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Net Addition and Long-Term Survival of Adult-Born Neurons in the Zebra Finch HVC: Why Replace When You Can Keep Them All?

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Net addition and long-term survival of adult-born neurons in the zebra finch HVC: why replace when you can keep them all?

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Net addition and long-term survival of adult-born neurons in the zebra finch HVC: why replace when you can keep them all?

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The study of neurogenesis in adult songbirds focused initially on the canary, a species that learns new song elements each year. Many neurons in the canary song control nucleus, HVC, are discarded every year, with cell loss peaking at the end of the breeding season. New neurons numerically replace those that have died and this replacement process occurs at a time when vocal output is most plastic, suggesting adult neurogenesis may have a role in the learning process. It is not known, however, whether spontaneous neuronal replacement accompanies recruitment of new HVC neurons in the adult zebra finch, a species that learns its song only once prior to sexual maturity.

To test for numerical replacement in the adult zebra finch, we conducted three experiments. First, we retrogradely labeled and counted the two populations of projection neurons in HVC in birds ranging in age from 90 days, when they are first sexually mature, to 11 years. We found that the neurons projecting from HVC to the robust nucleus of the arcopallium (HVC-RA neurons), which are known to be recruited after hatching and into adult life, substantially increased in number with age. In contrast, the number of neurons that project to Area X (proper name, HVC-X neurons) and are only produced during early development, remained constant throughout adulthood. Second, we retrogradely labeled the HVC-RA neurons

present at 95 days of age and found no loss in their numbers over the following 9 months, suggesting that recruitment of the HVC-RA neurons in adulthood is not accompanied by loss of the existing HVC-RA neurons. Third, we labeled neurons born at sexual maturity with a birthdate marker and tracked their survival from 1 month to 4 years. We found no loss of labeled neurons in HVC over this time period, showing that adult-born recruited to the zebra finch HVC can survive for the long-term.

Thus, the consequences of neuronal recruitment in the HVC of adult zebra finches are markedly different from those observed in adult canaries. New HVC-RA neurons are recruited without any appreciable loss in the existing neuron population and the recruited neurons survive for many years in HVC. Overall, these events result in a substantial net increase in the total number of HVC-RA neurons across adulthood. We speculated that a net addition of neurons to the vocal motor pathway in adulthood would have implications for adult song, specifically in song stereotypy which has been shown to improve with age after sexual maturity. However, we found that neuronal addition occurred as normal in deafened birds and that HVC-RA numbers were not correlated with song stereotypy or song stability after deafening. Thus, the functional role of neuron addition to the zebra finch song system is still unknown. Perhaps adult-born HVC-RA neurons have a perceptual rather than a motor function in this species. This research highlights that animal models for understanding the mechanism and function of adult neurogenesis must take into account the different lifestyles and needs of the species under investigation.

I dedicate this thesis to Joe Smale, my cousin, the bravest young man I know.

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I feel deeply privileged to have spent the last 5 years working under the guidance of the most wonderful advisor Fernando Nottebohm. His contributions to this thesis go far beyond his direct input to the text and the ideas it contains. He has taught me the most valuable lesson of scientific enquiry: “observe everything but believe nothing”. He has been incredibly generous with his time and wisdom throughout my studies and our lively discussions of data over the tea kettle will be remembered fondly for many years to come.

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GLOSSARY OF TERMS

Abbreviations / Acronyms

Song system:

HVC = used as the proper name, formerly “High Vocal Center”

RA = robust nucleus of the arcopallium

Area X = used as the proper name; homologue of the mammalian striatum

LMAN = lateral magnocellular nucleus of the anterior nidopallium

NXIIts = the tracheosyringeal portion of the nucleus of the twelfth cranial nerve

DLM = medial portion of the dorsolateral thalamus

HVC-RA neurons = neurons projecting from HVC to nucleus RA

HVC-X neurons = neurons projecting from HVC to Area X

LMAN-RA = neurons projecting from lMAN to nucleus RA

Methods:

CTB = cholera toxin B, a retrograde tracer

BrdU = 5-bromo-2-deoxyuridine, a marker of cell proliferation

SSI = syllable similarity index

Definitions

“Neurogenesis”

The production of neurons by division of stem cell progenitors

“Birthdate labeling”

The labeling of a cell as it is ‘born’ during cell division. A birthdate marker typically gets incorporated into new DNA during the S-phase of mitosis.

“Retrograde labeling”

Labeling a neuron by the transport of a tracer dye from the axon terminal backwards to its cell body.

“Posthatch day”

The age of a bird, in days, since the day of hatching

CHAPTER 1: INTRODUCTION

Neurogenesis in the adult brain: an overview

The brain is not a static structure but a highly malleable organ. It retains the lifelong ability to change based on new experiences and it is this flexibility that makes us so successful at adapting to an ever-changing world. As the demands we face evolve across a lifetime, our brains must constantly readjust to allow us to respond optimally to each new set of circumstances. We call this skill *neuroplasticity*. The key processes that enable an immature brain to develop and learn in response to new experiences are also at work in the mature organ. These processes facilitate new learning in the intact brain and are also invoked in the event of injury to enable other areas to compensate for lost function. Neuroplasticity can involve structural changes at many levels, from subtle changes in the strength of existing connections, to the creation of new synapses, or to the addition of completely new brain cells. The extent to which each of these processes contributes to neuroplasticity in the normal and damaged brain appears to differ greatly between species, particularly with regards to the production of new neurons.

As far as we know, mature neurons do not divide. In the developing vertebrate brain, new neurons arise from the division of non-neuronal progenitor cells that mostly reside in a specialized proliferative region adjacent to the ventricle called the ventricular zone (VZ; The Boulder Committee 1970). Cells born at the VZ migrate away to take up their final position and mature into neurons (reviewed by

Sidman & Rakic 1973). Towards the end of brain development in mammals, the majority of the VZ loses its proliferative potential and is transformed into a non-dividing ependymal layer (The Boulder Committee 1970). The notable lack of mitotic profiles in the VZ in the adult mammalian brain (Cajal & May 1959), combined with observations from neurologists that brain lesions in adulthood often had a poor prognosis for functional recovery, led to the widely held view that neurons were not regenerated in the adult brain.

However, after many decades of research, we now know that some regions of the brain do retain a population of active progenitor stem cells that continue to produce new neurons throughout adulthood. This phenomenon is known as “adult neurogenesis”. In mammals, the process has been most widely studied in rodents, where active progenitors are spatially restricted to two discreet regions: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) that sits just below the ependymal layer of the lateral ventricles (reviewed in Lledo et al. 2006). These two stem cell populations each generate one main type of neuron in adulthood; the SGZ progenitors contribute new neurons to the granule cell layer of the dentate gyrus and the SVZ progenitors produce new cells that travel to the olfactory bulb via the rostral migratory stream and become granular interneurons (Lledo et al. 2006). Thus, the majority of neuron types in the adult rodent brain are not normally produced in adulthood, which may explain why the phenomenon of adult neurogenesis remained elusive to neuroscientists for so many years. Adult neurogenesis has also been observed in

the brains of adult primates and humans, but it occurs to a lesser extent than in rodent species. New neurons have been observed in both the dentate gyrus and olfactory bulb of adult macaques (Koketsu et al. 2003; Kornack & Rakic 2001) and in the human hippocampus (Eriksson et al. 1998). It is still controversial whether new neurons are also added to the olfactory bulb in humans (Eriksson et al. 1998; Sanai et al. 2004; Sanai et al. 2007; Curtis et al. 2011).

In contrast to mammals, the brains of lower vertebrates, particularly teleost fish, show more prolific patterns of adult neurogenesis. For example, within any 2 hour period, 0.2% of all the cells in the brain of Brown ghost knifefish are undergoing mitosis (Zupanc & Horschke 1995). New neurons are added to most areas of the adult fish brain and although not all of them survive, some become permanent, leading to continuous growth of the entire brain in adulthood (reviewed in Zupanc 2009). Teleost fish brains also show a remarkable capacity for repairing damage: neurons lost through stab wounds to the cerebellum can be completely replaced by new neurons within a matter of weeks (Zupanc 1999). If the normally high level of neurogenesis in the healthy fish brain facilitates brain repair after damage, perhaps it is the apparent scarcity of neurogenesis in the adult human brain that accounts for its vulnerability to trauma, stroke and neurodegenerative disorders.

Despite its limited potential for regeneration, the human brain can still show significant repair after certain forms of damage. Depending on the brain region

affected, stroke patients sometimes show impressive recovery of lost functions after just months of rehabilitation. There are stories of patients that are almost paralyzed by a stroke in their sixties or seventies that go on to show dramatic recovery of movement, language and other faculties, even so late in life. Synaptic remodeling of the surviving neurons is sure to be a major factor in recovery after stroke, but there is also evidence from rodents to suggest that adult neurogenesis has a role in the repair process; inducing ischemic stroke in rats upregulates the production of new neurons and stimulates the recruitment of new cells to the damaged areas, even in regions that do not usually receive new neurons like the striatum (Arvidsson et al. 2002; Darsalia et al. 2005; and others, reviewed in Wiltrout et al. 2007). Thus, even though adult neurogenesis is restricted in the mammalian brain, there appears to be the potential to harness new neurons for regeneration of other areas, at least under some circumstances.

Adult neurogenesis may provide the brain with a repair mechanism under certain circumstances but it seems likely that the phenomenon persists in certain brains regions for reasons other than to enable repair after damage. New neurons can clearly provide a source of structural plasticity in the adult brain, but exactly what cognitive or behavioral functions this plasticity serves is not understood. The functional consequences of adult neurogenesis in the mammalian brain have been most heavily studied in the hippocampus, a structure involved in a complex set of behaviors relating to learning, memory and spatial navigation. In rats, new granule neurons are recruited into the dentate gyrus of the hippocampus continuously

throughout adulthood, resulting in an increase in their total number by about 40% between one month and one year of age (Bayer et al. 1982). This growth is likely to have functional implications for behaviors that rely on the hippocampus but due to their complex nature, it has been difficult to elucidate exactly how the new neurons are contributing to hippocampal-dependant learning and memory. Experiments to directly block the production of new neurons in the rodent hippocampus have yielded a confusing array of conflicting results that is as heterogeneous as the different labs, rodent species and behavioral paradigms used to generate them (recently reviewed in Deng et al 2010). Thus, the mammalian hippocampus does not appear to be the most useful system in which to understand the potential functions of new neurons in the adult brain.

More recently, the dentate gyrus of the hippocampus has been implicated in pattern separation, a specific form of hippocampal memory in which different episodes that occur close in space or time are encoded separately so they do not interfere with one another (Leutgeb et al. 2007; McHugh et al. 2007). Since adult hippocampal neurogenesis directly contributes new neurons to the granule layer of the dentate gyrus, it is possible that new neurons may play a role in pattern separation. One study suggests this might be the case, as mice with reduced hippocampal neurogenesis are impaired in their ability to discriminate between stimuli that are presented closely in space, but not stimuli that are presented at a distance apart (Clelland et al 2009). These preliminary findings need to be replicated with other behavioral testing paradigms before any firm conclusions can

be drawn about the contribution of new neurons to pattern separation. However, they do suggest that as we begin to unravel the complicated set of behaviors in which the dentate gyrus in involved, we may begin to understand what functional contribution adult-born neurons make to those behaviors.

A much more straightforward system in which to explore the functional role of adult neurogenesis is found in the song system of songbirds. Songbird brains fall somewhere between fish and mammals in terms of the amount of adult neurogenesis they experience: many more neurons are produced than in mammalian brains and the new neurons are not limited to a few brain areas but can integrate over much of the telencephalon (Alvarez-Buylla et al. 1994). However, neuron addition is not as prevalent as in teleost fish and bird brains do not continue to grow in adulthood. The avian song system is an interconnected set of discreet nuclei dedicated to the tasks of vocal imitation and song perception. New neurons integrate directly into HVC (originally 'high vocal center' but now used as the proper name), the major "cortical" nucleus required for song production, and many become part of the vocal motor pathway responsible for the pattern of learned song (Kirn et al. 1999; Hahnloser et al. 2002). Thus, because song is a clear and stereotyped motor output that can be easily analyzed and because new neurons are added directly to the relevant brain areas, the song system is proving to be more useful in studying the function of adult neurogenesis than the mammalian hippocampus.

The role of adult-born neurons in the avian song system was first explored in experiments conducted by Nottebohm and colleagues using the canary. Canaries are open-ended vocal learners that imitate the song of other adults during development; in future years they can modify existing syllables and add new ones (Nottebohm & Nottebohm 1986). In the adult canary, new neurons are recruited into HVC as part of a gradual replacement process (Kirn & Nottebohm 1993) and the rate at which new HVC neurons are recruited varies across the year. Peaks in cell death and neuronal recruitment coincide with seasonal periods of vocal instability that precede the appearance of new song syllables (Kirn et al. 1994). The temporal correlation between neuronal replacement and changes to the adult song suggests that new neurons in the canary HVC may have a role in vocal learning. However, partly due to a lack of appropriate experimental techniques in the canary, no experiments have yet shown that interrupting neuronal recruitment to the adult HVC hinders the learning of new songs.

In the last two decades another species of songbird, the zebra finch, has surpassed the canary as the main model system in the study of avian vocal learning, primarily due to its year-round fecundity, shorter generation time (3 months vs. 8 months in canaries) and shorter and more easily quantifiable song structure. Great progress has been made towards understanding the neural basis of song learning in the zebra finch (reviewed in Fee & Scharff 2010) and in the process several exciting genetic and optical techniques have been put to use in this system, including two-photon microscopy of HVC (Scott & Lois 2007), lentiviral-mediated gene delivery

(Schilz et al. 2010) and transgenesis (Agate et al. 2009). As in the canary, new neurons are added to the HVC of adult zebra finches and they become part of the vocal motor pathway for song (Nordeen & Nordeen 1988). Given our background knowledge of the zebra finch song system and the technologies now at our disposal, the stage is set to turn our attention once more to the functional role of neurogenesis in the adult avian brain.

However, there are key differences in singing behavior between canaries and zebra finches. While canaries can continue to modify their song in adulthood, zebra finches are “sensitive period” learners; they learn their song only once, before sexual maturity and the song mastered by that time is retained, with minor changes, for the remainder of their adult life (Immelmann 1969). Thus, a comparison of HVC neuronal recruitment in these two species may provide clues to the functional roles of adult-born neurons in an adult brain circuit. The fact that zebra finches continue to recruit new neurons to the adult HVC without participating in further song learning as adults is intriguing. Either adult neurogenesis in the song system provides different functions in canaries and zebra finches; or the role of new neurons in learning is more complex than was suggested by the early canary work; or perhaps there is, in fact, no functional relation between recruitment of new neurons into the adult HVC and song learning.

Most of our understanding of neuronal replacement in the avian song system comes from the study of canaries. In comparison, we know much less about the nature of events that occur in the adult zebra finch HVC. This thesis, therefore, aims to describe more fully the dynamics of adult neurogenesis in the zebra finch HVC in order to facilitate a comparison with the process in canaries. Also, given the new experimental toolbox available for use in this species, a better understanding of neuronal recruitment to the zebra finch HVC will provide the necessary framework for interpreting future functional studies.

The zebra finch song system: how does the brain master vocal learning?

Zebra finches are a social species and are rarely encountered alone in their natural habitats, the grasslands across most of Australia. They nest as mated pairs but it is common to find nests from several different pairs within the same tree or bush resulting in the formation of large colonies of between 20 to 1000 individuals. Although once they have mated zebra finches tend to stick with their partner, they have also been found to frequently engage in extra-pair copulations with other individuals in the colony (Birkhead et al. 1988). Breeding can occur at any time of year and is usually triggered by periods of heavy rain, which are harbingers of the seed crops that will soon follow. Both males and females vocalize frequently with contact calls but only the males sing. Song seems to serve several purposes; it attracts a female and induces hormonal changes that trigger her to adopt the copulatory position and stimulate ovulation (Zann 1996). In these contexts, song may act as a fitness indicator during mate selection as females show preferences for

different song characteristics (Clayton & Pröve 1989; Woolley & Doupe 2008; Neubauer 2000). One of these characteristics is stereotypy: females prefer song in which every rendition is sung the same way (Woolley & Doupe 2008). Since breeding can occur at any time of the year and the song is so crucial to courtship, both within mated pairs and when soliciting extra-pair copulations, a male's song must be stereotyped all year round. This is in stark contrast to seasonally breeding species like the canary whose song goes through periods of instability between breeding seasons. Vocal instability during the early autumn and early spring allows canaries to modify their songs by adding new syllables, which is an important part of the breeding preparation for a male because female canaries prefer males with more syllables in their vocal repertoire (Kroodsma 1976). This species difference in annual song stability places different demands on the song system of canaries and zebra finches which, as we will discuss later, is reflected in the neural behavior of the song circuit.

