apoptotic death of the pyramidal neurons in layers II/III causes transplanted immature neurons and multipotent neural precursors to selectively differentiate into pyramidal neurons and replace some of the killed neurons (Macklis, 1993; Sheen and Macklis, 1995; Hernit-Grant and Macklis, 1996; Snyder et al., 1997; Leavitt et al., 1999), although adult cortical neurons have not been observed to undergo replacement in mice. Reexpression of developmental control molecules normally downregulated in the adult cortex may play permissive and instructive roles in this process (Wang et al., 1998; Leavitt et al., 1999; Sheen et al., 1999).

Behavioral Recovery after HVC→RA Neuronal Death and Relationship to Neuronal Replacement

Functional recovery after injury to the central nervous system of higher vertebrates is usually quite limited. We observed a remarkable recovery of deficits in song production after death of the HVC→RA neurons in some animals but cannot tell from our data whether this recovery depended on the accompanying upregulated neuronal replacement. A detailed analysis of the time course of song recovery will be necessary to determine whether these two events are causally related. Since the majority

Table 3. Song Parameters in Experiment on Ablation of HVC→X Neurons in Adults

	Chlorin e_6 Only (n = 2)			Chlorin e_6 + Laser (n = 3)		
	Before Injection	1 Week after Injection	13 Weeks after Injection	Before Injection	1 Week after Injection	10 (11) Weeks after Laser (injection)
Number of notes	7.5 ± 0.71	7.5 ± 0.71	7.5 ± 0.71	8.5 ± 1.0	8.5 ± 1.0	8.0 ± 1.0
Intro notes	4.2 ± 0.22	2.9 ± 1.74	4.5 ± 0.00	3.6 ± 3.3	7.1 ± 0.00	5.3 ± 3.0
Linearity	0.74 ± 0.22	0.76 ± 0.18	0.88 ± 0.00	0.73 ± 0.04	0.83 ± 0.07	0.83 ± 0.06
Consistency	0.92 ± 0.09	0.91 ± 0.02	0.96 ± 0.00	0.86 ± 0.08	0.94 ± 0.02	0.92 ± 0.04
H value	2.9 ± 0.16	2.9 ± 0.02	3.0 ± 0.00	3.0 ± 0.34	3.0 ± 0.51	3.1 ± 0.37
Speed*	1.0 ± 0.00	1.0 ± 0.02	0.99 ± 0.00	1.0 ± 0.00	0.96 ± 0.04	0.96 ± 0.04

Numbers are group means, errors are standard deviation, and an average of 28 ± 14 motifs were counted.
*Speed normalized to motif length of preinjection = 1.

of new neurons recruited into the adult HVC acquire adult, postmigratory morphology ~2 weeks after being generated in the germinal ventricular zone (Kirn et al., 1999), any song recovery that was faster than this would have to be managed without the assistance of neurons born around the time of induced neuronal death. Alternatively, a study using [^{3}H]thymidine prelabeled neuronal precursors that have already migrated into the vicinity of HVC at the time of the induced neuronal death could track whether replacement and potential song recovery occurs even earlier. Ultimately, a more permanent prevention of neuronal incorporation after induction of neuronal death will have to address if and how birds sing in the absence of normal levels of neurogenesis. In the meantime, the recent finding that mice whose cerebellar Golgi cells were genetically ablated showed an acute motor disorder but gradually recovered partial motor function in the continued absence of this cell type highlights the existence of compensatory mechanisms other than neuronal addition (Watanabe et al., 1998).

Our results show that even in avian forebrain, in which adult, spontaneous neurogenesis is widespread, it still is highly selective. Neurons of some types are constantly produced and replaced; those of others are not. We now have learned that this specificity cannot be overcome just by creating vacancies of the "right" kind. However, this still does not tell us if bottlenecks on replacement result from restrictions at the stem cell level or limitations imposed by factors that regulate differentiation and survival. We infer that mechanisms of neuronal replacement in adult brain have not evolved as a generalized mechanism for repair, as occur for example in skin, muscle, bone, and liver. Instead, it is a capacity vested only in particular subsets of brain cells. We do not know yet what advantages this might confer, but it seems likely that an understanding of this issue will be of the greatest importance for understanding brain function and repair. The fact that loss of replaceable neurons and their replacement can be accompanied by behavioral deficits and their restoration is of key

