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HVC interneurons are not renewed in adult male zebra finches

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Abstract

Adult neurogenesis is a widespread phenomenon in many species, from invertebrates to humans. In songbirds, the telencephalic region, high vocal center (HVC), continuously integrates new neurons in adulthood. This nucleus consists of a heterogenous population of inhibitory interneurons (HVC_{IN}) and two populations of projection neurons that send axons towards either the robust nucleus of the arcopallium (HVC_{RA}) or the striatal nucleus area X (HVC_X). New HVC neurons were initially inferred to be interneurons, because they lacked retrograde labelling from the HVC's targets. Later studies using different tracers demonstrated that HVC_{RA} are replaced but HVC_X are not. Whether interneurons are also renewed became an open question. As the HVC's neuronal populations display different physiological properties and functions, we asked whether adult HVC indeed recruits two neuronal populations or whether only the HVC_{RA} undergo renewal in adult male zebra finches. We show that one month after being born in the lateral ventricle, 42% of the newborn HVC neurons were retrogradely labelled by tracer injections into the RA. However, the remaining 58% were not immunoreactive for the neurotransmitter GABA, nor for the calcium-binding proteins, parvalbumin (PA), calbindin (CB) and calretinin (CR) that characterize different classes of HVC_{IN}. We further established that simultaneous application of parvalbumin, calbindin and calretinin antibodies to HVC revealed approximately the same fraction of HVC neurons, i.e. 10%, as could be detected by GABA immunoreactivity. This implies that the sum of HVC_{IN} expressing the different calcium-binding proteins constitute all inhibitory HVC_{IN}. Together these results strongly suggest that only HVC_{RA} are recruited into the adult HVC.

Introduction

Many animals add new neurons to specific brain structures during adulthood. In zebra finches and other songbirds, stem cells residing along the lateral ventricle (Garcia-Verdugo *et al*., 2002) divide and commit to a neuronal phenotype shortly after becoming postmitotic (Barami *et al*., 1995). Thereafter, young neurons migrate and eventually reach many areas of the telencephalon including the high vocal center (HVC), a major auditory–vocal interface (Alvarez-Buylla & Nottebohm, 1988; Alvarez-Buylla *et al*., 1988a). Here they functionally integrate into existing circuits (Paton & Nottebohm, 1984; Burd & Nottebohm, 1985).

The HVC (Reiner *et al*., 2004) contains three types of anatomically and physiologically distinct neurons (Dutar *et al*., 1998). The HVC_X project to area X in the medial striatum, a nucleus of the anterior forebrain pathway involved in song learning and perception (Brainard & Doupe, 2000). The HVC_{RA} innervate the robust nucleus of the arcopallium (RA), part of the premotor pathway that controls singing (Nottebohm *et al*., 1976; Bottjer *et al*., 1989). The HVC_{RA} apparently 'orchestrate' the song temporally, as different ensembles of the HVC_{RA} produce a single short burst at a unique time during the song motif, generating a 'sparse code' (Hahnloser *et al*., 2002). A third population consists of local inhibitory interneurons, HVC_{IN} (Rosen & Mooney, 2003) that can be subdivided by type and number of the calcium-binding proteins they express, i.e. parvalbumin (PA), calbindin (CB) and calretinin (CR; Wild *et al*., 2005). Contrary to the HVC_{RA}, HVC_{IN} burst densely throughout the song (Hahnloser *et al*., 2002).

Among these cell types, the HVC_X are exclusively generated pre- and perinatally and adult-born neurons do not extend axons towards area X (Alvarez-Buylla *et al*., 1988b; Kirn *et al*., 1999; Scharff *et al*., 2000). When first discovered in songbirds, new neurons in HVC were interpreted to be local interneurons, because they lacked retrograde labelling ('backfilling') from HVCs target structures RA and area X (Paton *et al*., 1985). Unfortunately, the retrograde tracer horseradish peroxidase used in this study was probably not efficiently transported by the new neurons, leading to the false conclusion that only interneurons were renewed in adult HVC (Nottebohm, 2002). Subsequent studies, using fluorogold and other tracers for backfilling unequivocally demonstrated that the HVC_{RA} are in fact replaced in adult songbirds (Alvarez-Buylla *et al*., 1988b).