Adult zebra finch song is a highly stereotyped sequence of repeated "motifs" sung in succession. Each singing event is called a "bout" and is usually composed of several stuttered short "introductory notes" followed by the repeated motif, which is comprised of several distinct units of continuous sound called "syllables" (Figure 1.1). Zebra finches are vocal learners, meaning that their final adult song is acquired via imitative learning from an adult conspecific tutor, usually their father (Immelmann 1969). This process has attracted much attention from the scientific community because it has many similarities to the way human infants learn speech (Thorpe &

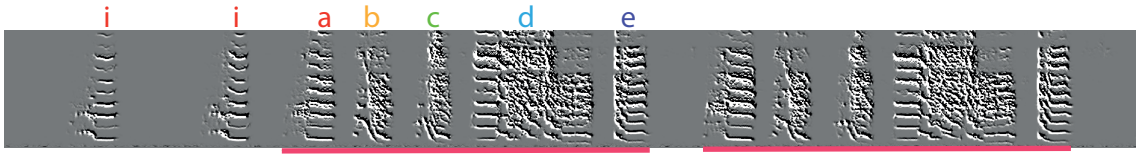


Figure 1.1: The structure of a typical adult zebra finch song.
This is the start of a typical song bout. There are two introductory notes (i) followed by two repetitions of the song motif which is underlined in pink. This bird also uses a facsimile of the introductory note as the first syllable of the motif, labeled a, followed by syllables b-e. Syllables are separated by silent intervals. Sound is represented in sonogram of spectral derivatives with frequency is on the vertical axis and time on the horizontal axis. The edge between the black and white components of each trace denotes the frequency of maximal amplitude (Tchernichovski et al. 2000).

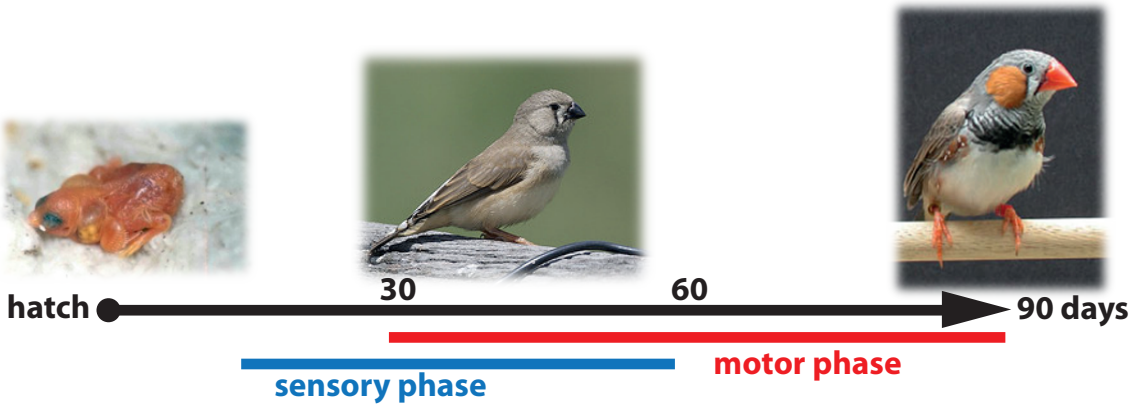


Figure 1.2: The stages of vocal learning in the zebra finch.
Zebra finches fledge the nest at 12-15 days of age. Memorization of a song model starts around day 20. The model can be the song of its father or of another nearby male. The sensitive period for model memorization ("sensory phase") in a socially reared bird closes at approximately 60 days of age and the juvenile will not imitate song elements presented after this time. The motor phase of song learning begins at approximately 30 days of age, when the young male starts to produce soft, variable sounds called subsong, which are then gradually shaped into the structured syllables characteristic of adult song. The adult song is said to 'crystallize' at 90 days of age, by which time the male is sexually mature.

Pilcher 1958; Marler 1970; Doupe & Kuhl 1999). Song learning occurs through a series of stages (Figure 1.2) that begin with a sensory phase where the young male listens to and internalizes the tutor song; this auditory memory later guides the attempt to imitate the external model, which can only be done if the birds hearing is intact (Konishi 1965). Around posthatch day 35 the young bird begins to practice with its vocal organ. It produces strings of noisy and unstructured vocalizations known as “subsong” which have been compared to the babbling of a 6 month old human infant (Thorpe 1955). Over the next two months, the juvenile uses auditory feedback to refine its own vocalizations making them more and more like those of the memorized song. The state of transition between subsong and learned song is called “plastic song”. During this transition, the rudimentary syllables of subsong become more structured and less variable and begin to appear more like the adult syllables of the tutor song. At the time of sexual maturity when the young male reaches about 90 days and is reproductively competent, its song is said to have “crystallized” into its adult form. Although song is learned by imitation, the copies are seldom perfect and therefore most adult male zebra finches will have their own unique song pattern.

The brain areas required for vocal learning and song production are much alike in canaries (Nottebohm et al. 1976; Nottebohm et al. 1982) and zebra finches (Bottjer et al. 1989; Scharff & Nottebohm 1991) and in all songbird species studied (Figure 1.3; Wild 1997). The song system is comprised of a connected set of discreet nuclei in the telecephalon, basal ganglia and thalamus that converge on a

subset of motor neurons of the hypoglossal nucleus (the tracheosyringeal portion of the nucleus of the twelfth cranial nerve, nXIIts). These motor neurons directly innervate the muscles of the syrinx, the vocal organ of the songbird, and work in concert with other motor neurons that control respiratory, laryngeal and jaw muscles to produce the sounds of song (reviewed in Wild 2004). The timing of song seems to originate in the telencephalic nucleus HVC (which initially stood for “high vocal center” but is now used as a proper name) because it has been shown that cooling it, but not other brain areas, with a peltier device uniformly stretches out all parts of the song motif (Long & Fee 2008). These timing signals are passed down to the nXIIts motor neurons via a second telencephalic nucleus RA (robust nucleus of the arcopallium) to make up the descending vocal motor branch of the song system (HVC $\rightarrow$ RA $\rightarrow$ nXIIts, see figure 1.3). The neurons connecting HVC to RA (subsequently referred to as HVC-RA neurons) are thought to encode the learned pattern for song; they exhibit a very sparse and stereotyped firing pattern with each HVC-RA neuron generating a single 6 millisecond burst at a specific time in the song motif (Hahnloser et al. 2002). RA neurons also generate stereotyped bursts during singing but they exhibit more complex bursting patterns than the HVC-RA neurons (Yu & Margoliash 1996). Each RA neuron produces a pattern of roughly 12 bursts lasting about 10 ms each, that is reproduced every time the bird sings its motif and unlike HVC-RA neurons, the same RA neurons can be active during different parts of the song motif (Leonardo & Fee 2005). Lesioning either HVC or RA or severing the connection between the two nuclei in an adult results in complete loss of the adult song (Scharff & Nottebohm 1991; Aronov et al. 2008) Thus, it is the

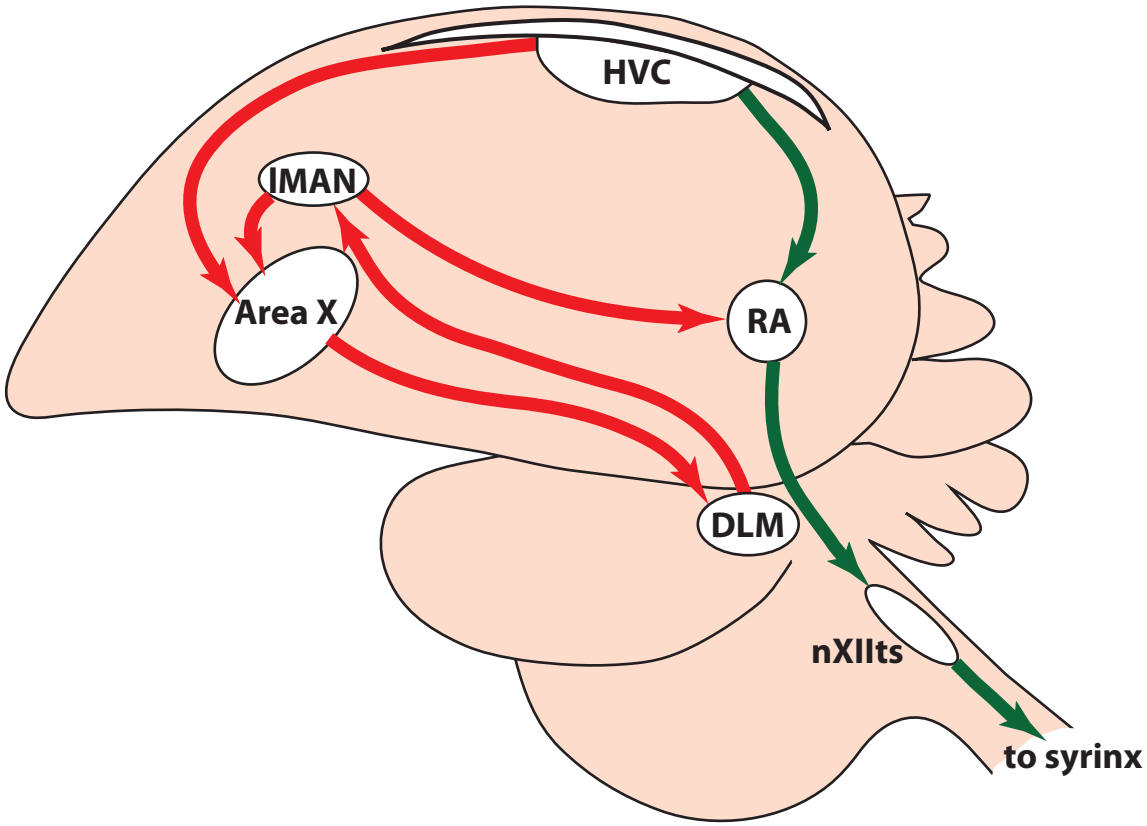


Figure 1.3: The avian song system.
Green arrows connect HVC, nucleus RA and nXIIts, the descending vocal motor pathway that is essential for the production of learned song. nXIIts in the brain stem contains motor neurons that directly innervate the muscles of the syrinx. Red arrows connect HVC, Area X, DLM, lMAN and RA, forming an anterior pathway essential for vocal learning but not for the production of adult song. HVC= originally 'high vocal center' but now used as a proper name, RA= robust nucleus of the arcopallium, nXIIts= the tracheosyringeal portion of the nucleus of the twelfth cranial nerve, Area X= proper name (homologous to the mammalian striatum), DLM= medial portion of the dorsolateral thalamus, lMAN= lateral magnocellular nucleus of the anterior nidopallium.

hierarchical efforts of HVC, RA and nXIIts neurons that largely determine the pattern of song produced by an adult male zebra finch.

In addition to the descending vocal motor pathway described above, HVC also gives rise to a second branch, the anterior forebrain pathway, that is essential for vocal learning but not for the production of learned song (Bottjer et al. 1989; Scharff & Nottebohm 1991). In the anterior forebrain pathway a second class of HVC projection neurons send axons anteriorly to Area X, an area that shows striking similarities with the striatum of the mammalian basal ganglia both in terms of neural composition and connectivity (Luo et al. 2001). Connections pass from Area X down to DLM (medial portion of the dorsolateral thalamus) in the thalamus, then back up to telencephalic lMAN (lateral magnocellular nucleus of the anterior nidopallium) and finally output on the motor pathway at RA (see Figure 1.3). The same lMAN neurons that project to RA also send axons back to Area X to form a feedback loop (Vates et al. 1997). Lesions of this pathway have severe effects on song learning in juveniles. Removing lMAN from the system during the juvenile stage causes premature crystallization and results in an impoverished song composed of a few repeated simple syllables (Bottjer et al. 1989; Scharff & Nottebohm 1991). In contrast, lesions of Area X result in an adult song that never appears to crystallize as it contains poorly structured syllables that are sung in a highly variable sequence (Scharff & Nottebohm 1991). In both cases, juveniles make very poor imitations of the tutor song.

Although the anterior forebrain pathway is not required for the production of adult song, it does still play a role in adult singing behavior. Males produce two kinds of song in different social contexts; they sing “directed” song towards a female where it is typically coupled with specific postures and head movements, and “undirected” song when alone or when not singing towards another individual (Zann 1996). The basic song motif and bout structure is identical in both social contexts but there are some differences between the two types of singing. Directed song is more of a refined performance than undirected song; it is sung faster, has more introductory notes, more motifs per bout and has reduced variability in the syllable fundamental frequency across motifs (Roland 1980; Kao et al. 2005). Electrophysiological recordings of lMAN and Area X neurons in adults show both areas increase their firing rates during singing but that undirected song elicits higher activity levels and a more variable firing pattern in both brain regions than directed song (Hessler & Doupe 1999). Permanent lesion or temporary pharmacological inhibition of lMAN reduces the variability and increases the tempo of undirected song to that of directed song but has no effect on the number of introductory notes or the number of motifs per bout (Brainard & Doupe 2001; Kao et al. 2005; Kao & Brainard 2006; Stepanek & Doupe 2010). Thus, the anterior forebrain pathway has a direct role in modulating song variability and speed in adults but its activity is not responsible for all context-driven differences in singing behavior.

Considering that by the time male zebra finches reach sexual maturity they are capable of producing highly stereotyped song towards a female, the need for variable undirected song is not obvious. Variability in motor output is important during song learning as it allows the juvenile to experiment with his vocal organ and explore acoustic space. Vocal imitation in songbirds is an example of trial-and-error learning where random variation in sound output is analyzed with regards to the tutor song and any improvements towards the desired outcome are permanently incorporated into the developing motor program (Troyer & Doupe 2000). The anterior forebrain pathway, and specifically lMAN provides the source of motor variability for song learning in juveniles (Olveczky et al. 2005), but the role, if any, of lMAN-generated variability in adult undirected song remains unclear. There is some evidence that it might be involved in correcting errors that could appear in the song as a result of aging or CNS damage (see below; Tumer & Brainard 2007; Andalman & Fee 2009).

Although song learning ends at sexual maturity in the zebra finch, adult song still shows signs of plasticity. For example, deafening in adulthood results in deterioration of the temporal and acoustic features of song over several weeks (Nordeen & Nordeen 1992), suggesting auditory feedback is used to actively maintain song structure in adult zebra finches. Furthermore, lesioning lMAN prior to deafening completely inhibits deterioration of adult song (Brainard & Doupe 2000), showing the anterior forebrain pathway plays an active role in the deterioration of song that follows deafening. Two recent studies have shown that the anterior

forebrain pathway also has a role in other forms of adult vocal plasticity. Both studies used noise playbacks during singing to disrupt auditory feedback in an attempt to trigger vocal learning in adults. By making the noise playback contingent on the pitch of a particular “trigger” syllable, birds will rapidly learn to modify the pitch of that syllable to avoid hearing the noise (Tumer & Brainard 2007; Andalman & Fee 2009). Presumably since the noise is played over the second half of the trigger syllable, it is detected in the auditory feedback process as an “error” signal and using trial-and-error learning the bird is able to bias the pitch of that syllable to escape playback and thus correct the perceived error. Pharmacological silencing of lMAN during this experimentally-induced learning procedure results in an immediate return in the pitch of the trigger syllable to baseline, indicating lMAN is active in the learning process. However, if multiple days of disrupted auditory feedback training are used to shift the pitch of the trigger syllable in a step-wise fashion each day, silencing lMAN results in the pitch returning to what it was at the beginning of the day of learning and not back to the original baseline (Andalman & Fee 2009). Therefore, lMAN is necessary for biasing the pitch within a 24 hour period but after a night of sleep the learning becomes lMAN-independant, presumably by becoming incorporated into the motor circuitry. These clever experiments have provided a direct demonstration of some of the neural components required for trial-and-error learning in the song system and have shown that even though adult zebra finches do not usually change their song beyond sexual maturity, they do retain the capacity to do so using the same neural circuitry that juveniles use for vocal learning.

Neurogenesis in the avian song system

As described earlier, the avian song system provides a unique opportunity to study the functional consequences of adding new neurons to the adult brain. Not only do birds show much more prominent levels of adult neurogenesis than mammals but new neurons integrate directly into brain areas responsible for producing a well-defined behavioral output. The brain areas and neuron types involved in song production are well described and song is a stereotyped and quantifiable behavior. Thus, by monitoring singing behavior and quantifying changes in the song pattern as new neurons integrate into the pathway, we may be able to get insights into their function.

The avian vocal motor pathway for song is unusual because unlike most vertebrate brain systems which are laid down in the embryo or during very early development, the connection between HVC and RA does not form until later. After hatching, neurons produced in the ventricular zone above HVC migrate in large numbers into this vocal control nucleus and send projections down to RA (Goldman & Nottebohm 1983; Scott & Lois 2007). Retrograde tracer injections into RA in canaries to label the HVC-RA neurons show that the pathway begins to form around 1 month after hatching and is fully formed by 4 months (Alvarez-Buylla et al. 1992). These cellular events coincide with vocal learning; around 1 month of age young canaries begin to produce early subsong and by 4 months most of the syllables that will be present during the first breeding season (~10 months of age) can already be identified (Nottebohm & Nottebohm 1986). Thus, the motor circuit physically

develops during the time the young bird is learning to utilize the pathway. Interestingly, not all the neuron types in HVC are laid down postnatally. Most of the HVC-X projection neurons are produced in the embryo or during the first few days after hatching (Alvarez-buylla & Theelen 1988) and so the late addition of HVC-RA neurons to the system is not just a developmental delay in the maturation of HVC. Perhaps the RA-projecting neurons require instructions from the song learning experience to correctly integrate into the motor pathway.

