# The Impact of Deafness to the Survival of the Newborn Cells in the Brain of Juvenile White-Rumped Munia, *Lonchura striata*

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**ABSTRACT**—In white-rumped munia, early auditory experience is critical for normal song development. New neurons are constantly added to the telencephalon in juveniles. We examined the potential role of auditory experience in regulating the developmental changes in the song nuclei and the survival of new-born cells. We chose two special days, postnatal day 23 and 37, at which we deafened the birds through bilateral cochlea removal. All birds were injected with the cell birth marker BrdU two weeks before the lesion surgeries, and then were killed two weeks or one month later. The BrdU-positive cells were distributed throughout the brain, including the high vocal center (HVC), Lobus parolfactorius and the ventricle zone (VZ) in telencephalon, the granular cell layer (GCL) of cerebellum. Moreover, these BrdU-positive cells in the GCL could self-renew. However, the nucleus robustus archistriatalis (RA) did not sprout new neurons in juvenile. In telencephalon except the VZ, 41 percent of BrdU-positive cells were NeuN-positive, too. Deafness had no significant effect on development of HVC and RA, the distribution of new cells, and the survival of new cells in telencephalon. From these data, we propose that auditory deprivation could not affect the survival of new cells of telencephalon within one month. Surprisingly, we found deafness had a complex and dramatic effect on the number of new cells in cerebellum. Deafness at postnatal day 23 could increase the number of new cells in the GCL, while deafness at postnatal day 37 decreased the number.

**Key words:** Juvenile white-rumped munia, deafness, HVC, RA, neurogenesis

## INTRODUCTION

Many passerine songbirds rely heavily on auditory input for normal song development (Immelmann, 1969; Price, 1979). Birds first listen to and memorize an acceptable song model (sensory learning) and then require auditory feedback from their own vocalizations to mimic the acquired model (sensorimotor learning). The mechanism of song learning in songbirds provides a good model to study speech learning in humans. In male zebra finch (*Poephila guttatta*), for instance, the sensitive period for song learning extends from 10 to 65 days after hatching (Immelmann, 1969; Price, 1979; Eales, 1985). Thereafter, song changes very little. For this reason zebra finch is referred to as a sensitive-period learner (Lombardino and Nottebohm, 2000).

Song in oscine birds is controlled by a system of telencephalon, midbrain, and brainstem nuclei (Nottebohm *et al.*, 1976; 1982; Bottjer *et al.*, 1989), which is referred as song

control system. The high vocal center (HVC) is critical for song production in adult birds (Nottebohm et al., 1976; Simpson and Vicario, 1990). During periods of song acquisition, many new neurons are added to HVC both in juveniles (Alvarez-Buylla *et al.*, 1988, 1990a; Nordeen and Nordeen, 1988; Nordeen *et al.*, 1989) and in adults (Kirn *et al.*, 1994), suggesting that new neurons might play an important role in the behavioral changes. Adult neuronal addition is a continuance of embryonic process. Exposure to environmental factors, including enriched environment, complicated experience and exercise, etc. influences the number of postnatal new neurons in hippocampus of birds (Barnea and Nottebohm, 1994; Rasika *et al.*, 1994; Patel *et al.*, 1997) and mammals (Kempermann *et al.*, 1997, 1998; Gould *et al.*, 1999; Kempermann and Gage, 1999; Nilsson *et al.*, 1999; van Praag *et al.*, 1999a, 1999b; Shors *et al.*, 2001).

Several studies have been done in attempts to determine the importance of auditory experience in songbirds. Deafness can deteriorate adult song (Nordeen and Nordeen, 1992; Lombardino and Nottebohm, 2000). Interestingly, auditory input is not necessary for developmental reg-