As retrograde tracer may never fill all projection neurons the question remains whether the percentage of new neurons not backfilled are interneurons or adult-formed HVC_{RA} that are not detected as such (Kirn *et al*., 1999; Scharff *et al*., 2000). We therefore analysed the phenotype of newly recruited neurons in HVC of adult zebra finches using both backfills and interneuron-specific markers. We show that 42.1 ± 2.7% of the new HVC neurons had connected to RA by one month after their birth, and that none of the 57.9 ± 2.7% remaining new neurons expressed any of the available HVC interneuron markers. Thus, in contrast to the cricket mushroom body

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(Cayre *et al*., 1994) and the mammalian olfactory bulb (Abrous *et al*., 2005) the HVC does not replace its interneurons.

Materials and methods

Subjects

Four-month-old male zebra finches (*Taeniopygia guttata*) used in this study were obtained from breeding colonies kept at the Max-Planck Institute for Molecular Genetics Berlin.

BrdU injections

Dividing cells were labelled with the cell birth marker, 5-bromo-2′-deoxyuridine (BrdU, Sigma). BrdU was administered intramuscularly (50 mg/kg, in 0.9% NaCl). Each bird received 12 BrdU injections (three times per day during four consecutive days) and labelled nuclei were detected 4 weeks later.

Retrograde tracer applications

Four days prior to killing, birds were anaesthetized with ketamine/xylazine and stereotactically injected with retrograde neuronal tracers using a stereotaxic apparatus (MyNeurolab, St Louis, USA). Rhodamine-conjugated microspheres (Lumafluor, Naples, USA) and Alexa fluor 647 cholera toxin subunit B conjugate (CTB Alexa 647; 0.2% diluted in 0.1 M phosphate buffer saline, Molecular Probe, Karlsruhe, Germany) were injected in RA (anterior/posterior, −1.4/−1.8; medio/lateral, ±2.4; dorso/ventral, −1.8/−2.1) and area X (anterior/posterior, +3.2/+3.5/+3.8; medio/lateral, ± 1.4; dorso/ventral, −4/−4.2), respectively.

Processing and immunochemistry

Each subject was deeply anaesthetized and perfused transcardially with saline (NaCl 0.9%) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS; pH 7.3). The brains were then excised and kept in PFA overnight at 4 °C. Frontal sections of 30 μm were serially cut using a vibrating microtome (VT1000S, Leica) and collected in PBS. All cytochemical reactions were carried out on free-floating sections. Newborn cells ($BrdU^+$) were identified with either rat anti-BrdU (ImmunologicalsDirect, Oxfordshire, UK) or mouse anti-BrdU (Dako, Denmark). Neurons were visualized with a mouse anti-HuC/D (Chemicon, Temecula, USA) and interneurons were phenotyped either with mouse anti-PV, rabbit anti-CB D28k, goat anti-CR (Swant, Bellinzona, Switzerland) or rabbit anti-GABA (Sigma). For BrdU staining, sections were permeabilized with 0.2% triton (PBS-TX) and DNA was denaturated with HCl (2 M, 30 min at 37 °C). Primary antibody incubations were carried out for 48 h (anti-HuC/D) or 72 h (interneuron markers) in PBS-TX containing 4% bovine serum albumin (BSA; Jackson ImmunoResearch, Hamburg, Germany) at 4 °C. Secondary antibody incubations lasted for 3 h in PBS-TX-BSA at room temperature. Negative controls involved the omission of one or more of the primary antibodies to check for cross-reactivity.

To determine the phenotype of the new neurons, double immunolabellings were performed. HVC was delimited by the presence of backfilled HVC_{RA}. Every fourth HVC section was selected for the different experiments and reacted with a combination of antibodies. Rat anti-BrdU (1 : 200) was combined with either the mouse anti-HuC/D (1 : 200), the mouse anti-PV (1 : 5000), the rabbit anti-CB (1 : 5000) or the rabbit anti-GABA (1 : 5000), and subsequently revealed with Alexa 488-labelled goat anti-rat IgG and either Alexa 568-labelled goat anti-mouse IgG or Alexa 568-labelled goat anti-rabbit IgG (each 1 : 200, Molecular Probe). Mouse anti-BrdU (1 : 200) was combined with the goat anti-CR (1 : 5000), and then revealed with Alexa 488-labelled donkey anti-mouse IgG and Alexa 568-labelled donkey anti-goat IgG (1 : 200, Molecular Probes).