Even though the HVC of male canaries reaches its adult size at 4 months, new HVC-RA neurons are added after then and throughout adulthood, but at a lower rate than in the early juvenile period. The neurons that continue to be added after the full adult complement is reached are thought to numerically replace older neurons because the adult HVC does not increase in size or total neuron number (Alvarez-buylla & Nottebohm 1988). Direct evidence for numerical replacement was obtained by labeling canary HVC-RA neurons present in April in one year olds with one colour of retrograde tracer and then using a second colour of tracer to label all the HVC-RA neurons present in October of the same year. In October, only 50% of the HVC-RA neurons were retrogradely labeled by both tracers, suggesting that half of the April neurons had been replaced over the 6 month period (Kirn & Nottebohm 1993). The inference that HVC-RA neurons are replaced in adulthood presents a bit of a paradox: if the neurons laid down in the juvenile period learn to control song, then surely replacing them would result in loss of that learning? A clue to the

potential function of neuronal replacement came from exploring the timing of cellular events in HVC. As mentioned above, canaries are open-ended vocal learners and can continue to modify their songs in adulthood (Nottebohm et al. 1986). During the fall of every year their songs undergo periods of vocal instability that precede the addition of new song syllables to their repertoire before the next round of spring breeding begins. Interestingly, the rate of neuronal replacement in the adult canary HVC varies annually; peaks in cell death in August and January are followed a few weeks later by peaks in the incorporation of new neurons, and the timing of these cellular events coincides with the appearance of new song syllables (Kirn et al. 1994). These correlational data suggests that neuronal replacement in the canary HVC may allow for adult song learning and was the first proposal of a possible role for adult neurogenesis in the vertebrate brain.

New neurons can be labeled by giving canaries systemic injections of ^{3}H-thymidine, a radioactive marker of cell proliferation that gets incorporated into newly synthesized DNA during cell division. Neurons born at the time of ^{3}H- thymidine injection will take up label and can be visualized in brain sections using autoradiography. Using this method, the timecourse for neuronal integration into the canary HVC was elucidated in a series of studies. Eight days after ^{3}H-thymidine injection, post-migratory labeled neurons begin to appear in HVC, with the number of labeled neurons peaking at around 2 weeks. Between the second and third week after injection, the number of labeled neurons in HVC is cut by half and this same number is present at 4 weeks (Kirn et al. 1999). From 3 weeks on, cells start to take

up retrograde tracers from RA, although only very few at this early stage (Kirn et al. 1999). After one month, 50% of new neurons in HVC can be labeled by retrograde tracer from RA and by 8 months, 75% of new neurons can be labeled in this manner (Kirn et al. 1991). New neurons have never been labeled with retrograde tracer injected into area X in adulthood. Therefore, the majority of new neurons that integrate into the canary HVC become RA-projecting neurons but there also appears to be addition of another type of neuron that does not take up tracer from RA or Area X. We know that new neurons are incorporated into functional circuits within HVC because one month after birth it is possible to elicit responses to auditory stimuli in some of them (Paton & Nottebohm 1984), in line with earlier reports that neurons in HVC respond to sound (Katz & Gurney 1981).

Culling of new HVC neurons does not end after the initial half disappear between survival weeks 2 and 3. The remaining new neurons are still vulnerable and their survival can be affected by use of the song circuit. If birds are injected with ^{3}H-thymidine and then after 30 days are prevented from singing for 8 days, the number of neurons present by survival day 38 is 40% fewer than the number present in birds that were able to sing unhindered during the 8 days (Li et al. 2000). Therefore, experience and use of the song circuit even 1 full month after neuron birth is important for survival of new neurons in HVC. As mentioned above, the time of year in which a neuron is born also affects its survival. Neurons born in September survive at least 8 months and are therefore around to perform in song during the following breeding season. In contrast, almost all new neurons born in May have

disappeared eight months later (Alvarez-Borda et al. 2004). This difference in survival could reflect seasonal hormone changes or seasonal differences in use of the circuit at the time of recruitment (Alvarez-Borda & Nottebohm 2002).

Although less research has been done on adult neurogenesis in the zebra finch song system, we do know that it shares many features of the process in canaries. The HVC-RA connection is first established during the juvenile song learning period (Nordeen & Nordeen 1988) and production of new HVC-RA neurons continues throughout adulthood, although the rate of new neuron recruitment declines after posthatch day 40 (Wilbrecht et al. 2002). The rate of neuron addition to HVC in an adult zebra finch is similar to that in a spring canary singing stable song (Alvarez-Buylla et al. 1990). As in canaries, HVC-X neurons do not seem to be produced in adulthood (Scotto-lomassese et al. 2007). After one month survival, only 42% of new neurons in the HVC of adult zebra finches can be backfilled with a retrograde tracer from RA, which is similar to the 50% seen in canaries. Presumably, longer survival times would result in an increasing proportion that can be labeled from RA. However, as in canaries, there seems to be a percentage of adult-born neurons in the zebra finch HVC that do not become HVC-RA neurons, or at least do not take up tracer from RA. It was thought that these cells become interneurons but a study in zebra finches has failed to find any neurons recruited into the adult HVC expressing interneuron markers (Scotto-lomassese et al. 2007). Thus, the exact nature of at least some of the neurons added to HVC in the adult remains an open question in both canaries and zebra finches.

What is the function of new neurons in the avian song system? From the canary literature it seems that new neurons are involved in motor learning: in both the juvenile and the adult new neurons integrate into HVC during periods of new syllable acquisition. But adult zebra finches do not learn new song elements beyond sexual maturity and yet their HVCs continue to incorporate new neurons throughout adulthood. Historically, this inconsistency has been explained by the lower levels of neurogenesis in adult zebra finches than canaries. Neuronal addition with presumed replacement occurs all year round in the canary, even during the spring breeding season when adult song is relatively stable, and the level of neuronal recruitment to the adult zebra finch HVC is similar to the level in a spring canary (Alvarez-Buylla et al. 1990). So perhaps lower levels of neuronal replacement can occur without significant changes to song. The paradox is that zebra finches replace HVC neurons at all, given that they do not make any significant changes to their song in adulthood. Perhaps there is redundancy in the way HVC-RA neurons encode song and so some of the neurons can be exchanged without an overall effect on vocal output. Or perhaps not all HVC-RA neurons fire during singing at any one time (see Fee at al. 2004) and it is these “non-singing” neurons that are replaced in adulthood. The latter hypothesis seems an unlikely explanation for the role of neurogenesis in adult zebra finches that retain a stable and stereotyped song during their entire adult life. If HVC-RA neurons were constantly being replaced, albeit at a low level, eventually one would expect some of the key neurons to be exchanged, unless of course there is a class of HVC-RA neuron that is never involved in song or if in fact replacement is not occurring at all. Clearly there are still many questions to be answered.

The ageing zebra finch

Although zebra finches do not modify their song motifs in adulthood, there are subtle changes occurring in song, and therefore in the song system, with age. The first is that the song structure becomes less dependent on auditory feedback as a bird gets older. As mentioned earlier, deafening in adulthood results in deterioration of the temporal and acoustic structure of song (Nordeen & Nordeen 1992). The rate at which song is disrupted is affected by the birds age at the time of deafening. While birds up to 5 months of age will show severe song deterioration within a couple of weeks after cochlea removal, birds that are older than 2 years at the time of deafening will retain their song structure for up to a year. Around 6 months of age there appears to be a transition point when song becomes much more resilient to disruption after auditory feedback is removed (Lombardino & Nottebohm 2000; Brainard & Doupe 2000).

Ageing also brings about an increase in the stereotypy of adult zebra finch song. Detailed analysis of undirected song shows there are fine-grained structural improvements in syllable structure with age. Songs from old adults (> 4 years) show significantly less variability in the syllable frequency than younger adults (< 6 months) (Kao & Brainard 2006). Also, recordings from the same bird at 4 and 15 months of age show a significant improvement in the motif stereotypy across multiple singing bouts with age (Pytte et al. 2007). Given than IMAN is a key source of motor variability in the song system, it is possible that the observed reduction in undirected song stereotypy is brought about by a reduction in the variable firing rate

of lMAN with age. Electrophysiological recordings from lMAN during undirected singing show that while lMAN firing variability is reduced in older birds, the decrease is not enough to explain the observed reduction in song variability (Kao & Brainard 2006). There is another source of increased motor stereotypy with age, perhaps yet undefined changes in the vocal motor pathway render it less susceptible to perturbations from lMAN or elsewhere. Lombardino and Nottebohm (2000) suggested that the more times a zebra finch sings its song, the more it becomes "ingrained" in the motor circuits that encode it, but they did not try to explain how this could occur.

One potential explanation for the stabilizing effects of age on song is an age-related decline in neurogenesis in HVC. As in the mammalian brain, the rate of adult neurogenesis decreases with age in the zebra finch (Wang et al. 2002). Although new neurons incorporate into the HVC of the oldest birds examined (Tony Lombardino, PhD thesis), they do so at a much lower rate than occurs in young adults. If neuronal incorporation into the zebra finch HVC acts as a source of variability in the motor program for song, then one could imagine that an age-related decline in neurogenesis would result in a reduction in song variability with age. Similarly, if the deterioration of song after deafening involves new neurons then a reduction in neurogenesis with age could explain why it takes much longer for deafness to induce song changes in older than in younger adults. This could be the case if, for example, new neurons require auditory feedback to learn their correct role in the motor pattern and accumulation of untrained neurons in HVC causes

normal song to be disrupted.

The issue is complicated further by the ability of lMAN lesions to block loss of song stereotypy after deafening (Brainard & Doupe 2000). If the accumulation of untrained neurons in HVC is responsible for song deterioration, then lMAN lesions must somehow interfere with the accumulation process. This hypothesis was tested directly by Scott, Nordeen and Nordeen in 2007 by comparing the survival of new neurons in HVC in birds that were deafened with or without prior bilateral lesion of lMAN. The authors found no differences in the survival of new neurons in HVC that were born in the week after deafening (Scott et al. 2007). While this experiment argues against a role of adult neurogenesis in the effects of deafening on song, it is possible that timing the BrdU injections to the week after deafening was too early to capture any effects of the surgical manipulations on the rate of neurogenesis or neuron survival. More experiments are needed to clarify the issue.

Goals of this thesis.

It is clear from the preceding review that there are many unanswered questions relating to the role of adult neurogenesis in the zebra finch song system. The aims of this thesis are to clarify some of the issues and provide a more thorough description of the process of neuron addition to the adult zebra finch HVC. Specifically, the body of work described here attempts to answer the following questions…

1. Do neurons added to the HVC of adult male zebra finches numerically replace older neurons or do they result in net addition?

2. What kinds of HVC neurons are born in the adult zebra finch?

3. How do age and the social environment affect neuron addition?

4. Is auditory feedback required for neuron addition to HVC?

5. Does neuron addition to the zebra finch HVC relate to changes in adult song?

6. Through a comparison with the previous canary work, are there systematic differences in the dynamics of HVC neuronal recruitment between adult canaries and adult zebra finches?

CHAPTER 2: NET NEURON ADDITION TO THE ADULT ZEBRA FINCH HVC

Counting the two types of projection neuron in HVC

Zebra finch males become sexually mature 80-90 days after hatching and by that time they have mastered the song they will sing for the remainder of their lives. Early reports of adult neurogenesis in sexually mature zebra finch males found that 0.26% of all neurons in HVC were labeled with radioactive ^{3}H-thymidine per day of ^{3}H-thymidine injection (Nottebohm 1984). This may seem like a small number but if every new neuron added to the adult HVC were to survive indefinitely, this rate would result in a doubling of the HVC neuron population within a year. Given the mounting evidence in the canary at the time, numerical replacement of old neurons by new ones seemed to be a more plausible scenario than net neuronal addition for the adult zebra finch HVC. However, despite a thorough exploration of adult neurogenesis in canaries, where it seems to be associated with replacement, there have been no data to tell whether replacement is also the predominant mode of neuron integration in the zebra finch HVC.

Characterization of the dynamics of neuron addition to the adult zebra finch HVC began with a simple experiment: we counted the number of neurons in HVC in adult males of different ages. Cholera toxin subunit B (CTB) is an efficient neuroanatomical tracer that is transported retrogradely from axon terminals back to the soma of projection neurons (Conte et al. 2009). This technique, which has been used before in the songbird brain (Lombardino et al. 2005; Scotto-lomassese et al.

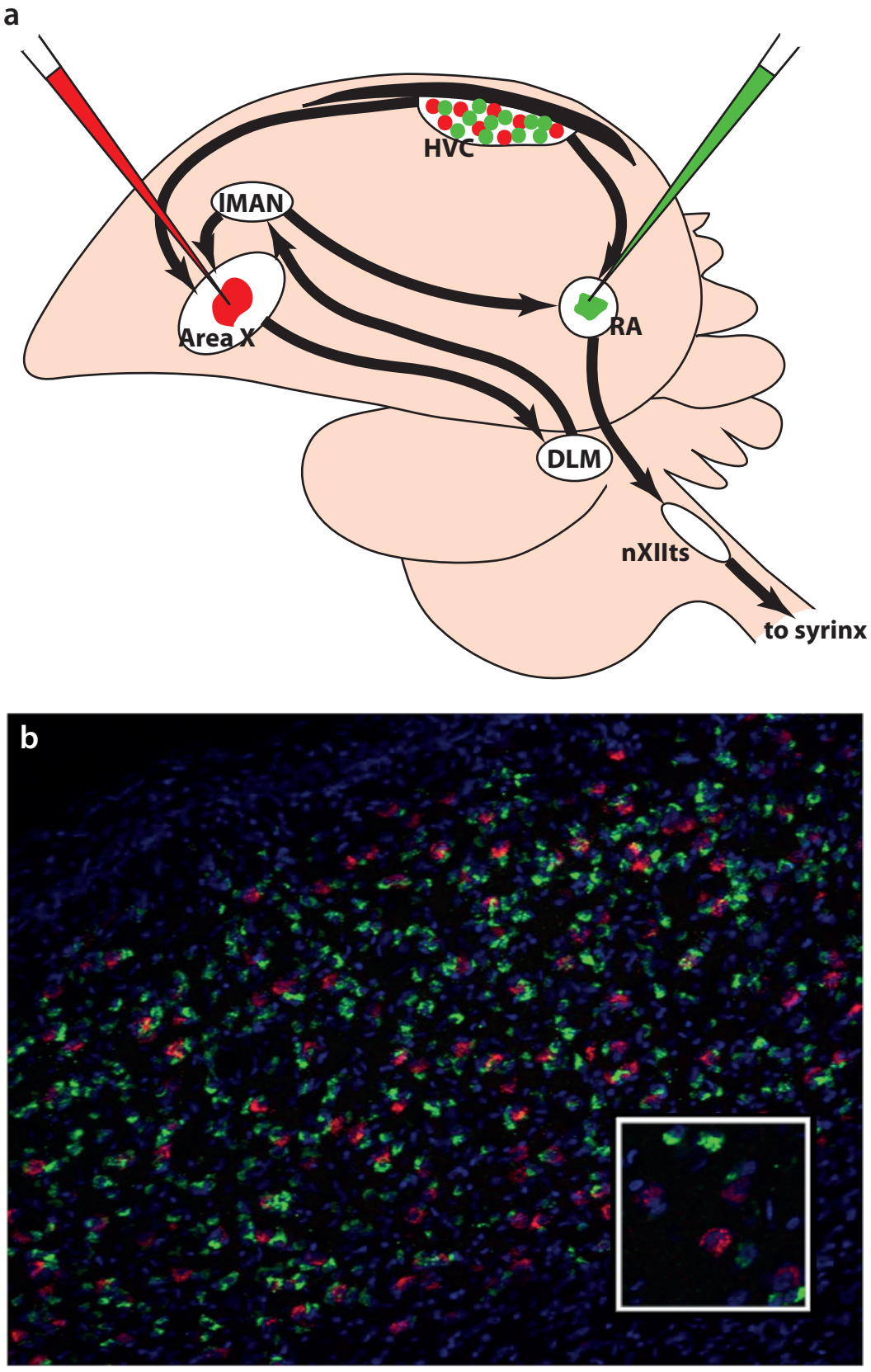
2007; Scott & Lois 2007), ensures proper identification of projection neurons based on the target brain nucleus that they innervate. By injecting CTB conjugated to green or red fluorescent dyes into nucleus RA and Area X, respectively, it was possible to label the two populations of projection neurons within HVC (Figure 2.1). Using birds ranging in age from 90 days to 11 years, we performed bilateral tracer injections, allowed one week for retrograde transport of CTB, dissected and sectioned the brains and imaged the sections on a confocal microscope (see methods for details). The total number of both neuron types was determined using the optical fractionator method: an unbiased stereological method for estimating the number of objects in a tissue by counting objects in a random but systematically sampled fraction of that tissue (West & Gundersen 1990; details in the methods section). This method counts objects within an array of 3D counting spaces contained within in thick tissue sections and is therefore less susceptible to the cell-splitting errors that arise when cell fragments are counted in thinner tissue sections. Number estimates derived using the optical fractionator method are also immune to any differences in tissue shrinkage that could result from the histological processing of samples because they do not rely on the density of objects in a small sample area but systematically sample across the entire region of interest. For an age-related neurological study, this is a key consideration because old brain tissue does not shrink as much during processing as young brain tissue and therefore estimates of neuron numbers that rely on sampling neuronal density can be biased (Haug et al. 1984). In fact, the long-standing misconception that the human cortex undergoes significant neuron loss with age partly resulted from counts of human cortical neurons that did not take into

Figure 2.1: Labeling the populations of projection neurons in HVC with retrograde tracers.

a. Schematic illustration of the double labeling surgery. Green fluorescent cholera toxin B (Alexa-488-CTB, Molecular Probes) is injected bilaterally into nucleus RA and red fluorescent cholera toxin B (Alexa-555-CTB, Molecular Probes) is injected bilaterally into Area X. Birds are killed after six days to allow retrograde transport of the tracers back to the cell bodies in HVC.

b. A view through a 20x magnified confocal stack taken of HVC showing the two intermingled populations of retrogradely-labeled projection neurons. Green= HVC-RA neurons, red= HVC-X neurons, blue= nuclear stain (To-Pro-3-iodide, Molecular probes). Inset: part of the image with 4x zoom to show the magnification used for cell counts.