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ulation of neuron number in song control system of young and adult zebra finches. Burek *et al.* (1991) deafened the male zebra finches at P10 (postnatal day 10, the birth day is P0), and then measured nuclear volume and neuron number at P25, 50, 65, and 120. They declared that in the zebra finch song system neither the growth of song nuclei nor the developmental regulation of neuron number depended on auditory learning during song development. However, in the next few years, some researchers deafened the adult (Wang *et al.*, 1999) and young (Wilbrecht *et al.*, 2002) zebra finches, and found deafening could alter the neuron turnover within HVC without altering the total neuron number. In these experiments, all of these researchers deafened the birds first, and then they injected the cell proliferation-specific marker ([$^3$H]thymidine or 5-bromodeoxyuridine, BrdU) to label the newborn cells. Nottebohm and his collaborators (Wilbrecht *et al.*, 2002) deafened zebra finches at P26, labeled new neurons in HVC at P60 by using BrdU, and then killed the birds at P90. So, what they saw were the integrative effects of deafness on neurogenesis, including the effects on production, migration and survival of the new cells. We wonder the extent to which the auditory experience is linked to the survival of new cells, casting aside the contribution of production to neurogenesis. In this context we administered BrdU two weeks before the deafened surgery. We report here on how early deafening affects developmental changes in the song nuclei and newborn cells. In this experiment we studied male white-rumped munia (*Lonchura striata*), a closely related species to zebra finch, whose sensitive period for song learning extends from P10 to P65 (Zeng *et al.*, 2002; Zuo *et al.*, 2002), too. We deafened the birds at the special ages, P23 and P37. P23 is the day at which the juvenile birds fledge from the nest and the time of onset of active vocal learning. At P37 the birds are in a sensorimotor learning period, but they could not sing. If these newborn cells play a role in auditory process, deafness at these ages has possibly some effects on the survival of these newborn cells.

## MATERIALS AND METHODS

### Animals

All white-rumped munias younger than P70 used in this study were bred and raised in our laboratory. Thirty-one male subjects were used for data analysis from our 41 subjects. Ten birds were female and they were cast aside. The gender confirmation was based on the autopsy. All birds were kept in standard cages (50×62×38cm) with their parents before P30 under a natural photoperiod of Beijing, with ad *libitum* access to dry seeds and water, and a mixture of freshly sprouted seeds and ground eggs with vitamins, supplied with greens twice a week and they were permitted to bathe once a week. The experiments were performed from March to July.

### Lesion surgeries

Lesion surgeries were performed on P23 and P37. Two weeks before surgeries, all birds were received intramuscular (pectoral muscles) injections of BrdU (Sigma), a thymidine analog, using injections of 0.08mg/g body weight at a concentration of 10mg/ml.

Injections were given twice, at an interval of 12hr (9:00 A.M. and 9:00 P.M.). Bilateral cochlea removal was performed following the procedure according to Lombardino and Nottebohm (2000). Birds were deeply anesthetized with intramuscular injections of sodium pentobarbital, their heads were secured, and an incision was made in the skin overlying the tympanic membrane. The columella was then removed and the tip of a finely hooked tungsten electrode was inserted into the oval window to pull out the cochlea. To confirm complete cochlear removal, we visually inspected each cochlea for an intact lagena under the dissecting microscope.

After surgery, the subjects were placed in an incubator to facilitate recovery and then returned to their home cages after one day. The deafened birds usually could perch freely and eat within 12 hr. Sham-operated birds were anesthetized only, since any further surgical manipulation risked at least temporary hearing impairment (Burek *et al.*, 1991). The normal control birds were unoperated. No systematic differences in the data derived from the two control procedures were found, so data were pooled as others did (Wang *et al.*, 1999). There was no evidence that any procedure used in this study caused sustained stress or discomfort.

### Survival time, perfusion, and fixation

Fifteen deafened and sixteen control birds were killed two weeks (short term, ST) or one month (long term, LT) after the deafened surgery. Our 15 deafened birds were assigned to one of four age groups, which were set up by reference to the age of deafening (P23 or P37) and the survival time (ST or LT). So, the above groups were named as, P23-ST, P23-LT, P37-ST and P37-LT, respectively.