For the quantification of the total number of HVC interneurons, double labelling ($GABA^+$ and Hu^+) and multilabelling (PV^+, CB^+, CR^+ and Hu^+) were carried out on adjacent sections. Primary antibodies were applied sequentially to minimize cross reactivity and secondary antibodies were all raised in donkey. Every fifth HVC section was incubated with either the three calcium-binding protein antibodies together (PV, CB and CR; each 1 : 5000) or the rabbit anti-GABA (1 : 5000) that were subsequently revealed with Alexa 568-labelled donkey IgG antibodies raised against the species in which the primary antibodies were made (each 1 : 200, Molecular Probes). Then, the slices were fixed in PFA for 10 min and incubated with the mouse anti-HuC/D (1 : 200), revealed afterwards with Alexa 488-labelled donkey anti-mouse IgG (1 : 200).

Image analysis and quantification

Immunofluorescent sections were counterstained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole; Serva, Heidelberg, Germany), mounted with fluoromount G (SouthernBiotech, Alabama, USA) and analysed using a Zeiss confocal microscope (LSM 510 META) equipped with an Argon laser (458, 477, 488, 514 nm), HeNe lasers (543 and 633 nm) and a Diode laser (405 nm). The meta scanning module is equipped with two single-channel detectors and the software allows the acquisition of multiple fluorescence per plane. Each plane of the confocal stack is 1 μm thick.

Statistics

Comparison between the number of GABA-expressing interneurons and the different interneuron subpopulations were carried out with a Wilcoxon Signed Ranks test. Levels of significance were set at $P < 0.05$.

Results

Four weeks after BrdU injections, an average density of 4165.6 ± 715.5 $BrdU^+$ cells per mm^3 was found in HVC ($n = 6$ birds), and 67.9 ± 6.8% of these new cells were immunopositive for the neuronal marker, Hu (Fig. 1A and B). Confocal analyses four days after the stereotactic injections of rhodamine-conjugated microspheres into RA (Fig. 2A and C) showed that 42.1 ± 2.7% of these new neurons represent HVC_{RA} (Fig. 1A and C–H). Moreover, none of the HVC_X neurons backfilled with the CTB Alexa 647 (Figs 1C–H, and 2B and C) incorporated BrdU, confirming that they are not renewed in adulthood (data not shown).

We then checked for the presence of different interneuron markers in the 57.9 ± 2.7% remaining non-HVC_{RA}. Frontal sections containing HVC were labelled with an antibody against the neurotransmitter GABA to visualize the entire population of inhibitory interneurons. No colocalization was observed between BrdU and GABA, suggesting that HVC interneurons are not renewed in adult songbirds (299 $BrdU^+$ cells analysed across three birds). However, as GABA immunostaining was also present in some non-neuronal cells (Fig. 3A–C), we employed additional interneuron markers.