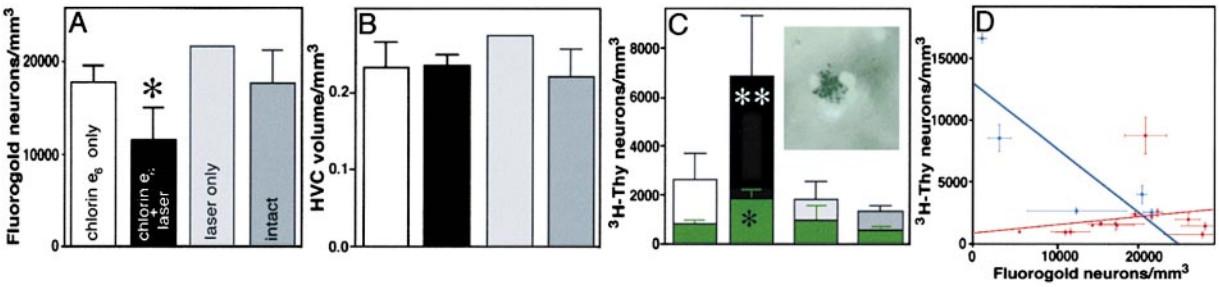

Figure 7. Induced Death of the HVC→X Neurons in Juveniles Increases the Incorporation of New HVC→RA Neurons

(A) The number of retrogradely labeled HVC→X neurons in the group that was injected with chlorin e_6 nanospheres plus laser illumination was significantly lower (by 40%) than in the three age-matched control groups combined ($p = 0.02$, 2-factor ANOVA, with hemispheres as repeated measures, $F = 6$ and 0.68, $n = 5$ and 12, respectively). The three control groups did not differ significantly from each other ($p = 0.8$, ANOVA, $F = 0.16$, $n = 6$ for chlorin e_6 plus laser group, $n = 7$ for chlorin e_6 only, $n = 1$ for laser only, $n = 6$ for intact). Mean values ± SEM are shown.

(B) HVC volumes did not differ significantly among the experimental (black) and the three control groups ($p = 0.8$, ANOVA, $F = 0.2$, $n = 6$, 5, 1, and 6 birds, respectively).

(C) The total number of new neurons (identified as [^{3}H]thymidine-labeled neurons in cresyl-stained material) added to HVC was significantly higher after targeted neuronal death of the HVC→X neurons (black bar, $n = 5$ birds) than in the three control groups (white, light gray, and dark gray bars; $n = 7$, 6, and 2 birds, respectively; $p = 0.01$). The control groups were not significantly different from each other ($p > 0.05$). The number of newly generated HVC→RA projection neurons (identified as double labeled with retrogradely transported green latex microspheres after injection into RA and with [^{3}H]thymidine; see inset) was also significantly higher in the experimental group than in the three control groups (green bars, ANOVA, $p = 0.0031$, $n = 6$, 6, 3, and 6 birds, respectively).

(D) After induced death of HVC→X neurons, there was an inverse relationship between the number of HVC→X projection neurons still present at the end of the experiment (x axis) and the number of new neurons recruited to HVC (y axis) (blue symbols and blue regression line, $R^2 = 0.64$, $p = 0.005$, $n = 10$ hemispheres). This relationship does not depend on the highest point, as it remains significant even if the bird with the highest [^{3}H]thymidine values is excluded ($R^2 = 0.70$, $p = 0.009$, $n = 8$ hemispheres). There was no such relationship in the control groups (red symbols and red regression line, $R^2 = 0.04$, $p > 0.1$, $n = 14$ birds). Each point represents the averaged value of the two hemispheres per bird, with vertical and horizontal error bars indicating the SEM between the hemispheres.

importance here, even if we do not yet have a full understanding of this phenomenon. It is, perhaps, equally noteworthy that the brain should be able to preserve the integrity of a learned behavior even though some neurons in the circuit that controls this behavior are constantly replaced.

Conclusion

Complex, learned behaviors are controlled by equally complex neural circuits in which heterogeneous, but intermingled, cell populations probably make unique functional contributions. In vertebrates, this complexity has hindered our understanding of brain–behavior relationships. Our results demonstrate that targeted neuronal degeneration is uniquely suited to functionally dissect individual song system components with a resolution previously not possible, leading to surprising insights.

There is now a clear fork in the road to further work. One leads to understanding the function of neuronal replacement in adult healthy brains. The other one, with a more therapeutic bent, will explore what can be done to overcome the restrictions that limit what kinds of neurons can be replaced, so that the brain's innate trove of neuronal precursors can be directed to repair broken circuits regardless of the type of neuron lost. For either path, the song system still seems superbly endowed to provide some answers.