Birds were quickly perfused via the left ventricle with 0.9% saline followed by cold 4% paraformaldehyde after an overdose of sodium pentobarbital. The brains were removed and stored in the same fixative for 4–6 hr at 4°C, then placed in a cryoprotective solution (30% sucrose in 0.1M phosphate buffer, pH7.4) at 4°C until immersion. They were then sectioned along the sagittal planes at 40 μm for one hemisphere brain and at 10 μm for the other hemisphere brain with a freezing microtome. Every third 40 μm-section was mounted on glass slides for Nissl staining and every eleventh 10 μm-section for immunofluorescence staining.

### Immunohistochemical staining

Immunofluorescence staining was processed according to the protocol recommended for Jackson immunofluorescent kits (Jackson ImmunoRes.). Briefly, after 2×3-min wash in 0.01M PBS, pH7.4, the slides were blocked with 3% normal goat serum for 1 hr, and incubated with mouse anti-NeuN (a neuron-specific marker) (1:500 in 0.3% Triton X-100/PBS, Sigma) overnight at 4°C. After 3×3-min wash in PBS, the specimen was incubated with FITC (fluorescein isothiocyanate)-goat anti-mouse (1:200, Jackson ImmunoRes.) for 2 hr at room temperature. To double label brain sections, the staining procedure described for the first staining was repeated, 1:2000 rat anti-BrdU (Accurate Chemicals) was added, and the sections were incubated overnight at 4°C. The anti-BrdU antibody was then detected by 2-hr incubation with rhodamine-goat anti-rat (1:200, Jackson ImmunoRes.) at room temperature. The detection of BrdU required treatment in 2N HCl at 37°C for 30min before the first antibody incubation, followed by 0.1M boric acid buffer (pH8.5) at 4°C for 25min. To avoid contaminating of the fluorescent signals from non-cells, sections were counterstained with Hoechst33342 (Sigma). Fluorescence signals were detected with an Olympus fluorescent microscope at excitation/emission wavelengths of 550/570 nm (rhodamine, red), 492/520 (FITC, green), and 360/400 (Hoechst33342, blue). Some sections for single labeling of BrdU were performed with the peroxidase method (ABC system, Vector) according to Bao *et al.* (2003).

In control, the primary antibody was omitted.

## Data quantification and analysis

The areas of HVC and RA (nucleus robustus archistriatalis) were measured every 120 μm throughout the nuclei. Then we chose the maximal areas of each animal for comparison. The mean diameter value of 30 neurons in HVC or RA was measured random for each animal.

Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and Image Pro Analysis Software (Media Cybernetics, L.P., Silver Spring, Maryland) were used to determine the density of BrdU-positive cells. BrdU$^+$ cells were counted at random in ten fields from each rhodamine-stained 10 μm-section. We counted five to seven sections per animal, spaced 100 μm apart, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdU$^+$ cells per square millimeter and reported as the mean ± SEM (standard error mean). Statistical comparisons were conducted with SPSS software. Differences between means were determined by independent samples $t$ test and considered significant when they exceeded 95% confidence level. All $p$ values reported are two-tailed.

## RESULTS

### 1. Dramatic effects of deafness on behavioral development

Normal male white-rumped munias, if trained by a tutor, start singing at around P50. In this experiment, on the other hand, the birds with bilateral cochlear removal became muted from the surgery until the end of experiments (i.e., over 2 months in the case of P37-LT). However, these deafened birds exhibited typical singing posture when confronted with mature females, similarly to normal un-operated birds. They opened the beaks and made neck muscles and feathers dither, but produced no audible sounds at all.

### 2. No significant effects of deafness on size of HVC and RA

Developmental changes in neuron diameters (data not shown) and the maximal sagittal areas of HVC and RA were largely unaffected by the absence of auditory input (as shown in Fig. 1, $p>0.05$) in all the groups. Both in HVC and RA, the maximal areas of the nucleus increased basically across both intact and deafened birds with age. However, these changes did not reach significant level.