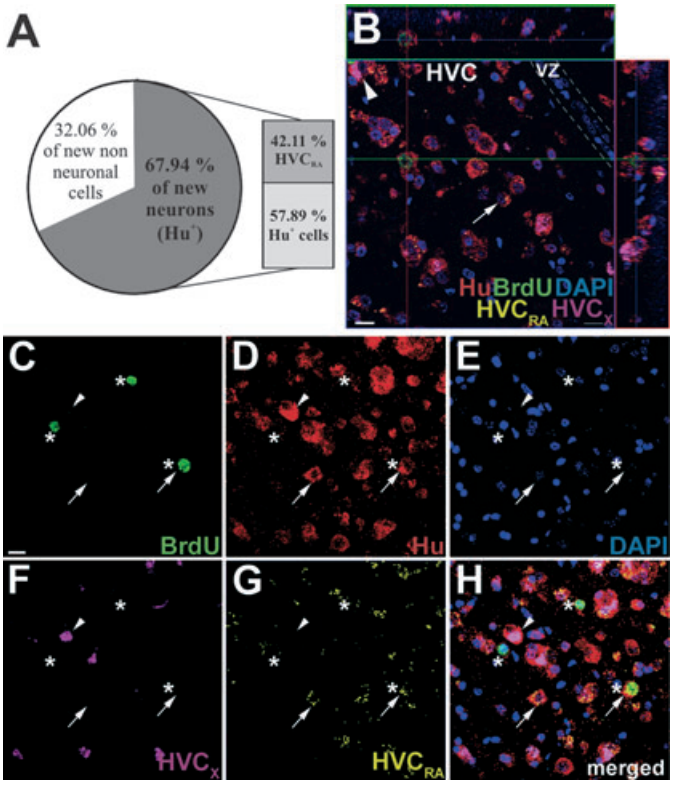

FIG. 1. Characteristics of neuronal recruitment in HVC. (A) Diagram illustrating the percentage of new cells in HVC that express the neuronal marker Hu. Forty-two per cent of newly recruited neurons are HVC_{RA}; the remaining 58% could be HVC_{IN} or HVC_{RA} that were not backfilled. Brains were analysed 4 weeks after birds received a total of 12 BrdU injections administered over the course of four days. (B) Three-dimensional confocal micrograph of a frontal HVC section immunocytochemically reacted for the cell-birth marker, BrdU (green; nuclear) and the neuronal marker, Hu (red; cytoplasmic), counterstained with DAPI (blue; nuclear), and retrogradely labelled either with CTB Alexa 647 (purple, arrowhead; cytoplasmic) identifying the HVC_X neurons or with rhodamine-conjugated microspheres (yellow dots, arrow; cytoplasmic) identifying the HVC_{RA} neurons. The green (*x–z*) and red (*y–z*) lines transect a new (i.e. $BrdU^+$) neuron (i.e. Hu^+) whose identity is undetermined because it is not retrogradely labelled by either tracer, which can also be seen on the reconstructed orthogonal projections presented in the *x–z* (top) and *y–z* (right) planes. Scale bar, 10 μm. (C–H) Projection of a confocal stack spanning 6 μm in the *z* dimension for individual (C–G) and merged markers (H). The arrowhead points towards a backfilled HVC_X neuron (purple, F), whereas the arrows indicate backfilled HVC_{RA} neurons (yellow dots, G). Three $BrdU^+$ cells are visible (asterisks, C). Although the Hu staining that surrounds the two $BrdU^+$ cells on the left part of the image appears weaker and more speckled than the Hu staining of the right one (asterisks, D), focusing through those cells under the microscope revealed that all of them were new neurons. The rhodamine-conjugated microspheres in the cytoplasm of the right-hand new neuron (asterisk and arrow, G) identify it as a new HVC_{RA} neuron. Scale bar, 10 μm.

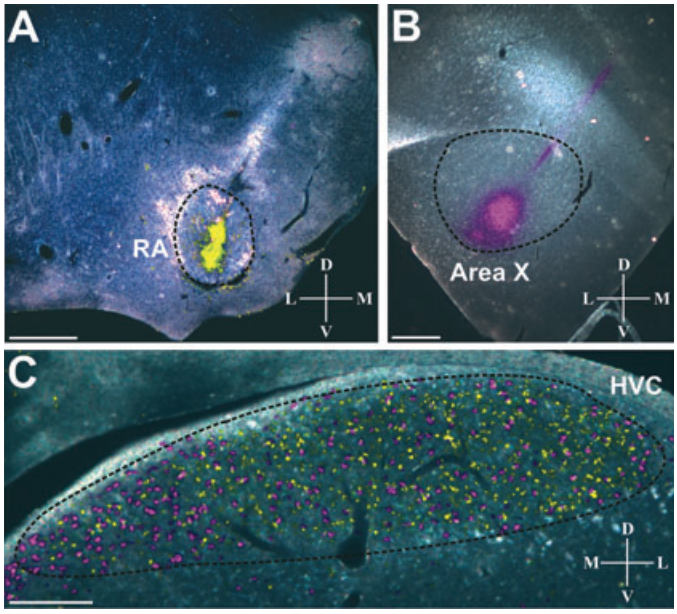

FIG. 2. Photomicrographs of the injection sites of (A) rhodamine-conjugated microspheres (yellow) into RA and (B) CTB Alexa 647 (purple) into area X shown in frontal brain sections. Scale bars, 500 μm. (C) Typical distribution and abundance of retrogradely backfilled HVC_{RA} (in yellow) and HVC_X (in purple) shown in a double-exposed photomicrograph of a frontal HVC section analysed four days after the stereotactic injections of the neuronal tracers. Scale bar, 100 μm.