Figure 2.1:



account age-dependent differences in tissue shrinkage (reviewed in Morrison & Hof 1997).

Using the optical fractionator method in the zebra finch HVC, we find that there is a significant increase in the total number of HVC-RA neurons between 90 days and 11 years of age (linear regression: R^2= 0.35,p< 0.0005, Figure 2.2a). In contrast, the number of HVC-X neurons, which are not produced in adulthood (Alvarez-buylla & Theelen 1988; Scotto-lomassese et al. 2007), show no change over the same span of years (linear regression: R^2=0.12, p=0.19, Figure 2.2a). Each point in Figure 2.2a represents the average neuron count from the left and right HVCs within an individual bird, but in the few cases where tracer injections were only correctly targeted in one hemisphere, the data represents the count from that HVC alone. There were no systematic differences in the number of either HVC neuron type between the left and right hemispheres.

HVC volume for each bird was estimated by measuring the cross-sectional area in every third section and multiplying the sum of areas by the section thickness and the sampling interval. We find that HVC volume does not change with age (Figure 2.2b) but there is, as described by others for both canaries (Kirn et al. 1999) and zebra finches (Airey et al. 2000), a considerable degree of heterogeneity in HVC volume between individuals of a given age. The reason for such high variability in HVC size is unknown but it has been correlated with the complexity of song, as measured by the number of unique syllables produced by an individual (MacDougall-

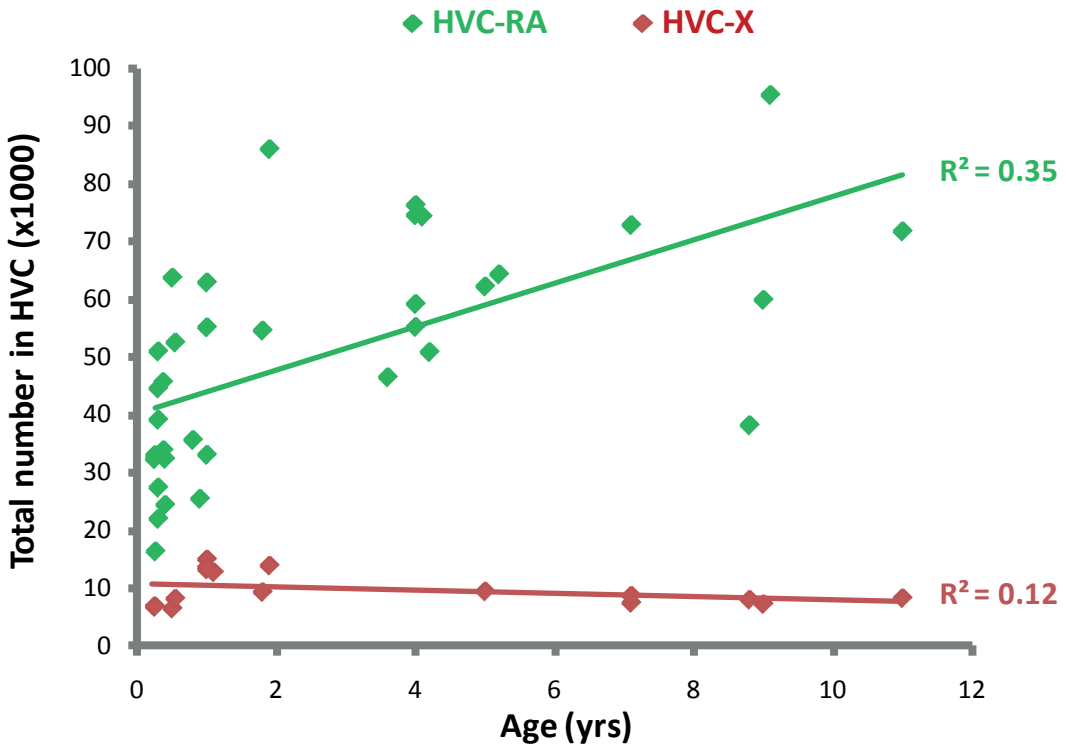
Figure 2.2: The number of HVC-RA neurons, but not the number of HVC-X neurons or the volume of HVC, change with age in the adult zebra finch.

a. Counts of the total number of retrogradely-labeled neurons of each projection type using the optical dissector method show there is a significant increase in the number of HVC-RA neurons from 90 days to 11 years of age (R^2= 0.35, $p<0.0005$) but there is no change in the number of HVC-X neurons across the same age range (R^2=0.12, p=0.19). Each point represents the average neuron count from the left and right HVC from an individual bird

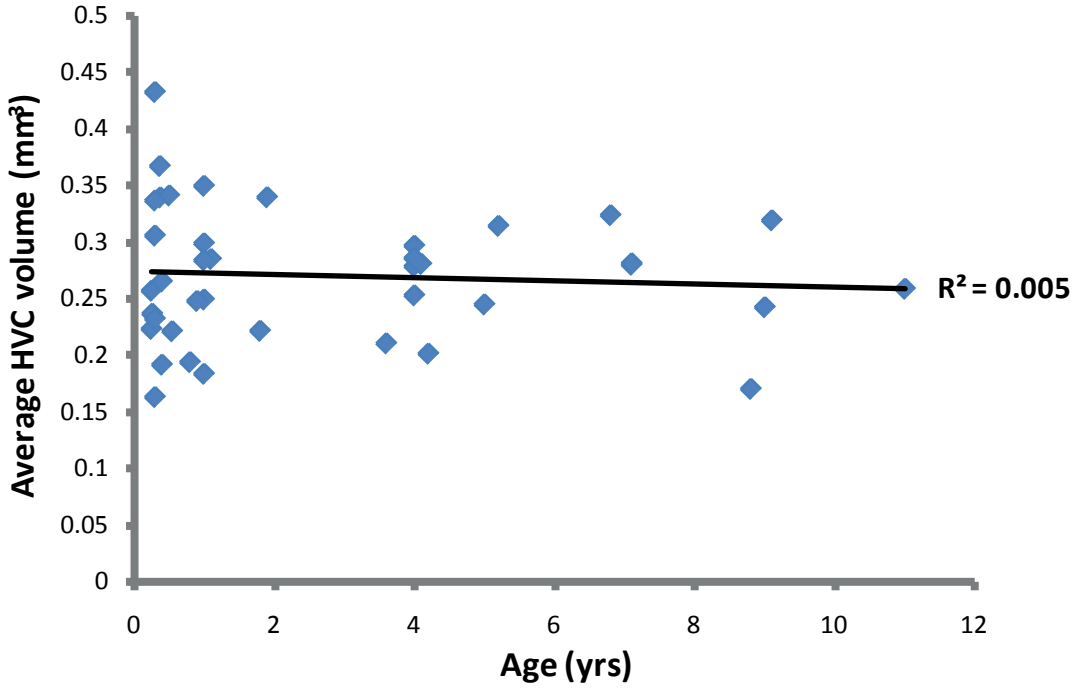
b. There is no change in the volume of HVC between 90 days and 11 years (R^2=0.005, NS). The mean HVC volume is 0.26 mm^3 but there is a great deal of variability between individuals, range = 0.15 mm^3 to 0.45 mm^3. Each point represents the average volume from left and right HVC within an individual bird.

Figure 2.2:

a


HVC-RA
HVC-X
Total number in HVC (x1000)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Age (yrs)
R² = 0.35
R² = 0.12

b


Average HVC volume (mm³)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
Age (yrs)
R² = 0.005

Shackleton et al. 1998) and also seems to be partly genetically determined (Airey & DeVoogd 2000). Since variation in HVC size is a significant source of variation in our data, we calculated the density of HVC-RA and HVC-X neurons for each bird by dividing the total number of neurons in each HVC by the volume of that HVC and then taking the average density between the left and right HVCs. The neuron density data showed less variability than total neuron number and as a result it revealed additional information about the relationship between HVC-RA neurons and age; there is highly significant increase in the density of HVC-RA neurons with age but this increase is described better by a logarithmic relationship than a linear one (log regression: R^2= 0.74, p< 0.0005, Figure 2.3). A logarithmic relationship indicates that the rate of increase slows with age, presumably as the rate of neuronal addition to HVC also slows (Wang et al. 2002). In concordance with the HVC-X total number data and the observation that no new HVC-X neurons are added in adulthood, there is no change in the density of HVC-X neurons across the adult lifespan (linear regression: R^2= 0.03, p= 0.56). From here on, neuron counts will always be presented as neuron density, the number of neurons per unit volume, rather than total neuron number in order to eliminate the variability inflicted by the natural diversity of HVC size. However, because the variability in HVC volume (and therefore in total neuron number) may be biologically relevant, statistical analyses will be conducted for both neuron density and total neuron number to ensure the same relationships are present in both data sets.

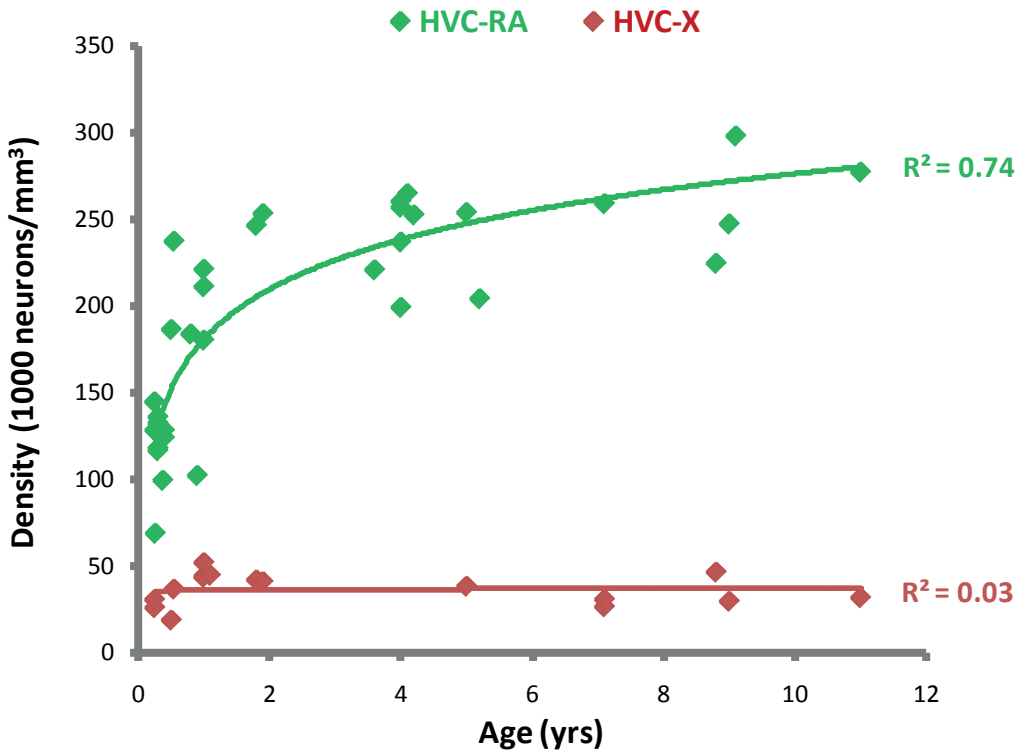


Figure 2.3: The density of HVC-RA neurons shows a significant but non-linear increase with age in the adult zebra finch.

The density of retrogradely-labeled HVC-RA neurons shows a significant increase between 90 days and 11 years of age and it is best modeled by a logarithmic relationship (R^2= 0.74, $p < 0.001$) indicating that the rate of increase is gradually slowing down with age. By contrast, the density of retrogradely-labeled HVC-X neurons does not change over the same age range (R^2= 0.03, p= 0.56). Each data point represents the average of left and right HVC from an individual bird.

Verifying the increase in HVC-RA neuron density with age

Our data show for the first time that there is net addition of RA-projecting neurons to the adult zebra finch song system. HVC-RA neurons are added in large numbers in early adulthood, resulting in an almost doubling of the neuron population between the ages of three months and two years. The rate of addition slows down with age, but the trend of increasing HVC-RA numbers continues across the adult lifespan. To verify that the increasing number of HVC-RA neurons results in a net increase in the total number of all neurons in HVC, we stained HVC sections from a sample of experimental birds with an antibody against Hu, a marker of neuronal cell fate, that has been previously tested in the songbird brain (Barami et al. 1995; Figure 2.4a) and used the optical fractionator method to count all HVC neurons in birds of different ages. If HVC-RA neurons are increasing, this should be reflected in the total neuron population of HVC since HVC-RA neurons make up about half of all HVC neurons (Nordeen & Nordeen 1988; Kirn et al 1991; Ward et al. 2001). Indeed, there is a significant increase in both the total number and the density of Hu-positive neurons in HVC with age (Figure 2.4b; for total number, linear regression: R^2= 0.28, p= 0.036; for density, linear regression: R^2= 0.63, p= 0.0002).

To further characterize the changes in HVC-RA neuron density with age, we examined whether the neurons were getting smaller or closer together in space. To get an indication of neuron size we measured the nuclear diameter of 40 randomly selected HVC-RA neurons per bird (20 from each HVC) across the range of ages from 90 days to 11 years. We find a 10% reduction in average nuclear diameter

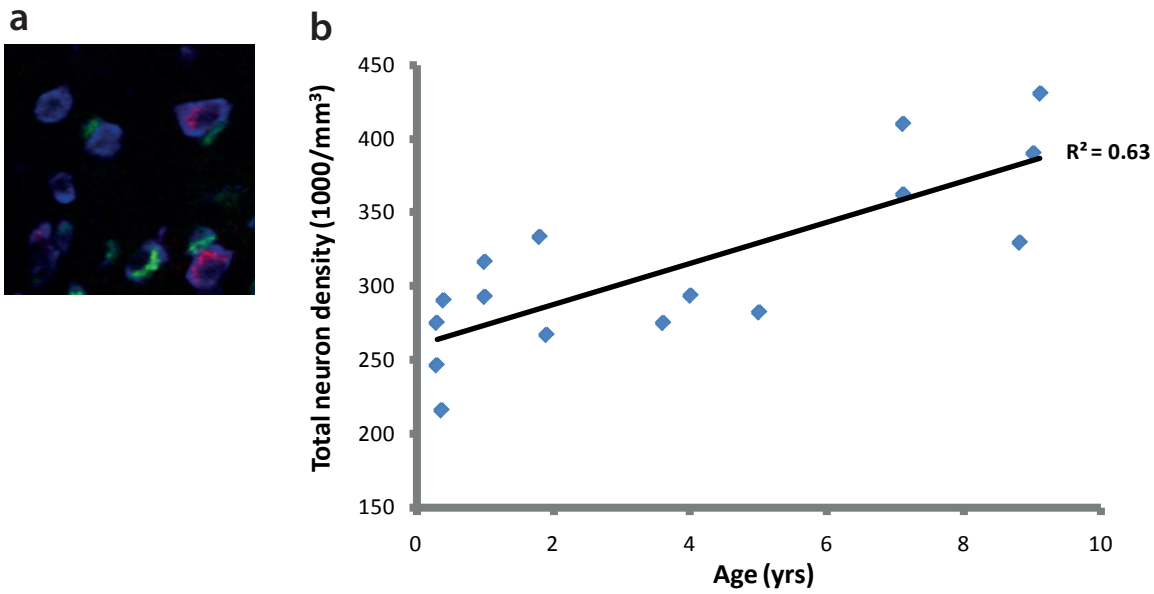


Figure 2.4: There is an increase in the total HVC neuron density with age in the adult zebra finch.

a. An image of HVC stained with an antibody for the neuronal marker Hu C/D (blue) in a bird that had received injections of fluorescent cholera toxin B to RA (green) and Area X (red). Hu stains the neuron cytoplasm leaving a dimly stained nucleus.

b. There is a significant increase in the density of total HVC neurons, determined by staining for Hu C/D, between 90 days and 10 years of age in the adult zebra finch (R^2=0.28, p=0.036).

between young adults (90-120 days) and older birds (4+ years, Figure 2.5a; unpaired t-test with equal variance: $t(240)= 5.63$, $p< 0.0001$) and this nuclear shrinkage is linear with age (Figure 2.5b; linear regression: $R^2= 0.04$, $p=0.003$). A reduction in the nucleus size of adultborn neurons has been previously shown in the canary: one month old HVC-RA neurons were significantly larger than 240 day old HVC-RA neurons (Kirn et al. 1991) and the difference was on a similar scale to the shrinkage we have observed here. Although the significance of nucleus shrinkage is unclear, evidence from rat motorneurons suggests nuclear and cytoplasmic shrinkage are correlated and that both are correlated with increased neural activity (Geinismann et al. 1971).