### 3. The distribution of the new-born cells throughout the brain

The BrdU-positive cells were distributed throughout the brain, including telencephalon (the anterior pole of the telen-

cephalon, all hyperstriatal regions, and caudal neostriatum), midbrain and cerebellum (Fig. 2A, Fig. 2B) across both intact and deafened birds. In the song control system of telencephalon, the density of newborn cells was uniform across the HVC, DLM (Nucleus dorsolateralis anterior thalami, pars medialis) and area X in Lobus parolfactorius (LPO, an area of high neuronal density) and other areas. However, we found no new neurons within RA at all the groups. BrdU labeling in the ventricle zone (VZ) was discretely localized to a narrow band adjacent to the ventricle (Fig. 2C) where all cells were NeuN-negative. The lateral ventricle zone and the third ventricle zone had the largest BrdU-labeled density except the cerebellum.

Many NeuN and BrdU double-immunopositive cells were observed in telencephalon of intact and deafened birds (Fig. 2D). BrdU-immunoreactive nuclei, generally having condensed grains and exhibiting variable shapes (Fig. 2B), were smaller than the nuclei with NeuN-positive staining (Fig. 2D). All NeuN-positive cells were double labeled with Hoechst33342 (Fig. 2E). The NeuN and BrdU double labeling of VZ and cerebellum failed to detect. We found that many BrdU$^+$ cells within the telencephalon were distributed as "doublets" (Fig. 2F).

### 4. No change of the number of new-born cells in the forebrain after deafness

In HVC, area X and DLM, deafness had no significantly effect on the number of new cells. The maximal density was 94.1 BrdU-positive cells per square millimeter in HVC. Fig. 3 showed the average number of BrdU-positive cells per square millimeter in telencephalon. There were more new cells in the treated birds at P23 than their control partners. By contrast, the telencephalon sprouted less new cells in deafened birds than control at P37. However, neither of they reached the significant level (Fig. 3A, $p>0.05$). After long survival time (P23-LT and P37-LT), about one-third to half of newborn cells disappeared compared with the short survival time (P23-ST and P37-ST), respectively.

In HVC and LPO of telencephalon, 36.1–48.1% of the new cells were neurons, that is, labeled by NeuN (the average was forty-one percent). This ratio was similar at all the
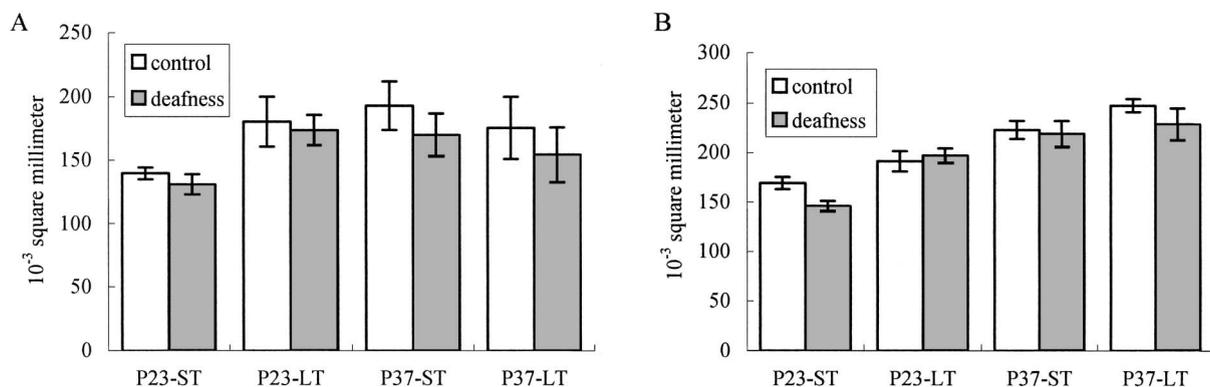