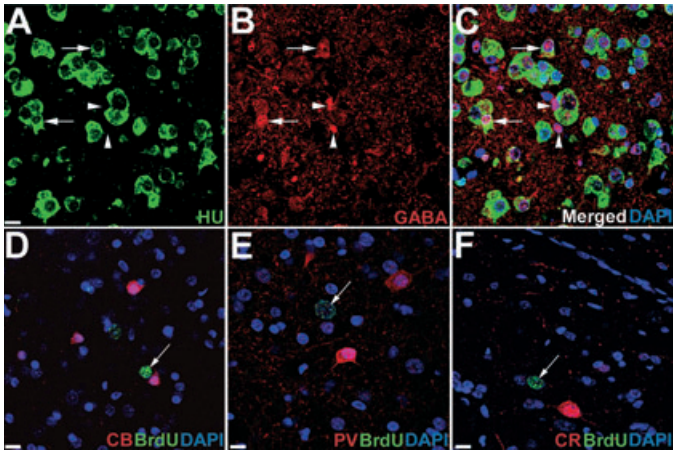

FIG. 3. New neurons not retrogradely labelled from RA are not inhibitory interneurons. (A–C) Immunoreactivity against neurotransmitter GABA (red) labels Hu-positive HVC_{IN} (green, arrows), but also non-neuronal cells (Hu-negative, arrowheads), shown in confocal photomicrographs of HVC frontal sections counterstained with nuclear dye DAPI (blue). Scale bar, 10 μm. (D–F) Confocal photomicrographs of HVC neurons expressing calcium-binding proteins (D) calbindin (CB, red), (E) parvalbumin (PV, red) and (F) calretinin (CR, red) that were not $BrdU^+$ (green, arrows) as shown in DAPI-counterstained brain sections. Scale bars, 10 μm.

The GABA synthetic enzyme, glutamic acid decarboxylase (GAD), is the most commonly used to identify GABA expressing-neurons and an antibody against this enzyme was shown to work on cryostat sections of zebra finch brains (Luo & Perkel, 1999). However, this particular antibody (AB108, Chemicon) is no longer available and other clones used (MAB5406, AB1511, Chemicon) failed on our vibratome brain sections (data not shown). Fortunately, a recent paper demonstrated that the neuronal markers PV, CB and CR define different subpopulations of the HVC interneurons in zebra finches (Wild *et al*., 2005). For this reason, we determined whether the new neurons expressed any of these three calcium-binding proteins. When sections were double-stained for BrdU and CB, the most abundant calcium-binding protein in HVC (Wild *et al*., 2005), no colocalization was observed (1019 $BrdU^+$ cells analysed across 5 birds; Fig. 3D). The same results were obtained when BrdU and PV were investigated (1279 $BrdU^+$ cells analysed across five birds; Fig. 3E) or BrdU and CR (1489 $BrdU^+$ cells analysed across five birds; Fig. 3F). These results suggest that none of the interneurons detected with antibodies

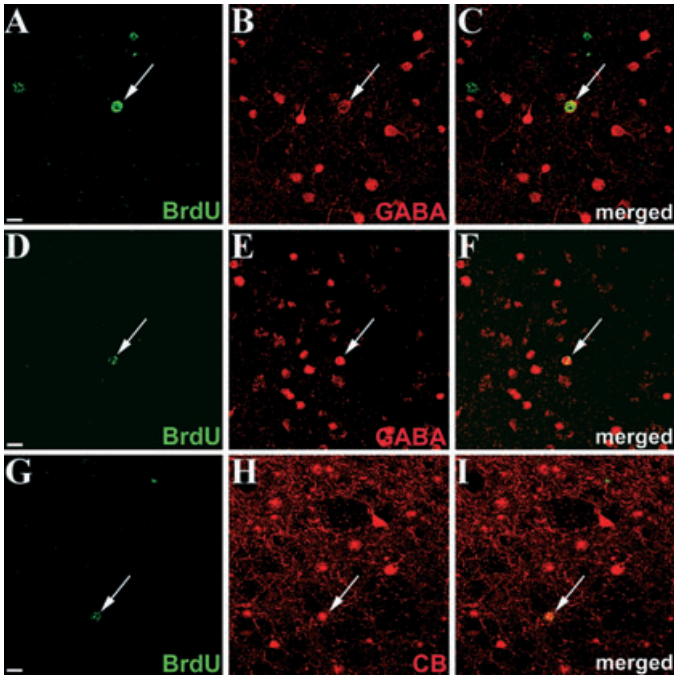

FIG. 4. New interneurons are observed in areas outside of HVC (frontal sections). (A–I) Projection of confocal stacks spanning 2 μm in the *z* dimension for individual and merged markers (A, D, G, BrdU; B, E, GABA; H, CB; C, F, I, merged images). These cells were detected (A–C and G–I) in the nidopallium and (D–F) in the striatum. The arrows point towards $BrdU^+$ cells that colocalize with either GABA (A–F) or CB (G–I). Scale bar, 10 μm.