Experimental Procedures

Animals

Zebra finches (*Taenopygia guttata*) were obtained from the breeding colony at the Rockefeller University Field Research Center (Millbrook, NY) and were kept on a 12 hr/12 hr light/dark cycle. Seed and water were provided ad libitum. A total of 148 birds were used in six separate series of experiments, performed over the course of 3 years. Due to the multistep nature of the experiments (see below), only 64 birds met the criteria to be included in the final analysis. In each experiment, animals from both experimental and control groups were processed simultaneously to minimize the impact of procedural variation. The age of our birds ranged from 30 days to 3.3 years. The number of animals used in each experiment is shown in Table 1.

Stereotaxic Surgery and Laser Illumination

Birds were anesthetized with intramuscular injections of 0.03 ml (20 mg/ml) xylazine (Rompun, Haver) and 0.025 ml (100 mg/ml) ketamine (Ketalar, Parke-Davis) per 10 g of body weight. All surgical procedures were performed bilaterally. Chlorin e_6–conjugated rhodamine-labeled nanospheres (prepared with slight modifications from Madison and Macklis, 1993), FG (Fluorochrome), and green fluorescent microspheres (Lumafluor) were injected through glass micropipettes with a tip diameter of 30 μm using stereotaxic coordinates modified from the canary atlas (Stokes et al., 1974). At this time, a small opening in the skull was also incised over HVC, to facilitate subsequent access during the laser illumination. Chlorin e_6–conjugated nanospheres were prepared fresh for each series of experiments. After allowing sufficient time (Table 1) for retrograde transport of the chlorin e_6–conjugated rhodamine-labeled nanospheres from the point of injection back to HVC, all animals were transported by car to one author's laboratory (J. D. M.) in Boston where the experimental group then underwent laser illumination. For this, animals were anesthetized as above, and using a stereotaxic apparatus the HVC of each hemisphere was exposed noninvasively to collimated laser illumination of 674 nm wavelength with a total energy flux of ~150 J/cm^2 over a period of 12 min. One or two days after surgery, when the birds had fully recovered from the anesthesia, they were driven back to New York, where all subsequent experimental manipulations were performed.

Thymidine Injections

Birds received intramuscular (M. pectoralis) injections of the cell birth marker [^{3}H]thymidine (6.7 Ci/mM, New England Nuclear; 2.5 μCi/g body weight) on the second, fourth, sixth, eighth, and tenth day after laser illumination. For the adult birds that had undergone death of HVC→X neurons, the protocol was slightly different in that the first injection was given 1 day after surgery and then every 48 hr over the course of days 1–10, 11–20, or 21–30 following laser illumination. Since the numbers of [^{3}H]thymidine-labeled cells were comparable for each of these subgroups, data were pooled.

Perfusion and Tissue Processing

Birds were perfused intracardially under deep anesthesia (2× dose used for surgery) with 60 ml of PBS and 60 ml of 4% paraformaldehyde. After postfixation, brains were cryoprotected by sequential infiltration with 15% and 30% sucrose/PBS. Three series of frozen 14 μm sections were cut in the sagittal plane on a freezing sliding microtome and mounted onto chromalum-subbed slides. One series was processed for autoradiography, as described previously (Alvarez-Buylla et al., 1992). Sections in this series were sometimes lightly counterstained with a fluorescent Nissl stain (Alvarez-Buylla et al., 1990b). This series was subsequently analyzed for FG-positive/[^{3}H]thymidine-positive double-labeled cells (i.e., newly formed HVC→X or HVC→RA projecting neurons) as well as for FG-negative/[^{3}H]thymidine-positive neurons. The second series was left unstained and was analyzed for total number of FG-labeled and/or chlorin e_6–labeled cells in HVC. The third series was stained with 0.1% cresyl violet acetate (Sigma) and used to measure HVC volume and to assess the targeting of injection sites. As an independent confirmation of successful tracer injection into Area X, we always found neurons retrogradely labeled with chlorin e_6 and/or FG in the Area Ventralis of Tsai (AVT), known to project to Area X.