**Fig. 1.** Developmental changes in the maximal sagittal area of HVC (A) and RA (B). Left panels show means of three to five animals per group, with SEM indicated by error bars.
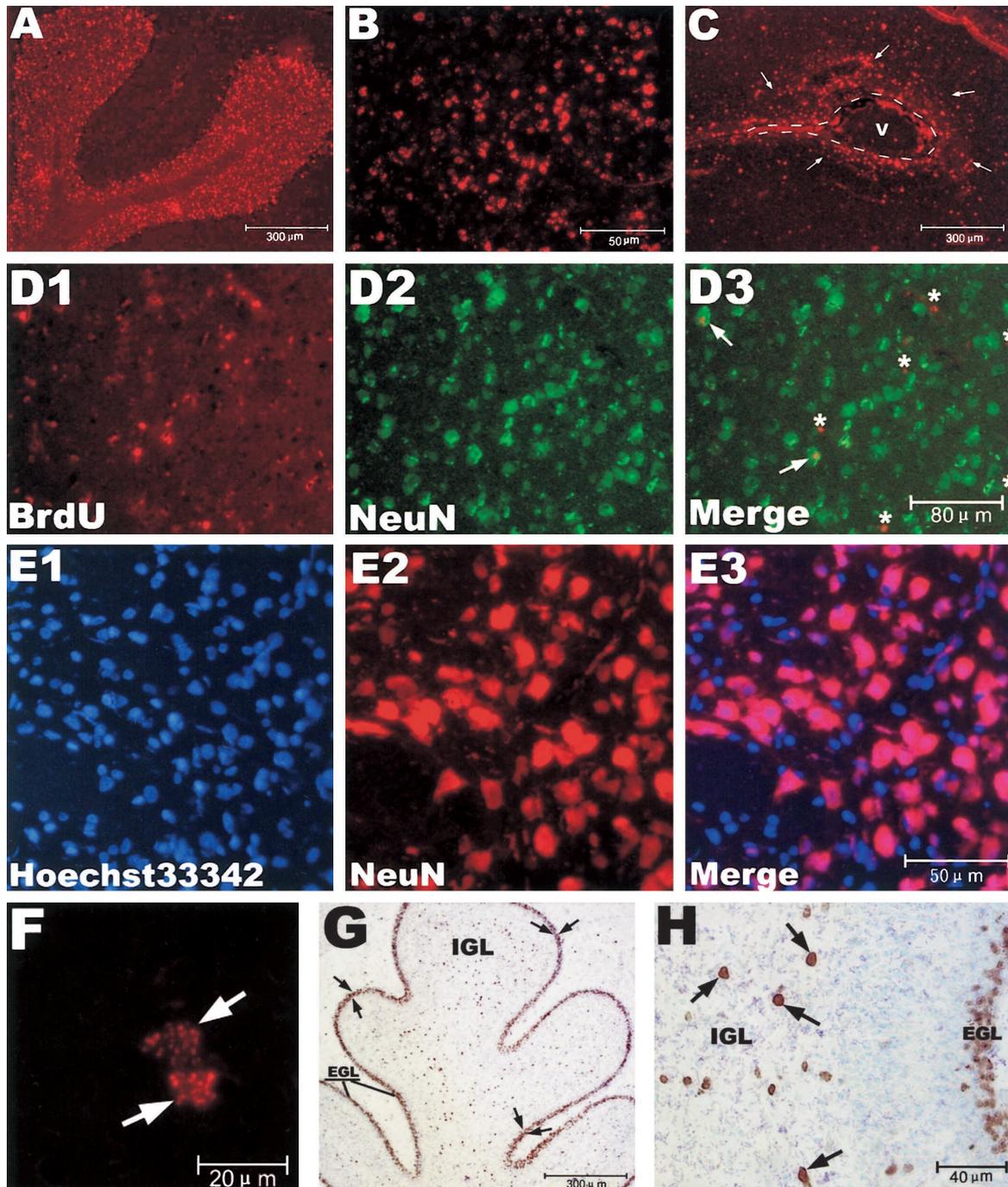
**Fig. 2** A: Low power photomicrograph illustrated dense BrdU-positive cells (red) in the GCL of the cerebellum, which was from P23-ST (The birds were deafened at P23, injected with BrdU two weeks before surgeries, and then killed two weeks later.). B: High power immunofluorescent micrograph of BrdU-positive cell (red) in cerebellar GCL showed that BrdU-positive nuclei were condensed grains and exhibited variable shapes. C: Arrows showed that BrdU labeling (red) in the VZ was discretely localized to a narrow band adjacent to the ventricle (V), indicated by broken white lines. D: Relationship between BrdU incorporation and NeuN expression. Double immunolabeling for BrdU (D1, red) and NeuN (D2, green) was performed in telencephalon of deafened birds of P23-LT (The birds were deafened at P23, injected with BrdU two weeks before surgeries, and then killed one month later.). Double-labeled cells (yellow indicated by arrowheads in merged image, D3) showed the colocalization of BrdU with neuronal marker NeuN and BrdU-immunoreactive nuclei were smaller than NeuN-positive nuclei. Some newborn cells were NeuN negative (*asterisk*). E: Relationship between