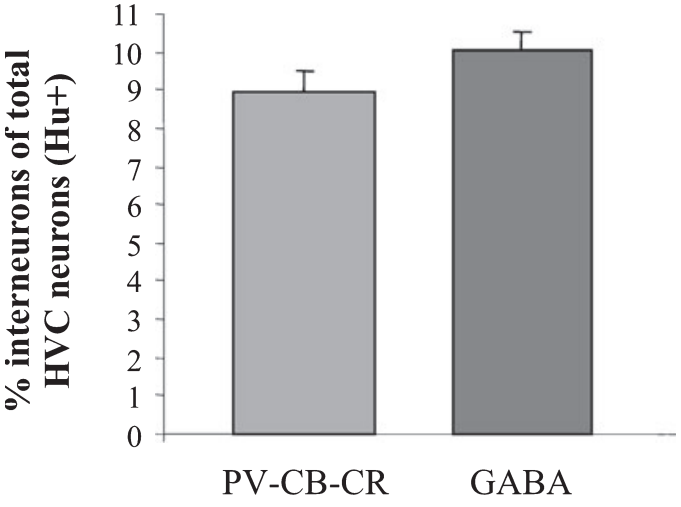

FIG. 5. The fraction of HVC neurons (Hu^+) that express GABA is not statistically different from the fraction of HVC neurons comprising the sum of cells that express PV and/or CB and/or CR. Data are means ± SEM.

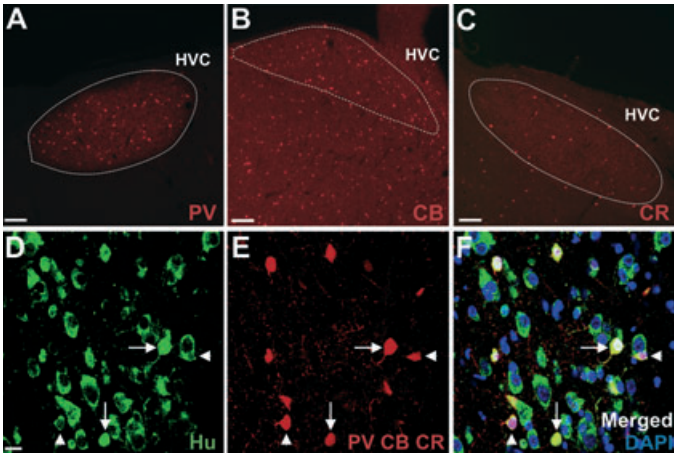

FIG. 6. Immunocytochemical detection of the calcium-binding proteins PV, CB and CR to label the ensemble of HVC_{IN}. (A–C) Photomicrographs of HVC frontal sections showing the expression pattern of (A) parvalbumin, (B) calbindin and (C) calretinin in HVC and surrounding nidopallium (red). Scale bars, 100 μm. (D–F) Confocal photomicrographs of HVC frontal sections DAPI-counterstained and labelled for Hu (green) and the three calcium-binding proteins PV-CB-CR (red). (D) Hu expression is limited to the cytoplasm, whereas (E) PV, CB and CR are localized in the nucleus and the cytoplasm. As the antibodies against PV and Hu markers both derive from mouse, unspecific detection of the Hu antibody in the nucleus could be observed in the PV^+ interneurons (arrows). Such cross reactivity did not occur between the rabbit anti-CB or the goat anti-CR and the mouse anti-Hu (arrowheads). Scale bar, 10 μm.

against PV, CB or CR were newly recruited HVC neurons. To address the possibility that maturation of adult-born interneurons is a slow process and takes longer than 4 weeks, we searched for such cells outside of the HVC and found a small number of $BrdU^+/GABA^+$ (Fig. 4A–F) and $BrdU^+/CB^+$ (Fig. 4G–I) in the nidopallium and the striatum. This finding also rules out the possibility that technical limitations could prevent the detection of new interneurons.