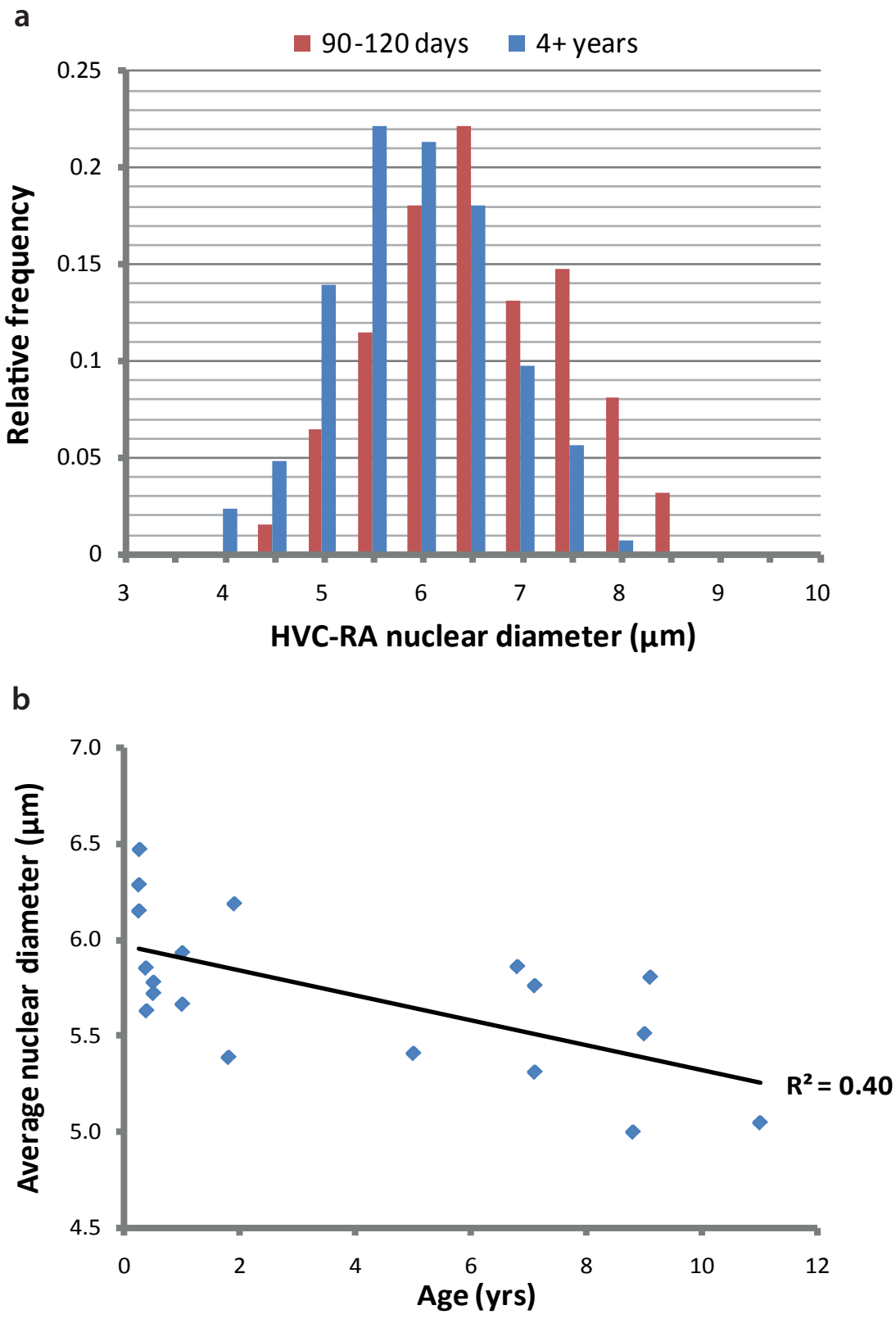
Even though the HVC-RA neurons are getting smaller with age, the decrease in nucleus size is modest and therefore cell shrinkage is unlikely to fully compensate for the increase in HVC-RA neuron density that we have observed. It seems likely that HVC-RA neurons would also be getting closer together in space as new neurons are added to HVC. To test this directly the distance between HVC-RA neurons was estimated by measuring the distance to a cell's three nearest neighbouring cells in a 2D image of HVC. Measurements were taken from the center of the nucleus of a CTB-labeled HVC-RA cell to the center of the nucleus of the three nearest CTB-labeled HVC-RA neurons for 30 randomly chosen HVC-RA cells per bird (Figure 2.6a). On average, HVC-RA neurons get together with age and the decrease in inter-neuronal distance is greater for younger adults as the data fits a logarithmic decay relationship better than a linear one (Figure 2.6b; log regression:

Figure 2.5: The nuclei of HVC-RA neurons get smaller with age in the adult zebra finch.

a. Histogram showing the distribution of HVC-RA nuclear diameters in young (aged 90-120 days) and old (aged 4 years or older) adults. Young adults show a significant shift towards smaller nuclei (t-test $p < 0.0001$). The distributions of nuclear diameters within each group are similar showing all neurons are getting smaller, not just a subset of HVC-RA neurons. The measurements were made in neurons identified by retrograde labeling with CTB from RA and the nuclear stain To-Pro-3-iodide.

b. The decrease in HVC-RA nuclear diameters is linear with age ($R^2=0.40$, $p=0.003$). Each data point represents that average of 20 HVC-RA neurons in the left HVC and 20 HVC-RA neurons in the right for each bird.

Figure 2.5:


a
90-120 days
4+ years
Relative frequency
0.25
0.2
0.15
0.1
0.05
0
3
4
5
6
7
8
9
10
HVC-RA nuclear diameter (µm)
b
Average nuclear diameter (µm)
7.0
6.5
6.0
5.5
5.0
4.5
0
2
4
6
8
10
12
Age (yrs)
R² = 0.40

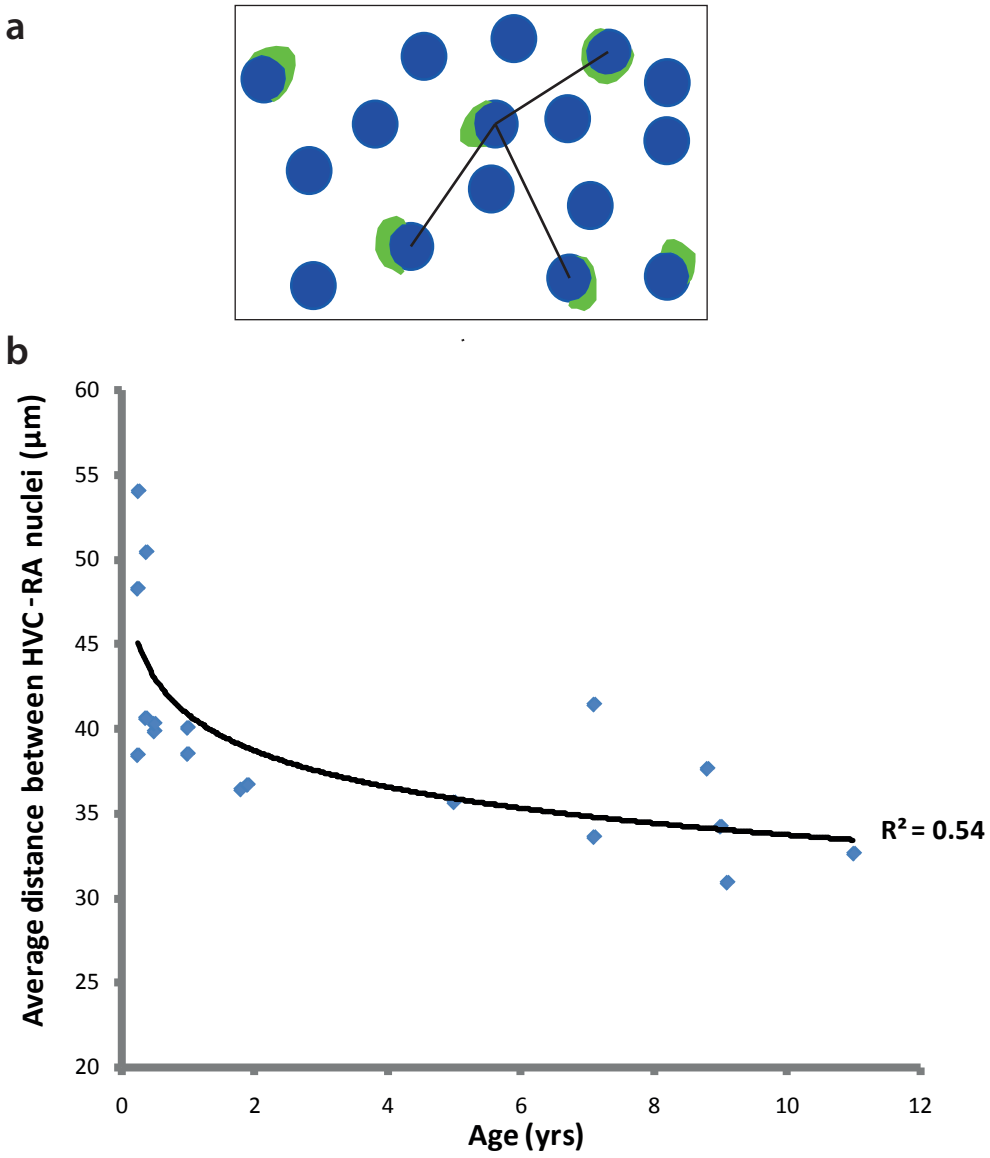


Figure 2.6: The distance between HVC-RA nuclei decreases with age.

a. A schematic of how we measured the internuclear distance between HVC-RA neurons. Retrogradely-labeled HVC-RA neurons were selected at random and the distance from the center of the ToPro-positive nucleus to the center of the nuclei of its three nearest retrogradely-labeled neighbours was measured. 20 neurons were selected in the left and right HVC and then the measurements for all 40 neurons were averaged for each bird.

b. The average internuclear distance between retrogradely-labeled HVC-RA neurons decreased between 90 days and 11 years of age. The data are described by a logarithmic relationship (log regression: R^2= 0.54, p= 0.0005) so the greatest change in distance occurs in younger birds.

R^2= 0.54, p= 0.0005; linear regression: R^2= 0.40, p= 0.004). The observed relationship is the inverse of the change in HVC-RA density with age observed by counting neurons in HVC. Overall, HVC-RA neurons increase their packing density within HVC by both getting smaller and getting closer together.

Although the HVC-RA neurons used to measure nuclear diameter and inter-nuclear distance were selected at random, we were worried that an unconscious selection bias may have affected the results. To confirm the two relationships with age we developed an automated workflow for CellProfiler image analysis software (Carpenter et al. 2006) to identify all the CTB-labeled HVC-RA neurons within a 2D confocal image and to measure the nuclear area for each identified neuron (see Figure 7a and methods). These data were then used to get an average nucleus size for each bird and also to find the total percentage of the image area containing HVC-RA nuclei, which is another way of measuring density of the HVC-RA neurons whilst taking into account any nuclear shrinkage. Results from the automated image analysis confirm the manually-generated data by showing a significant decrease in nucleus size with age (Figure 2.7b, linear regression: R^2= 0.19, p= 0.029) and a significant increase in the packing density of HVC-RA nuclei with age (Figure 2.7c, linear regression: R^2= 0.41, p= 0.001).

Modeling the net addition of HVC-RA neurons in adulthood

There are two possible ways to get net addition of neurons: either all neurons adding to HVC will survive and therefore the rate of population growth equals the

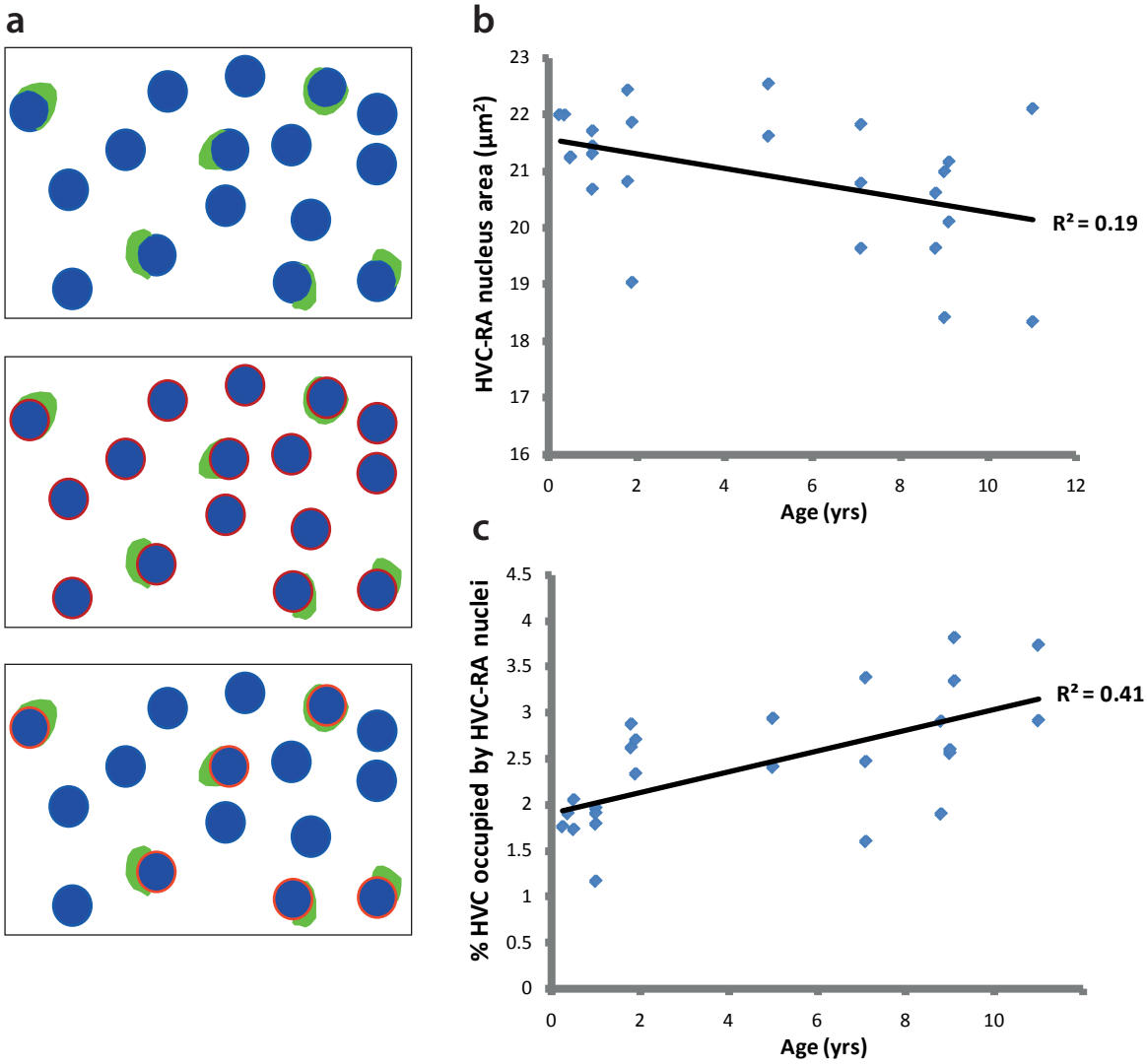
Figure 2.7: Automated image analysis confirms that HVC-RA nuclei get smaller and more densely packed with age in the adult zebra finch.

a. Automated image analysis was performed using CellProfiler software (Carpenter et al. 2006). Single images from the confocal stacks were used first to identify all nuclei (blue, using the ToPro channel), then to identify the subset of nuclei that belonged to HVC-RA cells (blue adjacent to green). The area of each HVC-RA nucleus in this latter class was measured in the blue channel.

b. The average nuclear area of HVC-RA neurons decreases between 90 days and 11 years of age (R^2= 0.19, p= 0.029). Each data point represents the average of all HVC-RA neurons identified in a single image from one HVC. Left and right HVCs are represented as separate data points.

c. The area of all identified HVC-RA nuclei was summed and the total was divided by the area of the image to find the percentage of the image occupied by HVC-RA nuclei. The percentage of the image filled with HVC-RA nuclei increased between 90 days and 11 years of age (R^2= 0.41,p= 0.001) .