Hoechst33342 labeling and NeuN expression. Double labeling for Hoechst33342 (E1, blue) and NeuN (E2, red) showed that all NeuN-positive nuclei were all cells, which were pink in merge image (E3). F: Immunofluorescent image of BrdU-labeled cells in the telencephalon. BrdU-labeled cells in this area were often distributed as "doublets" (arrows). G: Light micrograph of BrdU-positive cells from the cerebellum of P7. To assess proliferation, animals received a single injection of BrdU 2 hr before they were killed. Color development was achieved with $H_2O_2$ and DAB. The picture showed that there was a dense, narrow BrdU-positive band (arrows) in the external granular cell layer (EGL) and some newborn cells sparely distributed in the internal granule cell layer (IGL). H: High power micrograph of Fig. 2G. It showed there were fewer and larger BrdU-positive cells (arrows, brown) in the IGL than in the EGL (labelling in the right). The section was lightly counterstained with cresyl violet acetate.
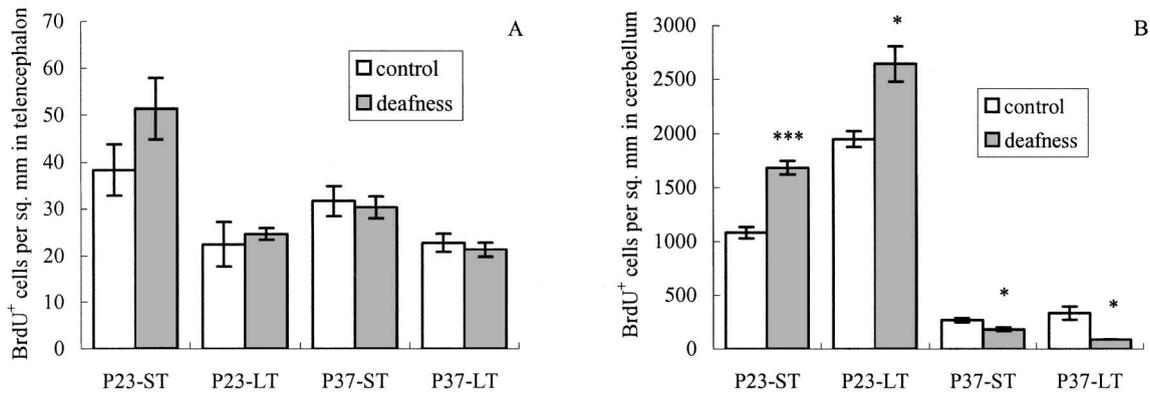
**Fig. 3.** The average BrdU-positive cells per square millimeter in telencephalon (A) and in cerebellum (B). Left panels show means of three to five animals per group, with SEM indicated by error bars. *Asterisk* above the bar indicates a significant difference from its deafened partners and control birds (*$p<0.05$, ***$p<0.001$, *t* test).