We next analysed whether the detection of the PV^+, CB^+ or CR^+ cells was sufficient to visualize the entire population of HVC interneurons, or whether another subpopulation of interneurons not expressing any of these three calcium-binding proteins might be recruited into adult HVC. To address this possibility, the number of HVC interneurons stained for either GABA or the three calcium-binding proteins (PV^+, CB^+ and CR^+), were compared to the total number of HVC neurons (Hu^+). The quantification of the relative proportion of $GABA^+/Hu^+$ showed that interneurons represent 10.1 ± 0.6% of the HVC neurons ($n = 3$ birds; Fig. 5). However, as the HVC interneurons contain one or more of the three calcium-binding proteins (Wild *et al*., 2005), the relative proportion of PV^+/Hu^+, CB^+/Hu^+, and CR^+/Hu^+ would be higher than the relative proportion of $GABA^+/Hu^+$. To avoid overestimating the total number of the different interneuron subpopulations, we reacted HVC sections concurrently with antibodies against PV^+, CB^+ and CR^+ and detected them with secondary antibodies all labelled with the same fluorophore. Controls omitting two of the three primary antibodies but using all three secondaries verified that the expression pattern of each calcium-binding protein remained the same as obtained with a single primary and its corresponding secondary antibody. PV immunoreactivity was restricted to the HVC (Fig. 6A), whereas the CB^+ and CR^+ cells were ubiquitously distributed throughout the nidopallium, although the densely distributed CB^+ cells outnumbered the scattered CR^+ cells (Fig. 6B and C). For the accurate labelling of PV, CB, CR with one fluorophore and Hu with another, sections were sequentially reacted first against PV, CB, CR and afterwards against Hu. This was necessary because both PV and Hu antibodies were raised in mouse, thus resulting in cross-reactivity of the secondary antibodies. As the PV^+ neurons constitute a subpopulation of all Hu^+ neurons, labelling for PV before Hu was essential. The resulting immunoreactivity shows some Hu^+ neurons with non-specific nuclear staining (arrows in Fig. 6D), which is due to the prior reaction against PV (arrows in Fig. 6E). Such cross reactivity did not occur between the rabbit anti-CB or the goat anti-CR and the mouse anti-Hu (arrowheads in

Fig. 6D–F). The quantification of the relative proportion of PV^+–CB^+–CR^+/Hu^+ showed that 8.9 ± 0.6% of the interneurons expressed one or several of these calcium-binding proteins ($n = 3$ birds; Fig. 5). This proportion is not significantly different from the relative proportion of $GABA^+$/Hu^+ ($P = 0.109$), suggesting that the sum of the PV^+, CB^+ and CR^+ cells reflects the entirety of the HVC inhibitory interneurons. Together, these results imply that inhibitory interneurons are not renewed in HVC of adult male zebra finches.

Discussion

The present findings strongly suggest that HVC of adult male zebra finches does not recruit inhibitory interneurons. New HVC neurons were not immunopositive for GABA, CB, PV or CR. Our results cannot rule out the possibility that expression of GABA or CB in new interneurons takes longer than 4 weeks, but we think this is unlikely because new neurons outside of the HVC were found to be $GABA^+$ or CB^+. In addition, Kirn *et al*. (1999) observed that by 15 days after birth, new cells in the HVC canary already display a mature appearance, and some of them start establishing their efferent connections with RA, suggesting that the molecular events that control the survival and the differentiation of the new cells in the HVC can potentially occur during a short time frame. Already, Paton *et al*. (1986) observed that the newly generated neurons did not show GABA-like immunoreactivity, leading them to hypothesize that, as the projection neurons were not observed to be renewed (Paton *et al*., 1986), two populations of local interneurons existed in the HVC, those generated during adulthood and those expressing GABA. GABA is also not detected in the HVC of female zebra finches (Grisham & Arnold, 1994), which incorporates new neurons, albeit fewer than males (Nordeen & Nordeen, 1988; Kirn & DeVoogd, 1989). Lending further support to the notion that the new HVC neurons are not inhibitory interneurons is the observation that while the soma size of the adult-formed neurons is initially closer in diameter to the larger HVC_X or HVC_{IN}, it gradually becomes smaller and more characteristic of HVC_{RA} (Kirn *et al*., 1991).