Data Acquisition and Analysis

HVC volumes and number of labeled neurons were quantified using a computer interfaced mapping microscope (Alvarez-Buylla and Vicario, 1988). Analysis of labeled cells was done blind with respect to treatment, using a 63× objective and eight to ten evenly spaced sections per hemisphere (total hemispheres analyzed for FG: 102; for fluorescein microspheres: 20; for [^{3}H]thymidine: 81). For each bird, HVCs in both hemispheres were quantified, except in rare cases when this was impossible due to poor tissue preservation in one hemisphere. Depending on the experiment, HVC neurons labeled with different markers were assigned to different categories and counted as follows: (1) FG only, (2) [^{3}H]thymidine only, (3) FG and [^{3}H]thymidine, and (4) fluorescein-labeled microspheres ("green beads") and [^{3}H]thymidine. Densities of labeled cells per mm^3 were calculated as the sum of labeled cells divided by the sum of the measured areas multiplied by section thickness, i.e., 14 μm, and sampling interval, i.e., four. HVC volumes were estimated by measuring the area of every fourth 14 μm thick, cresyl-stained HVC section throughout the medial–lateral extent of the nucleus and multiplying by section thickness and sampling interval. There were no significant differences in HVC volumes among the control and experimental group in any of the experiments (ANOVA, $p > 0.05$). This justified expression of all neuron counts per mm^3, which afforded us to include data of a few hemispheres where HVC volume could not be estimated reliably (either because of suboptimal staining or because the entire medial–lateral extent of HVC was not represented). All data presented in the text for mm^3 were, however, also calculated per HVC, and the findings reported in the results are qualitatively equivalent. For cell counts, only neurons whose nucleoli were clearly in focus were counted. [^{3}H]thymidine-labeled cells that had 20 times background levels of silver grains were considered labeled (minimum of seven grains per nucleus). Placement of tracer injection sites of all animals included in the final analysis was on target. Despite injecting a fixed volume of tracer for each injection, the size of injection sites varied in different birds. However, there was no systematic relationship between the size of injection and the number of retrogradely labeled neurons, similar to the findings of Iyengar et al. (1999). Regardless, the variance in the numbers of retrogradely labeled projection neurons was similar in the three different experiments (HVC→X in adults, HVC→X in juveniles, and HVC→RA) and

across the three control treatments (laser only, chlorin e_6 only, intact), indicating that any heterogeneity in retrograde filling efficacy affected the groups equally.

Statistical Analysis

Typically, both hemispheres in each animal were analyzed and averaged (so that each n represents the mean of two values). One-way ANOVA was used to analyze differences between groups, and post hoc Fisher PSLD tests determined differences between individual groups. To compare song performance at multiple recording times, repeated measure ANOVA was employed. In some experiments, the variability in labeled neurons among hemispheres in the same brain was considerable, probably due to the independent and multistep nature of the experiments; the two hemispheres in each bird received three different types of surgeries consecutively: first, injection of chlorin e_6 nanospheres; then, laser illumination to induce neuronal death; and finally, retrograde tracer injection before perfusion. Since each of these procedures varies in effectiveness depending on slight differences in accuracy of injection and laser targeting and amounts of tracers deposited, but since we wished to avoid arbitrary post hoc exclusion of animals, we sometimes considered values from both hemispheres as repeated measures in an ANOVA. When this was done, it is clearly stated in the text. When group sizes were very small or data were nonnormally distributed we used nonparametric statistics.

Song Recording and Sound Analysis

In experiments that started with adult birds, song was recorded at least once before chlorin e_6 nanosphere injection, at least once after injection, and at least twice after laser illumination. Whenever possible, we recorded song directed at females (directed song) and song when the male was by himself (undirected song). Results presented are always from recordings in the same context. Software used to analyze and depict sonograms was SoundEdit 16 vs2 (Macromedia) and Canary 1.2.1 (Cornell Bioaccoustics Laboratory). Terminology and definitions of song, recording equipment, and analysis are as described before (Scharff and Nottebohm, 1991). Essentially, this method assesses various song parameters, including the stereotypy of note order within song motifs and the morphology of notes. To assess stereotypy of song, the following parameters were measured blind with respect to treatment: (1) *sequence linearity*, which reflects the different ways the notes are ordered in a song, resulting in slightly different motifs; (2) *sequence consistency*, which does not address *how* the notes are ordered but how *often* each particular motif is sung; and (3) *Shannon information H values*, which determine the frequency with which each note is sung within a bout, as used by Woolley and Rubel (1997). To quantitate *song speed*, motif length was measured. To assess *note morphology*, sonograms of a minimum of five song motifs each were compared between recording sessions. Different notes were labeled with numbers by one experimenter. Note identity was based on sound-spectrographic appearance and order within the motif. Printed sonograms were then cut into individual notes (to eliminate the sequence information of song), and all notes of a bird recorded in two different recording sessions were intermingled. Another experimenter who had not seen song of these animals before was asked to sort all notes into two categories, "typical" zebra finch note morphology and "atypical" note morphology. None of the songs of control animals were found to have "atypical" note morphology. A second test consisted in trying to sort notes of the same identity into two bins, i.e., the two different recording sessions. Of all animals so analyzed, only the four animals with targeted death of the HVC→RA neurons contained notes that were correctly identified (75% correct) to belong to two different recording sessions.

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