groups we measured (P23-LT and P37-ST).

**5. Complex and dramatic effects of deafness on new-born cells in the cerebellum**

In cerebellum, the BrdU-positive cells located in the GCL (granular cell layer) (Fig. 2A). In GCLs of the intact birds, there were four to six times more BrdU-positive cells per square millimeter in P23-ST (control vs. deafness: 1084.8±50.9 vs. 1683.5±63.0) and P23-LT (control vs. deafness: 1950.5±73.3 vs. 2642.6±163.9 cells per square millimeter) than that in P37-ST (control vs. deafness: 269.5±19.7 vs. 184.3±17.1) and P37-LT (control vs. deafness: 334.7±60.8 vs. 87.7±3.2) respectively (Fig. 3B). The group P23-LT had 1.6-1.8 times more new cells than P23-ST (Fig. 3B) and this difference reached significant level ($p<0.05$). In the controls of P37-ST and P37-LT, there was similar result (that is, longer survivors have more new cell than the shorter). However, this increase in the number did not reach significant difference. The birds deafened at P23 had many more new cells than controls across P23-ST ($p<0.001$) and P23-LT ($p<0.05$). However, deafness decreased the new cells of GCL both in P37-ST and P37-LT (Fig. 3B, $p<0.05$).

## DISCUSSIONS

We compared the body weight of BrdU-treated bird with that of non-treated bird; however, no detectable effects on development of the animals were found. Deafening at P23 and P37 precludes the sensorimotor learning; birds lack the opportunity to use their own auditory feedback to perfect those song characteristics that are independent of external song models. Hence, when the birds with deafness in this experiment were executed, they could not sing at all. However, the normal male white-rumped munia trained with a tutor usually can sing around P50 and the volume of their song control nuclei reaches the maximum around P60. In contrast to the drastic impacts of deafening on song behavior, developmental changes of HVC and RA in size were only slightly affected by deafness, which was similar to

another report (Burek *et al.*, 1991). The ages of all the birds to be killed in this experiment were from P37 to P67. During this period, the areas of HVC and RA only increased slightly (Fig. 1). Contrast to this result, the volumes of HVC and RA increased most dramatically between P25 and P50 in zebra finch (Burek *et al.*, 1991) and between P25 and P40 in white-rumped munia (Zeng *et al.*, 2002). The ages from P37 to P67 might be beyond the period of maximal increase for these nucleus developments in white-rumped munia. Collectively, we know that the sensitive periods of these two kinds of bird were identical.

In HVC of younger birds in this experiment, the density of BrdU-positive cells could reach 94.1 per square millimeter and forty-one percent were colocated by NeuN. So, the maximal density of new neuron in our experiment was about 40 per square millimeter and this result was consistent with previous report in zebra finch, which added new neurons at a rate of 10.2–58.9 per square millimeter at the same age (Wilbrecht *et al.*, 2002). We failed to find new neurons within RA even at the youngest group and the result was consistent with other studies of neuronal birth (Konishi and Akutagawa, 1990; Alvarez-Buylla *et al.*, 1994). They declared the only region in the telencephalon that stopped receiving new neurons during juvenile development and thereafter was the archistriatum. Most of the neurons in archistriatum were born at the early stages of embryonic development. The fact that the VZ had the largest labelled density in telencephalon was not to be surprised, because the VZ was the pool of cell proliferation in postnatals (Alvarez-Buylla *et al.*, 1990b). We found that many BrdU+ cells within the telencephalon were distributed as "doublets" which were likely the daughter cells of a mitotic event within this area.

As we know, neurogenesis is a continuous process that consists of several major phases: proliferation, migration, differentiation and survival. Nottebohm and his collaborators deafened zebra finches first at P26, and then the birds were treated with BrdU to label new neurons in HVC and killed after survival some days (Wilbrecht *et al.*, 2002). So, they chose the term "recruitment" to refer to the number of new

neurons present at any one time, without attempting to distinguish the contributions of production, migration, and survival. They found that the number of labeled HVC neurons was not significantly different from that in age-matched intact controls if birds were only deafened. Our design was to detect the effect of deafness on the present BrdU-positive cells in telencephalon, which had survived for two weeks, without respect to the production. If we only want to know the effect of deafness on the production of new cells, we could deafen the animals first, and then label the new cells with single BrdU injection. Before the new cells begin migrating, kill the animals. Considering this, we labeled the new cells two weeks before the columella removal. Although deafening early after hatching completely disrupted normal song development, deafened and hearing-intact birds did not differ significantly in either the maximal area of HVC and RA or the number of new cells in telencephalon, in agreement with previous thionin-stained data (Burek *et al.*, 1991). Deafness at adult could deteriorate song progressively and had dramatic effects on the incorporation of new HVC neurons in male zebra finches without altering total neuron number (Nordeen and Nordeen, 1992; Wang *et al.*, 1999). Collectively, these observations would seem to close the door for a possible role of auditory experience in recruitment of new HVC neurons. From the data of P23-ST or P23-LT in telencephalon and cerebellum, we inferred deafening augmented the migration of new cells to these areas. However, this effect did not reach significant level in telencephalon.