Figure 2.7:


a
b
HVC-RA nucleus area (µm²)
23
22
21
20
19
18
17
16
0
2
4
6
8
10
12
Age (yrs)
R² = 0.19
c
% HVC occupied by HVC-RA nuclei
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
Age (yrs)
R² = 0.41

rate of new neuron incorporation, or, if some neuronal replacement is occurring, then the rate of new neuron incorporation is greater than the rate of neuron death. To explore these two possible scenarios for the zebra finch HVC we used previously published data to predict how the HVC-RA population would grow if all new neurons were surviving indefinitely. In 2002, the lab of John Kirn published data showing that the number of new HVC-RA neurons recruited to the adult HVC decreases as the zebra finch gets older. They injected birds ranging in age from 90 days to 3 years with ^{3}H-thymidine, waited 4 months, injected a retrograde tracer into nucleus RA to label the HVC-RA neurons and then counted the number of ^{3}H-positive HVC-RA neurons surviving in HVC. They found a decline in the number of labeled neurons as zebra finches got older and that the decline was not linear with respect to age (Wang et al. 2002; Figure 2.8). These data did not distinguish between an age-related decline in the production of new neurons or an age-related decline in the survival of new neurons in HVC; they just tell us that the number of neurons that will be still be present in HVC after 4 months declines with age. We took these data and used non-linear regression to derive the following exponential decay rate constant for the change in new neuron survival with age:

$$\boldsymbol{R_t = 111e^{-0.002t}}$$

where $\boldsymbol{t}$ is the posthatch age in days and $\boldsymbol{R_t}$ is the rate of HVC-RA neuron addition per day at posthatch day $\boldsymbol{t}$. This equation describes the number of new HVC-RA neurons that will be added to HVC and survive for 4 months on a given posthatch day. By making the assumption that every HVC-RA neuron surviving in HVC for 4 months will survive indefinitely, one can predict how the HVC-RA population will

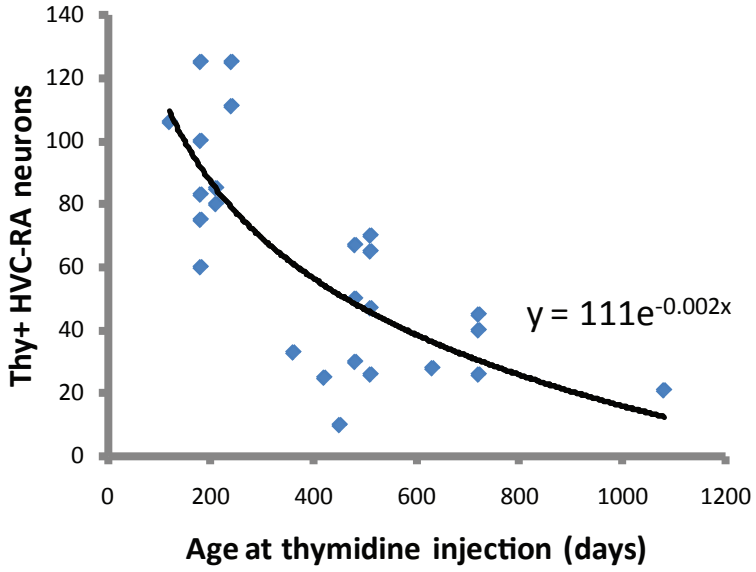


Figure 2.8: Data extracted from figure 3 in Wang et al. (2002) J Neurosci. 22: 10864-10870.

The authors injected adult zebra finches of different ages (horizontal axis) with ^{3}H-thymidine and killed the birds 4 months later. A retrograde tracer was injected a few days before killing to label HVC-RA neurons. The number of ^{3}H-thymidine-positive HVC-RA neurons present after four months decreased as the age at the time of ^{3}H-thymidine injection increased. We used non-linear regression to find the exponential decay equation that best described the data: $y= 111e^{-0.002x}$ ($R^2= 0.44$, $p= 0.0003$).

change with age by integrating the rate equation to find total number of new HVC-RA neurons adding to HVC in a given time period. Since the study from Wang et al. (2002) only uses adult birds aged 90 days or older, the integration was performed from posthatch day 90 to day $\boldsymbol{i}$, as the dynamics of neuron addition to HVC could be very different in the juvenile period. The integral equation used was

$$\boldsymbol{\tau_i} = \left(\int_{\mathbf{90}}^{\boldsymbol{i}} \mathbf{111} \boldsymbol{e}^{-\mathbf{0.002}\boldsymbol{t}} \, \boldsymbol{dt} \right) + \boldsymbol{c}$$

where $\boldsymbol{\tau_i}$ is the total number of HVC-RA neurons present at posthatch day $\boldsymbol{i}$ (which in this prediction must be greater than posthatch day 90) and $\boldsymbol{c}$ is the number of HVC-RA neurons present at posthatch day 90.

Using this integral equation and an experimentally derived value for the average number of HVC-RA neurons in a 90 day old, we generated a set of predicted values for the number of HVC-RA neurons expected at a given age if our assumption of complete neuronal survival is taken to be correct. Using an average value for HVC volume, these predicted values were converted to HVC-RA neuron densities and compared to the observed data for HVC-RA neuron density and age. The integral equation quite accurately describes the observed data and gives an R^2 value of 0.65 (Figure 2.9), which is impressive given it was derived from experimental data produced by a different lab using a different set of birds. The asymptote of the predicted data appears to be higher than the true asymptote which could be the result of some neuron death occurring in older birds but also could just

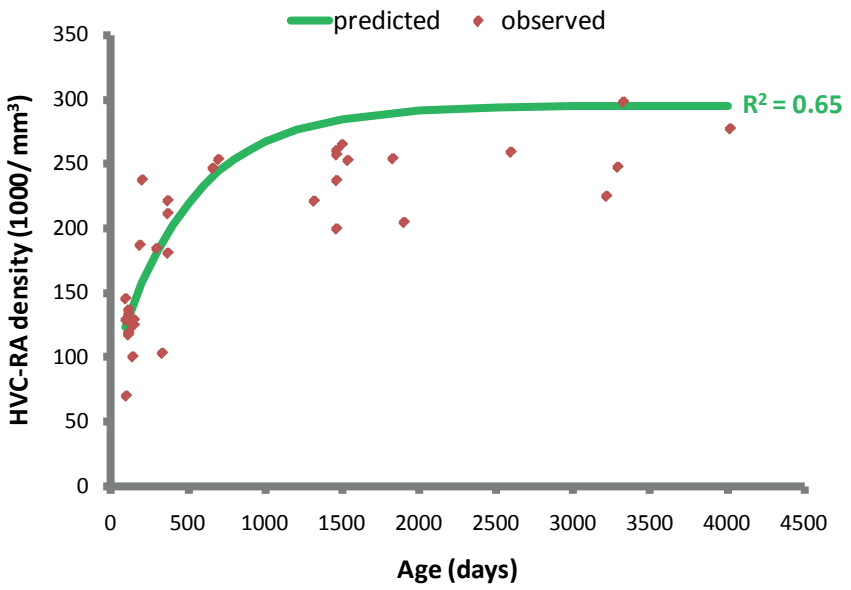


Figure 2.9: A model that assumes complete survival of new HVC-RA neurons approximates the age-related changes we see in the HVC-RA neuron density with age.

Wang et al. (2002) reported that the number of neurons surviving for four months in HVC decreases with the age of the bird (see Figure 2.8). We used the rate equation derived from these data to generate a set of predicted values for how the HVC-RA population would change with age if all the HVC-RA neurons that survived for four months in Wang's data were to survive indefinitely (see methods). The predicted values for how the HVC-RA neuron density would change with age under these conditions comes close to the real change in HVC-RA density with age that we have measured (R^2= 0.65).

reflect differences in the methods used between the two data sets, for example Wang et al. (2002) used fluorogold as a retrograde tracer and we used CTB and the labeling efficiency of these two tracers may differ. Alternatively, the different asymptotes could be due to a baseline difference in the rate of neuronal recruitment to HVC between the two cohorts of birds. We cannot distinguish between these three possibilities. However, even if our assumption of complete neuron survival is incorrect and there is some death of the HVC-RA neurons, the relative similarity between predicted and observed curves suggests that most new neurons added to HVC after day 90 survive for the long-term and that neurogenesis in the adult zebra finch HVC results, predominantly, in the net addition of neurons. This is in stark contrast to observations made in the canary where new HVC neurons are often short-lived and numerically replace older neurons that have died. In the adult zebra finch HVC neurogenesis results in net growth in the number of HVC-RA neurons whereas in the canary it appears to just maintain a fixed HVC-RA population size.

Comparing our results to past literature.

It is surprising that the dramatic increase in the number of HVC neurons we see here has not been previously described given the large amount of attention that the zebra finch HVC has received over the years. Examination of the literature revealed that the only study to have looked at HVC neuron number at different ages in the adult zebra finch was that of Wang et al. (2002). This study, which used fluorogold to retrogradely label HVC-RA neurons in birds aged between 4 months

and 3 years, reported no age-dependent increase in the total number of HVC neurons or in the number of HVC-RA neurons specifically. There could be several reasons why this study failed to find a relationship between HVC neuron number and bird age. The first is that the authors looked at the relationship between age and total neuron number but not neuron density, which as mentioned above, shows high variability due to individual differences in HVC size. The second is that their study included only one bird over 2 years of age so it had less power to reliably identify an accumulation of neurons with age than our study that included many older birds. The third is the way the counts were performed: Wang et al. (2002) identified neurons in thin 6 μm sections using morphological features of neurons (a large clear nucleus containing 2 nucleoli) and counted in 4 sample areas placed within HVC. Neuron density was determined by dividing counts by the sample volume and this density was then extrapolated to the whole volume of HVC. As mentioned above, density counts depend on how much the brain tissue shrinks during processing and shrinkage can be affected by age. However, tissue shrinking would presumably also affect HVC volume and because the Wang et al. (2002) study reported no change in HVC volume with age, as we also find here, it seems unlikely that an age-dependent difference in tissue shrinkage would have been a significant contribution of error to their data. Another possible source of error in the counts made by Wang et al .(2002) is that neuron profiles were counted in thin sections but no corrections were applied for cell-splitting errors, which could result in an undercount of the smaller HVC-RA neurons found in older birds. The authors applied no corrections to their data because they found no systematic change in nucleus size with age. Again, the

inclusion of only one bird above 2 years of age may have reduced the power of their study to reveal the age-related nuclear shrinkage that we report here. Overall, it seems possible that the relationship between HVC neuron number and age was lost in the Wang et al. (2002) study because of the individual variability in HVC volumes, the manner in which neuron numbers were estimated and the under-representation of older birds.

It is important to note that this study is the same one that provided data for our model of net addition. Despite the conflicts in the total neuron counts and counts of retrogradely labeled HVC-RA neurons between this study and ours, we still felt it was appropriate to use their counts of 4-month old ^{3}H-positive neurons at various adult ages for our model. Due to the scarcity of ^{3}H-positive neurons, the authors used a different strategy to count these cells than they used for counts of total neurons. For each section counted, the complete area of HVC was scanned for ^{3}H-positive neurons and therefore total ^{3}H-positive numbers were assessed directly rather than extrapolating from density estimates made in small sample areas. In addition, all the ^{3}H-positive neurons were 4 months old at the time of counting and therefore probably had comparable nuclear sizes in birds of all ages. Thus, errors resulting from tissue shrinkage and cell-splitting were less likely to affect the counts of 3H-positive neurons than the counts of either total neurons or fluorogold-positive HVC-RA neurons.

Summing up.

The unbiased stereological counts performed here show a net increase in the total number and density of HVC-RA neurons across the lifespan of the adult male zebra finch. This increase is present both in counts of CTB-labeled HVC-RA neurons and in counts of all Hu-positive neurons in HVC and is verified using an automated image analysis of confocal HVC images. Our modeling efforts in which HVC-RA neurons surviving for 4 months in HVC are assumed to survive indefinitely are quite close to our real observations of the HVC-RA population. Thus, we conclude that, unlike in canaries, net addition is the primary consequence of adult neurogenesis in the zebra finch HVC. However, although the modeling data suggests a case for total new neuron survival, there could also be some level of neuronal replacement occurring in HVC that is lower than the rate of new neuron incorporation and thus still results in a net increase in total neuron numbers. Because of this possibility, we directly tested for neuronal replacement in the adult zebra finch HVC and the results are discussed in the following chapter.

CHAPTER 3: ARE NEURONS REPLACED IN THE ADULT ZEBRA FINCH HVC?

A review of the canary evidence for neuronal replacement.

The most compelling demonstration of neuronal replacement in the canary HVC involved retrogradely-labeling HVC-RA neurons with fluorescent latex microspheres in April and comparing the number of labeled neurons after 20 days and 6 months. A second retrograde tracer (fluorogold) was injected into RA 3 days before the birds were killed in order to provide an independent count of the HVC-RA neurons present. At 20 days, 90% of the fluorogold-positive neurons were also labeled with microspheres but by October, 50% of the fluorogold-positive neurons were negative for microspheres, suggesting that most of them had been recruited to HVC after the initial microsphere injection surgery. There was no overall reduction in the total number of HVC-RA neurons retrogradely labeled by fluorogold, implying that about a 40% of the HVC-RA neurons present in April had been numerically replaced by new adult-born neurons over the 6 months from late April to October (Kirn & Nottebohm 1993).

Alternative evidence for neuronal replacement in the canary comes from following the survival of birthdate-labeled new neurons born at different times of the year. Adult-born neurons were birthdate-labeled with systemic injections of ^{3}H-thymidine in either May or October and their survival in HVC was tracked over the next 8 months by sacrificing birds at 0.5, 1, 4 or 8 months after ^{3}H-thymidine injection. For the birds injected in May, the number of ^{3}H-positive neurons present in HVC dramatically declined with survival time such that almost all of them had

disappeared by 8 months (Alvarez-Borda et al. 2004). Thus, the adult-born neurons added to HVC in the spring are very short-lived and their transient presence in HVC is indicative of a replacement process. In contrast, the birds injected in October showed a 50% reduction in the number of ^{3}H-positive neurons between the 0.5 and 1 month survival time but beyond that there was no further loss of neurons; they had the same number of ^{3}H-Thymidine neurons present 1, 4 and 8 months after ^{3}H-thymidine injection. Therefore, not all adult-born neurons added to the canary HVC are so transient; the time of year in which a neuron is born dramatically affects its survival. Given earlier reports that the total number of neurons in HVC is stable in adult canaries (Kirn et al. 1991), the continued recruitment of new neurons to HVC and the rapid demise of some of these neurons suggests a continuing process of neuronal recruitment and turnover is at work in the canary HVC.

Lastly, a study by Kirn et al. (1994) looked directly at cell death in the canary HVC at different times of the year. They found a temporal correlation between seasonal peaks of cell death in HVC and peaks in the recruitment of ^{3}H-thymidine labeled neurons to HVC suggesting that death of older neurons precedes the addition of new ones (Kirn et al. 1994). Taken together, the results from the three experiments with canaries reviewed above show that the stable number of neurons observed in the HVC of adult males (Kirn et al. 1991) masks a constant process of recruitment and turnover, with more neurons being added and removed during some seasons than at others (Kirn et al. 1994). Unfortunately no published study on canaries shows what percentage of the neurons born in the fall would still be present

1, 2, or 3 years later. Although many neurons recruited to the adult canary HVC appear to be short-lived (Alvarez-Borda et al. 2004), it is possible that some of the neurons added survive for a much longer period. The above studies are useful, too, in what they tell us about methods. For example, they suggest that the injection of latex microspheres into RA and their incorporation into HVC-RA neurons does not, by itself, result in the demise of these cells; rather, survival regimes are perhaps determined by other variables such as how the cell is connected and to what extent it is used, which may depend on time of year when the cell is born. The fact that ^{3}H-labeled HVC neurons born at different times of year have different survival profiles also argues in favor of the counts of persisting neurons being influenced by seasonal changes in recruitment and death and not a reflection of inherent toxicity in the markers used.

Does neuronal replacement occur in the zebra finch HVC?