We found that GABA labelled both neuronal and non-neuronal cells, consistent with reports in mammals (Reynolds & Herschkowitz, 1987; Blomqvist & Broman, 1988; Stuckey *et al*., 2005), thus forcing us to resort to the additional and more specific inhibitory interneuron markers, PV, CB and CR (Wild *et al*., 2005). We show that the interneuron population represents ≈10% of the HVC neurons, irrespective of whether quantified with GABA or with combined PV/CB/CR immunostaining. This implies that all inhibitory interneurons express at least one of these calcium-binding proteins. We found the same expression pattern of the three calcium-binding proteins as previously described (Wild *et al*., 2005); nidopallial interneurons immunopositive for CB and PV were more numerous than CR^+ neurons. Whereas CB^+ and CR^+ neurons were distributed throughout this region, including HVC, PV^+ neurons were limited to HVC.

Four weeks after BrdU administration, approximately half of the new neurons were retrogradely labelled from RA, leaving the other half of unknown identity. As those are definitely not HVC_X (Alvarez-Buylla *et al*., 1988b; Kirn *et al*., 1999; Scharff *et al*., 2000; present study) and as suggested here, not inhibitory neurons either, it is most likely that some newly arrived HVC_{RA} are not as effective in transporting retrograde tracers from the periphery to the soma, or alternatively have not yet connected to their target, RA. This was found to be the case in canaries. One month after the injections of the cell birth marker, [^{3}H] thymidine, approximately half of the new neurons were backfilled from RA, i.e. a fraction equivalent to the one we found in zebra finches (Kirn *et al*., 1991, 1999). In canaries, this fraction then increased by 50% over the following 8 months while the total number of new neurons stayed stable (Kirn *et al*., 1991). A precedent for HVC_{RA} waiting before synapsing with their target apparently also exists in juvenile zebra finches where HVC_{RA} nerve terminals arrive along the dorsal border of RA but delay innervation for as much as 15 days (Konishi & Akutagawa, 1985). It is thus conceivable that some adult-born HVC projection neurons may likewise linger before connecting to RA. Unfortunately, an increase of new HVC_{RA} over time would be difficult to observe experimentally in zebra finches, because four months after having been generated only 25% of the new HVC neurons survive (Nottebohm *et al*., 1994; Wang *et al*., 1999). In sum, while we cannot formally exclude the possibility that the adult-recruited neurons belong to a fourth, so far undescribed neuronal population in HVC, we consider the hypothesis that all new HVC neurons eventually either project to RA or die more parsimonious.

Many questions about the function of adult neurogenesis in song behaviour remain, especially how the HVC can be instrumental in producing a stable song while new neurons continuously integrate into its microcircuitry. Current knowledge about the HVC neuronal populations suggests that the HVC_{RA} orchestrate the temporal sequence of the song (Hahnloser *et al*., 2002), whereas the HVC_X send a copy of the premotor signals towards the basal ganglia pathway (Hessler & Doupe, 1999; Mooney, 2000). Both HVC_X and HVC_{RA} are selectively tuned to the bird's own song (Rosen & Mooney, 2006). Activity of the projection neurons is shaped by the strong inhibition of the HVC interneurons (Mooney & Prather, 2005). Given that HVC_{RA} are replaced, it will be interesting to determine how they acquire their sparse song-related firing (Hahnloser *et al*., 2002). Do old neurons instruct new ones or are prespecified new neurons selected according to their 'firing profile? It is curious that adult-born neurons in insects and rodents are limited to brain structures involved in sensory processing and/or learning + memory, whereas in songbird HVC the new neurons integrate into a premotor circuit. From a functional point of view it might be more interesting to point out a common feature though; the hippocampus and olfactory bulb of rodents, the mushroom body of certain insects and HVC of songbirds, all sites of adult neurogenesis, exhibit a sparse coding strategy (Hahnloser *et al*., 2002; Perez-Orive *et al*., 2002; Chawla *et al*., 2005; Rinberg *et al*., 2006), a property thought to bestow a number of advantages on neural networks (Olshausen & Field, 2004). Perhaps ensembles consisting of sparsely coding neurons are also particularly well suited to influence adult behavioural function by incorporating new neurons into their midst.

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Abbreviations

BrdU, 5-bromo-2′-deoxyuridine; CB, calbindin; CR, calretinin; CTB, cholera toxin B; HVC, high vocal center; RA, robustus nucleus of the arcopallium; PV, parvalbumin.

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