Yet, more and more studies showed that experience and enriched environment could increase neurogenesis. When the birds in this experiment were deafened, the BrdU-positive cells had survived two weeks at all the groups. Our results implied if the auditory experience had some impact on the survival of new cells in telencephalon, this impact only lasted for two weeks. We guessed that the new cell was sensitive to the experience for a specific period, and the later experience had very few effects on the survival of a cell if not within this sensitive period. Taken together, these observations might expose that during neurogenesis, cell proliferation and survival might be regulated independently (Rochefort *et al.*, 2002). Consistent with this, enriched environment affects only cell survival (Kempermann *et al.*, 1997), whereas exercise increases both cell production and survival (van Praag *et al.*, 1999b) in the hippocampus of rodents. Alternatively, the effects of deafness on the cell survival were not reflected among two weeks or one month. If we prolonged the survival time, the effects would display. However, this guess requires further experiments to test.

The phenomenon that the groups of long survival time had more new cells than the groups of short survival time in cerebellum indicated these new cells could have an ability of self-renew in GCL. If so, we recognized some new cells which underwent a few more mitosis, because the method we adopted happened to be somewhat hypersensitive. However, in telencephalon one-third to half of the new cells could not survive within the same two weeks. These results implied the different fates of the cells in different regions. The new cells in GCL of cerebellum were considered immature granular neurons that have not yet begun to express NeuN or cells with other appearances such as astrocyte, because we could not positively identify these cells colabeled for BrdU and NeuN. Previous studies in mouse (Laemmli, 1970; Gillardon, *et al.*, 1995; Migheli, *et al.*, 1999) showed that in the Purkinje and internal granule cell layer (IGL), numerous PCNA (the proliferating cell nuclear antigen)-positive cells were proliferating astrocytes, and mature granule cells were PCNA-negative. In another report (Alvarez-Buylla, *et al.*, 1994), they showed large numbers of cells with glial morphology were labelled throughout the hindbrain during juvenile time.

To determine the developmental change of cerebellum in songbird, we injected BrdU at P7, and then killed 2 hr later. We found the dense BrdU-positive band was in the external granular cell layer and a few cells were in the internal granule cell layer (Fig. 2G, Fig. 2H). All the data demonstrate that the postnatal early development of the vertebrate cerebellum is a conservative model, similar with rodents (Altman and Bayer, 1996; Migheli *et al.*, 1999). Contrary to the very few effects on telencephalon, deafness had a remarkable and complex effect on immature granule cells in cerebellum. Deafening augmented the production, which could account for our results of P23-ST and P23-LT in cerebellum. Alternatively, it might be a compensatory adaptive response to deafness. On the contrary, deafness augmented the death of new cells at groups of P37-ST and P37-LT (Fig. 3B). Differential effect of deafness on the telencephalon and on cerebellum could seem surprising. In fact, there existed a similar tendency in telencephalon. However, this effect did not reach significant level. This more detailed approach will be necessary to determine how the survival of new cells in cerebellum is related to the deafness. If the birds with unilateral cochlear removal were similar to intact controls in the number of new cells in the cerebellum, together with our results from bilateral cochlear removal in this experiment, we could suggest that auditory experience do influence the neurogenesis in the cerebellum, because the birds with unilateral cochlear removal could hear as the normal animals do. In this case, the different results derived from unilateral or bilateral cochlear removal could be very interesting and valuable to further study.

In conclusion, deafness at P23 and P37 had not obvious effects on the survival of new cells born before deafening in telencephalon, thus, auditory experience may play a limited role in the survival of cells produced in control-vocal system of the songbird. However, this cannot exclude the possibility that longer auditory deprivation will influence the final fates of these cells.

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