To test for neuronal replacement in the zebra finch HVC we employed some of the same techniques used to show neuronal replacement in the canary. First we labeled HVC-RA neurons present at the beginning of adulthood with a retrograde tracer and followed their survival over the next 9 months. If HVC-RA neurons are being numerically replaced, we would expect to see some loss of the original population over this period as they get replaced by new unlabeled neurons. Forty males received bilateral injections of red fluorescent latex microspheres into RA at around posthatch day 95 and were killed 1, 3, 6 or 9 months later (10 birds at each survival time, Figure 3.1). In this experiment, as in the canary work, fluorescent latex

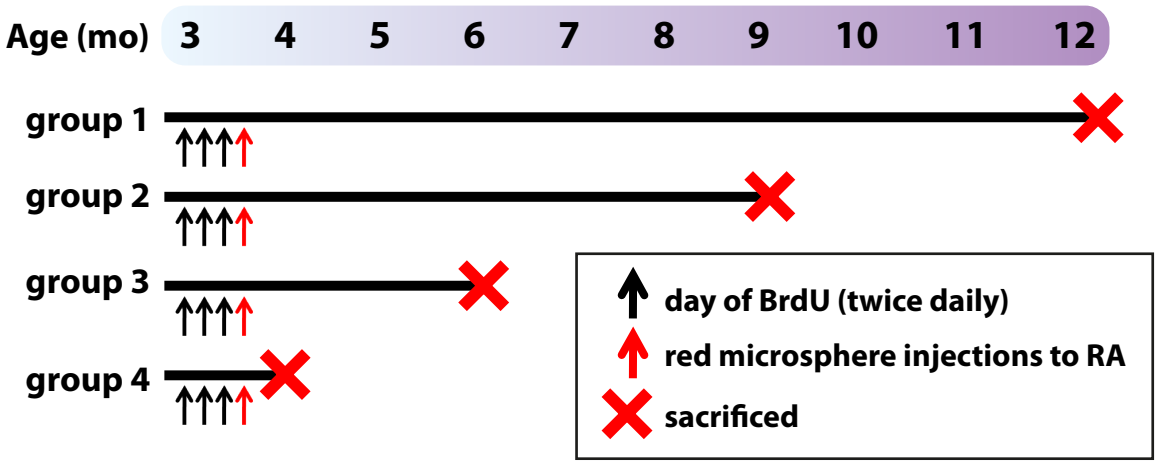


Figure 3.1: Experimental procedure: are HVC-RA neurons lost from the adult zebra finch HVC?

Four groups of ten birds between 90 and 98 days of age were given twice-daily injections of BrdU for three consecutive days. On the fourth day they received bilateral injections of red latex microspheres into RA to retrogradely label HVC-RA neurons. The groups were sacrificed either 1, 3, 6 or 9 months later and the number of HVC-RA neurons and BrdU-positive neurons in HVC were counted.

microspheres were used as the retrograde tracer because microsphere-labeling persists for at least a year *in vivo* (Katz et al. 1984).

During the 3 days prior to the tracer injection surgery, the experimental birds received twice-daily intramuscular injections of 5-bromo-2-deoxyuridine (BrdU) in order to label new neurons born between posthatch day 92-94. BrdU acts like ^{3}H-thymidine; it is a modified nucleotide base that gets incorporated into newly synthesized DNA during the S-phase of mitosis and can be visualized in tissue with a fluorescently-tagged antibody. Accordingly, it can be used to identify all new neurons born during the period of BrdU injections. In recent years BrdU has been favored over ^{3}H-thymidine because it avoids the hazards of working with radioactive material. The combination of retrograde tracer injections to RA and birthdate labeling with BrdU allows us to examine the survival of 2 different cohorts of HVC neurons: HVC-RA neurons retrogradely labeled at 95 days of age (and therefore born during the juvenile period) and neurons labeled with BrdU and therefore born after 90 days of age. In addition to the 4 survival groups, we included a fifth group of 6 birds that received the same protocol of BrdU injections at sexual maturity (posthatch day 90) and were then killed 4 years later. These birds did not receive the latex microsphere injections and so only provide a fifth time point for the survival of BrdU-labeled adult-born neurons.

To count neurons, every third brain section containing HVC was stained with antibodies against BrdU and the neuronal marker Hu and 3-color confocal image

stacks were collected showing the red latex microspheres, green BrdU and far-red Hu. HVC-RA neurons labeled with red latex microspheres were identified by the presence of punctate inclusions of red microspheres in a Hu-positive cytoplasm, usually clustered around a negatively-stained nucleus (Figure 3.2a) and they were counted using the optical fractionator method. Visual inspection of HVC at the various survival times revealed no obvious loss of microsphere labeling (Figure 3.2b) and stereological counts confirmed there was no change in the total number or density of retrogradely labeled HVC-RA neurons across the 4 survival groups (Figure 3.3; for density, one-way ANOVA: $F(3,27)=0.42$, $p=0.74$; for total number, one-way ANOVA: $F(3, 27)=0.91$, $p=0.45$). Thus, unlike in the canary where there was almost a 50% loss of retrogradely labeled neurons over a 7 month period (Kirn & Nottebohm 1993), there was, in our adult zebra finches, no appreciable loss of HVC-RA neurons retrogradely labeled at posthatch day 95 and counted 1, 3, 6 and 9 months later.

It is possible that the HVC-RA neurons present in a 90 day old and therefore born during the juvenile period behave differently to HVC-RA neurons born in adulthood. HVC-RA neurons born and connected during song learning in the juvenile are likely to be involved in coding the song pattern and so could represent a permanent population that is not be replaced. HVC-RA neurons born in later, adult life could serve another role and conceivably be part of a more transient and replaceable neuron population. To look for neuronal replacement of the adult-born neurons, we counted the number of neurons labeled with BrdU at 92-94 days of age

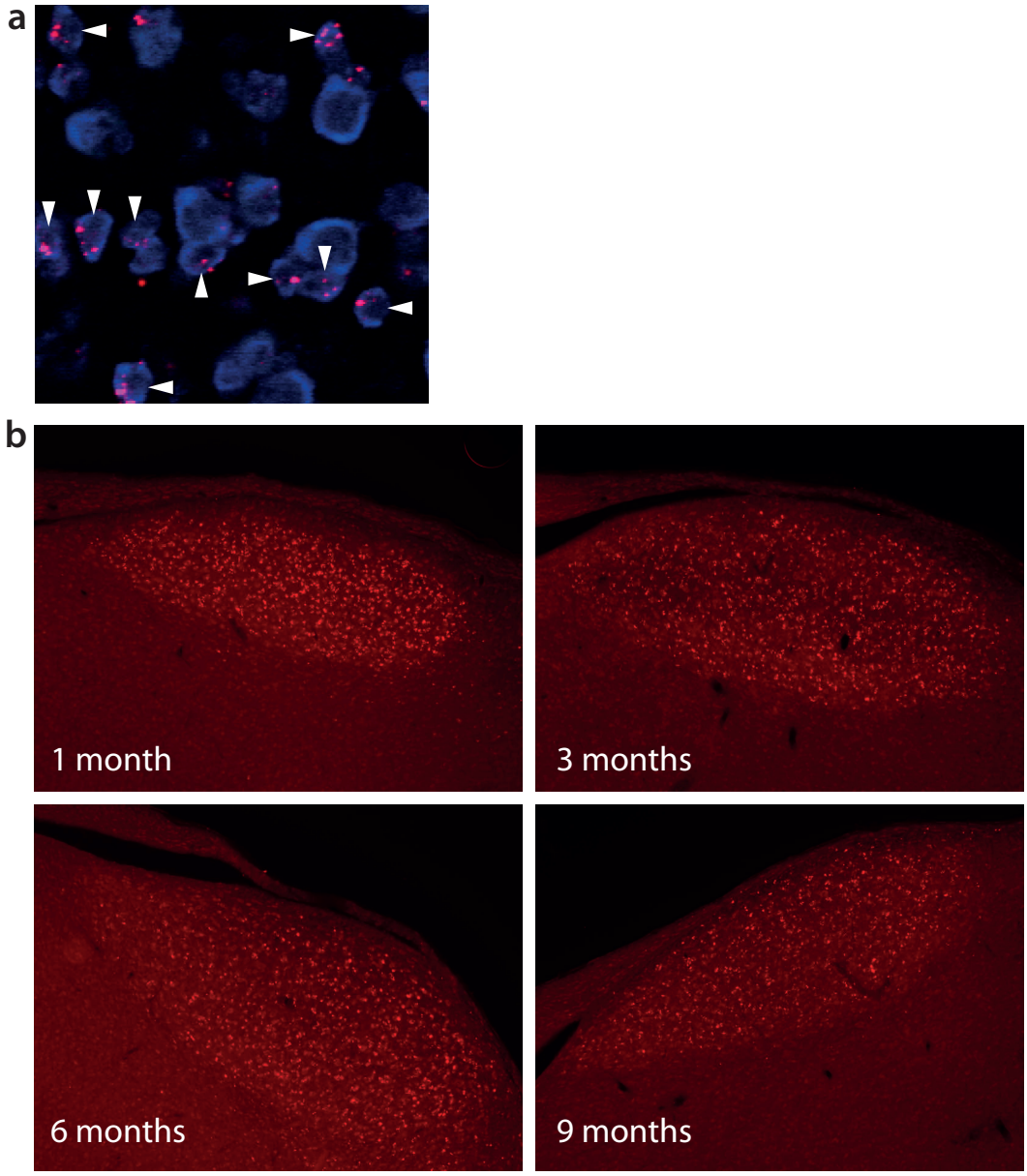


Figure 3.2:

Retrogradely-labeled HVC-RA neurons persist in HVC for 9 months.

a. A confocal image showing HVC neurons stained with an antibody for Hu C/D (blue) and the presence of red latex beads in a subset of these neurons. For neuron counts, HVC-RA neurons were identified by presence of punctate inclusions of red microspheres in a Hu-positive cytoplasm clustered around a negatively-stained nucleus. All the HVC-RA neurons in this particular optical plane are identified with arrowheads.

b. An example of HVC from each survival group showing that many retrogradely-labeled HVC-RA neurons are present at 1, 3, 6 and 9 months after injection of red latex microspheres to RA.

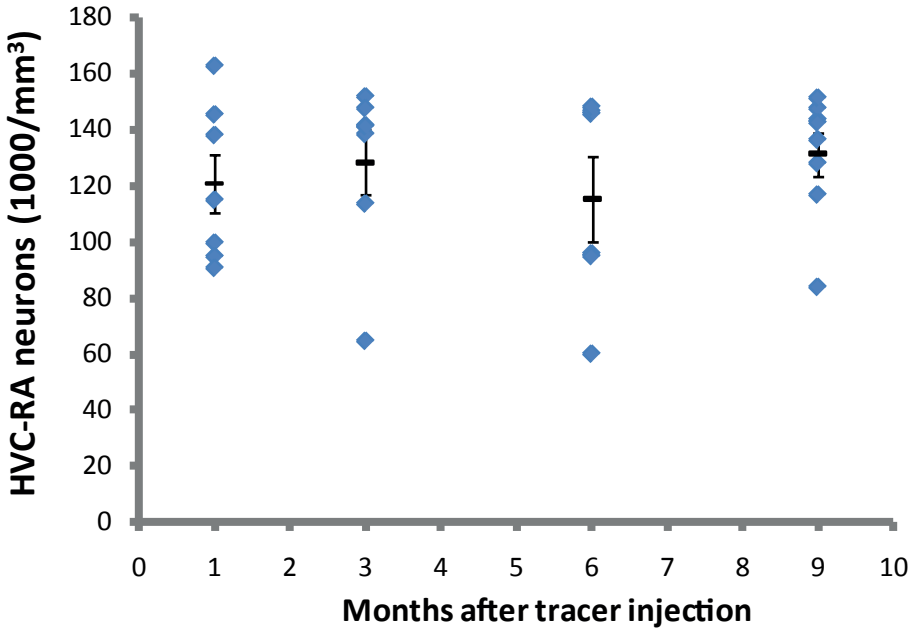


Figure 3.3: The number HVC-RA neurons retrogradely-labeled at 90 days of age remained unaltered during the following nine months.

Counts of retrogradely-labeled HVC-RA neurons showed there was no difference in the density (or total number) of labeled neurons between survival groups, suggesting that there was no significant loss of HVC-RA neurons between 90 days and 1 year of age. Each point represents the average of left and right HVC in an individual bird. Group means are represented as black bars with SEM error bars. Final group sizes were lower than ten birds because not all retrograde tracer injections were successfully on target. Final group sizes were: 8, 8, 6 and 9 birds for 1, 3, 6 and 9 month survival, respectively.

surviving in HVC at 1, 3, 6, 9 and 48 months after the BrdU injections. In the first 4 survival groups, BrdU-positive neurons were identified by a green BrdU-positive nucleus in the middle of a far-red Hu cytoplasm (Figure 3.4a). As part of the experiment discussed in the following chapter, the 6 four-year survival birds were given injections of green CTB to nucleus RA 5 days prior to sacrifice in order to determine what proportion of the surviving adult-born neurons project to RA. As a result, these birds had to have BrdU-staining in red and thus positive neurons were identified by a red BrdU-labeled nucleus in the middle of a Hu-positive cytoplasm (Figure 3.4b).

We were surprised to find BrdU-positive neurons present in the HVCs of the 4 year old birds that had received BrdU injections at 90 days of age. Many of these BrdU-positive neurons were also labeled with CTB from injections made into RA (Figure 3.4b). This is the first time that adult born neurons have been shown to persist for many years in the song bird HVC. To estimate the number of these long-term surviving neurons, we counted all the BrdU/Hu double positive cells within HVC in every third brain section and multiplied the number by 3. The same counts were performed in the 1, 3, 6 and 9 months survival birds and all counts were converted to neuron density using the measured volumes for each HVC. Comparing across all survival groups we found no difference in the total number or density of BrdU-positive neurons in HVC (Figure 3.5; for total number, one-way ANOVA: $F(4, 41)= 1.89$, $p= 0.13$; for density, one-way ANOVA: $F(4,41)= 1.35$, $p=0.27$). Thus, there was no significant loss of adult-born neurons in these birds; apparently new neurons born

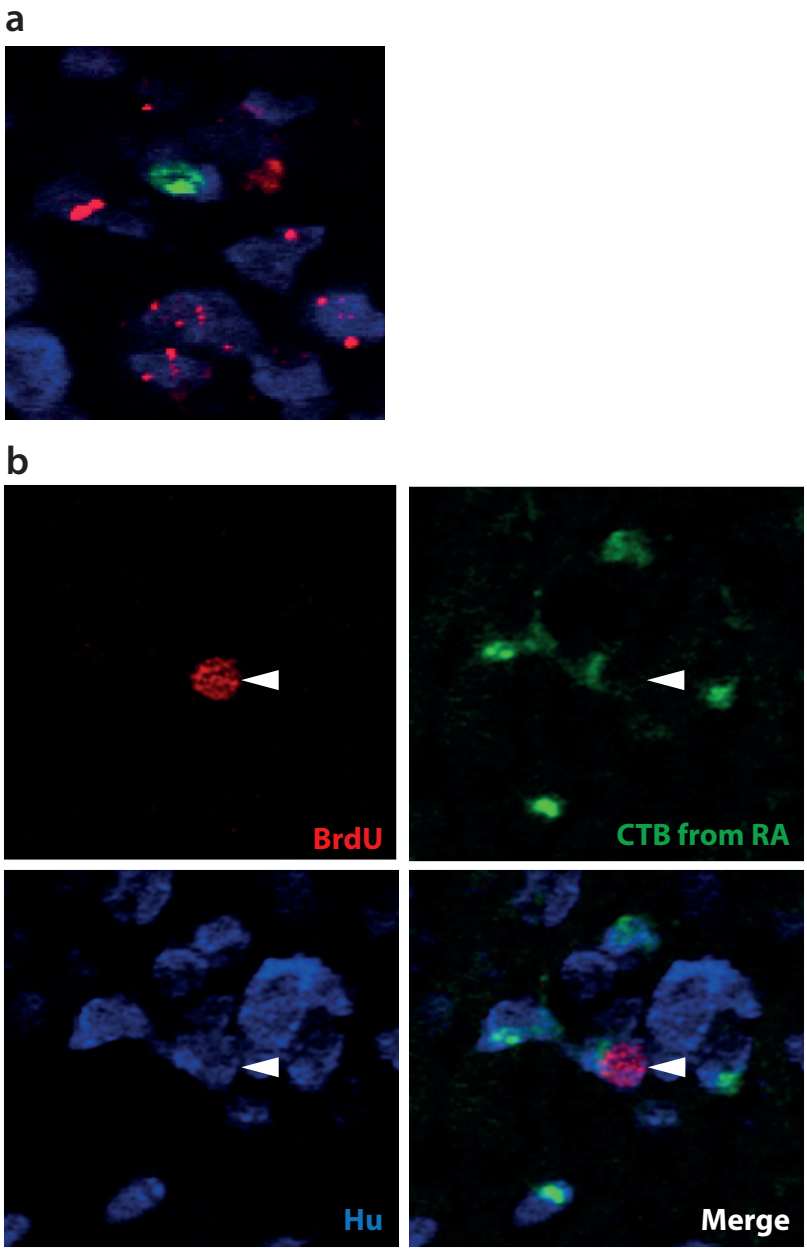


Figure 3.4: Identifying BrdU-positive neurons in HVC.

a. A BrdU-positive neuron in HVC identified by a green BrdU-positive nucleus in the center of a blue Hu-positive cytoplasm. Red latex microspheres that have been transported from RA are visible in other neurons but in this image the BrdU-positive neuron is not retrogradely-labeled.

b. A four year old BrdU-positive HVC-RA neuron identified by a red BrdU-positive nucleus in the center of a blue Hu-positive cytoplasm. The neuron in this figure is also retrogradely-labeled with green CTB from RA.

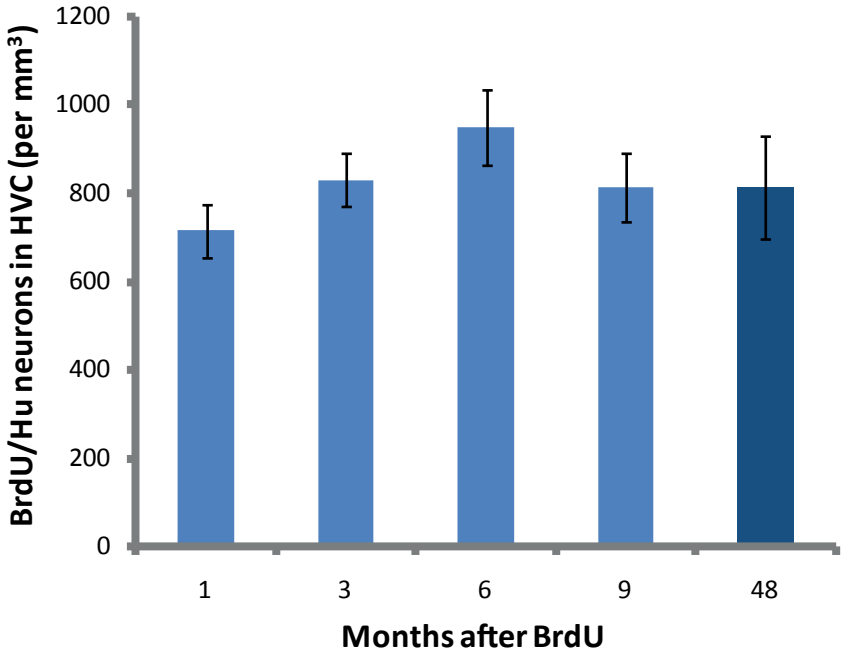


Figure 3.5:

Long-term survival of adult-born neurons in the zebra finch HVC.

There was no difference in the number of BrdU-positive neurons surviving in HVC between 1 month and 4 years after BrdU injections at 90 days of age. Error bars are SEM. Final group sizes were 9, 10, 10, 9 and 6 birds for 1, 3, 6, 9 and 48 months survival, respectively.

at approximately 90 days of age and that survive for 1 month in HVC were present 4 years later. This outcome is compatible with the observation that adult-born neurons accumulate in the HVC of adult zebra finches, a process of net gain with little evidence of replacement.

A caveat in the interpretation of our data.

We must, however, always be mindful of the limitations inherent in the techniques we use. The method of using retrograde tracers to determine the age of a neuron is confounded by the possibility that tracer at the injection site remains available for transport back to HVC after the original injection. New neurons picking up tracer weeks or even months after its injection into RA could mask any loss of some of the original retrogradely labeled cohort. We found some evidence for this possibility. Some of the BrdU-positive neurons born immediately prior to the injection of latex microspheres to RA were found to have transported microspheres back to their cell bodies in HVC (Figure 3.6a) even though it takes (in canaries at least) 2 weeks or more for the axons of adult-born neurons to innervate RA (Kirn et al. 1999). This observation suggests that a deposit of tracer remains in RA that can be taken up by neurons connecting after the initial injection surgery. This does not pose a problem for the neuron counts presented in Chapter 1 since those birds were sacrificed 1 week after tracer injection and so there was not much time for new neurons to be added to HVC, take up tracer and get counted. However, in the current experiment the last group of bird survived for 9 months after injection of the microspheres and the addition of neurons after the tracer injection that could have

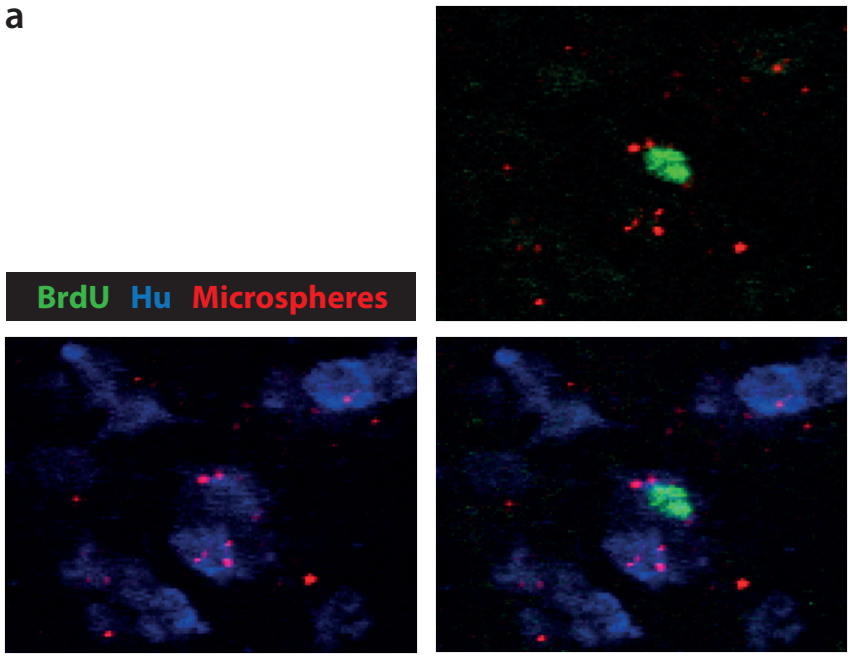
Figure 3.6: A subset of new neurons that reach HVC after injection of retrograde tracer to RA can become labeled.

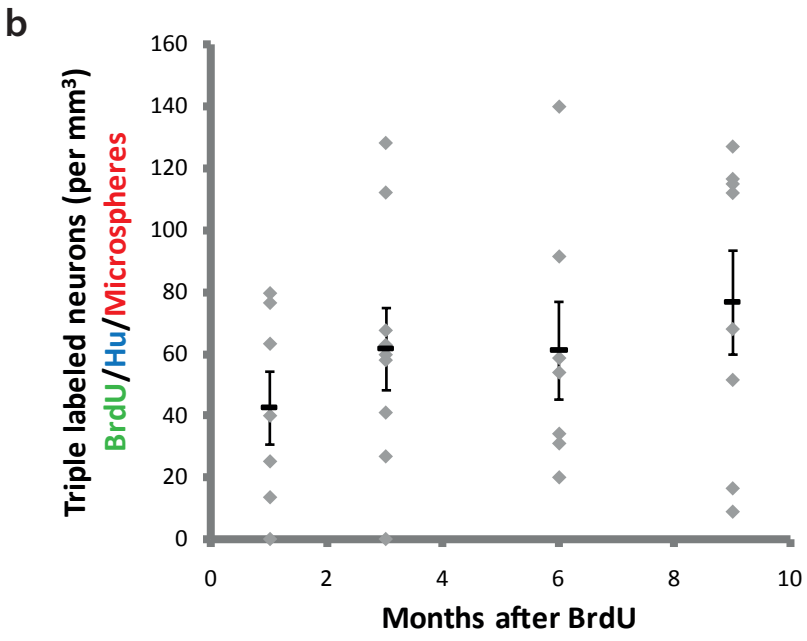
Some of the new BrdU-positive neurons in HVC were retrogradely-labeled with red latex microspheres from RA even though though their axons could not have reached HVC by the time the microspheres were injected into RA. Thus, tracer remains available for transport after the initial injection so new neurons adding to HVC after the tracer injection could be added to the originally labeled cohort.

a. A triple-labeled neuron in HVC identified by a green BrdU-positive nucleus in the center of a blue Hu-positive cytoplasm that contains punctate inclusions of red microspheres.

b. There was no significant difference in the number of triple-labeled neurons across the 4 survival groups. Each data point represents the average number between left and right HVC for one bird. Group means are represented as black bars with SEM error bars.

Figure 3.6:


a
BrdU Hu Microspheres


b
Triple labeled neurons (per mm³)
BrdU/Hu/Microspheres
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Months after BrdU

also taken up microspheres could be significant enough to conceal a loss of members of the original cohort of labeled HVC-RA neurons.

We tried to estimate the extent to which HVC-RA neuron loss could have been masked by neurons becoming labeled after the tracer injection as follows. We counted the number of BrdU/Hu/microsphere triple positive neurons in each survival group and found that the mean number of triple labeled neurons was not significantly different across all 4 survival times (Figure 3.6b; one-way ANOVA: $F(3,29)= 0.89$, $p= 0.46$). Next, we calculated the percentage of all the BrdU/Hu positive neurons that were also positive for microspheres and found that it was also not significantly different between the 4 survival times (Mean ± SEM: 5.3 ± 1.2% at 1 month, 9.5 ± 1.7% at 3 months, 6.3 ± 1.0% at 6 months, 8.8 ± 1.6% at 9 months; one-way ANOVA: $F(3,29)= 1.84$, $p= 0.14$). Because there was no change in the percentage of BrdU-positive neurons that had taken up microspheres between the different survival times, we calculated an average percentage across all 4 groups. On average, 7.5% of all the BrdU-labeled neurons were also labeled with microspheres from RA. This value provides a rough indication of the probability that a new neuron recruited to HVC after the injection of microspheres will take up tracer to become labeled and therefore get included in our counts of microsphere-labeled HVC-RA neurons.

In this experiment, birds in the 9 month survival group will have added many more new neurons to their HVCs since the initial injection of microspheres than birds in the 1 month survival group. If 7.5% of all these extra neurons become retrogradely

labeled with microspheres then the final counts of microsphere-positive HVC-RA neurons in the 9 month group will include more of these confounding extra neurons than counts from the 1 month survival group. To estimate the number of confounding neurons that could have been included in the HVC-RA neuron counts from each survival group, we used the integration equation derived from the data in Wang et al. (2002) and described in Chapter 2. This equation provides an estimate of the number of additional HVC-RA neurons that would have been added between the time of the microsphere injection and the day the bird was killed for each survival time. The estimates were then multiplied by 0.075, the likelihood that each new neuron would have taken up microspheres from the deposit in RA, to give the total number of 'extra' retrogradely labeled neurons that could have been included in the final HVC-RA counts for each survival group. Subtracting these final estimates from the actual counts of retrogradely labeled HVC-RA neurons provided new total HVC-RA numbers that are more likely to represent the true number of the originally labeled HVC-RA cohort present in each survival group. Figure 3.7 showed the corrected counts of retrogradely labeled HVC-RA neurons after removing the potential masking effect of newly-labeled neurons. There is still no significant difference in the total number of HVC-RA neurons between survival groups (one-way ANOVA: $F(3,27)=0.69$, $p=0.57$). We appreciate that because these estimates are based on experimentally derived data from a different lab using different birds (Wang et al. 2002) that they may not be very accurate at representing the actual number of newly-labeled neurons that could have been counted in our experimental birds. However, because the difference between the raw HVC-RA neuron counts

Figure 3.7: Retrograde label uptake by neurons born after retrograde label injection into RA probably has a minor effect on counts of HVC-RA neurons made up to 9 months later.

The real counts of HVC-RA neurons retrogradely-labeled with microspheres (raw counts) were corrected for the potential confounds of neurons added to HVC after microsphere injection that could have taken up microspheres from a deposit in RA (counts minus 'extra' neurons). The number of 'extra' neurons that could have been added for each survival group was estimated using published data for the rate of neuronal recruitment to HVC (Wang et al., 2002) and a likelihood of 0.075 that a neuron added after the original microsphere injection could become retrogradely-labeled (see text for details). Even correcting for this potential source of error, there is no significant difference between the total number of retrogradely-labeled HVC-RA neurons present at 1, 3, 6 or 9 months survival time after microsphere injection at day 95 ($p = 0.57$).

and the corrected counts is so small, even if these estimates are not entirely accurate, we would argue that any masking effect of retrogradely labeled neurons added after the original microsphere injection is negligible in comparison to the levels of net addition we describe in Chapter 2. Thus, even accounting for the potential limitations of the experimental method, we report no appreciable loss of the HVC-RA neurons present at posthatch day 95 over the next 9 months of life in adult zebra finches.

There is a similar limitation to interpreting the counts of BrdU-positive neurons at different survival times after injection of BrdU. The assumption is that BrdU-positive neurons present in HVC were born during the three days of BrdU injections. However, in reality, it is possible that the neuronal progenitor cells dividing in the ventricular zone at the time of BrdU exposure retain BrdU in their DNA and continue to produce BrdU-positive daughter neurons after the time of BrdU injections. Thus, as described above for using retrograde tracers to define the age of a neuron population, the same confounding factor is possible with birthdate markers. In this experiment, any loss of the original cohort of BrdU-labeled neurons could have been masked by a continuous influx of BrdU-positive neurons to HVC. In the early canary work, this caveat was overcome by counting the number of silver grains above a ^{3}H-thymidine positive neuron in the exposed autoradiograph. Because ^{3}H-labeled DNA is diluted in half every time a progenitor divides, the mean amount of radiolabel in a neuron, and thus the number of silver grains it generates, indicates whether it was labeled in the first or in subsequent rounds of progenitor division. Due to the way

BrdU is visualized and the many factors that can affect the labeling intensity of BrdU staining using antibodies, it is not possible to apply such intensity criteria to make inferences about the amount of BrdU present in the DNA of a BrdU-positive neuron. Moreover, we do not have enough information to estimate how much of an effect these extra BrdU-positive neurons could have on the total counts at each survival time. To do so we would need to know how often the progenitor cells divide to produce new neurons, whether this rate changes with age and also how many divisions can occur before the BrdU is diluted enough that a daughter neuron no longer appears BrdU-positive.

To shed some light on the issue we estimated the number of labeled progenitor cells present at each survival time by counting the number of BrdU-positive cells lining the ventricular zone above HVC in our confocal stacks. Labeled progenitors were counted if they had a BrdU-positive nucleus, were negative for Hu-staining and were found positioned on the edge of the ventricle (Figure 3.8a). The number of labeled ventricular zone cells decreases rapidly with survival time (Figure 3.8b), suggesting that either these cells are dying or more likely are continuing to divide until the BrdU is too diluted to meet our labeling criteria for a BrdU-positive cell. The fact that the number of labeled cells in the ventricle above HVC drops off so quickly with the survival time agrees well with earlier observation by Alvarez-Buylla and Nottebohm (1988) who, working on another part of the zebra finch forebrain, also saw a precipitous drop in the number of labeled ventricular zone cells by one month after exposure to the birth-date marker. Thus, because we are dealing with

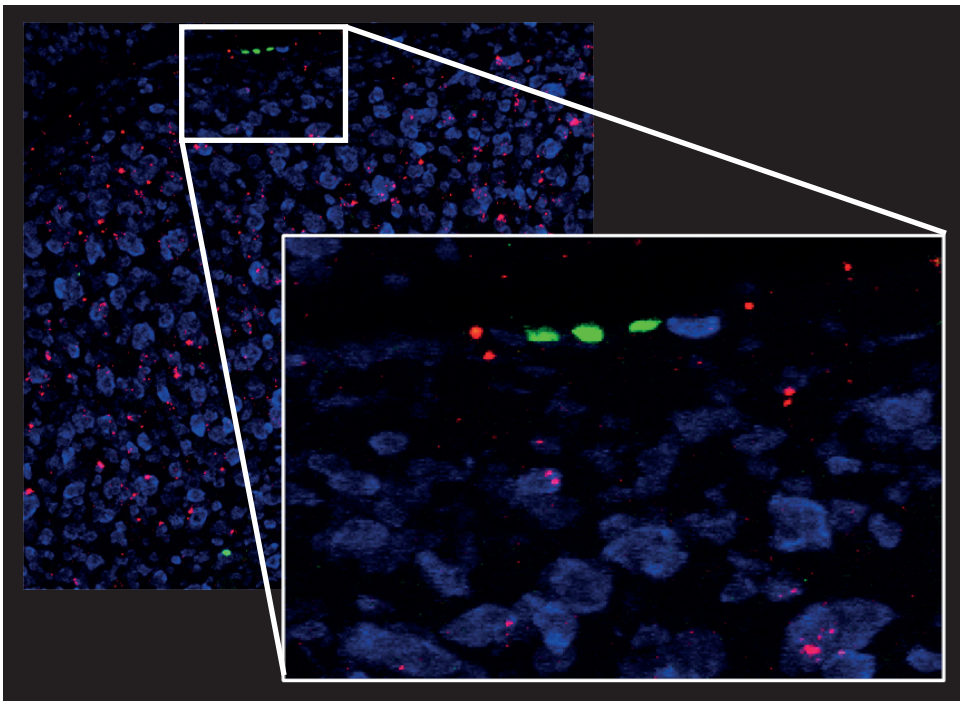
Figure 3.8: The number of BrdU-labeled ventricular zone cells declines with survival time after BrdU injection.

a. BrdU-positive ventricular zone cells were identified as green BrdU-positive nuclei without any blue Hu staining and lining the ventral ventricular surface above HVC. Three positive cells can be seen in this example.

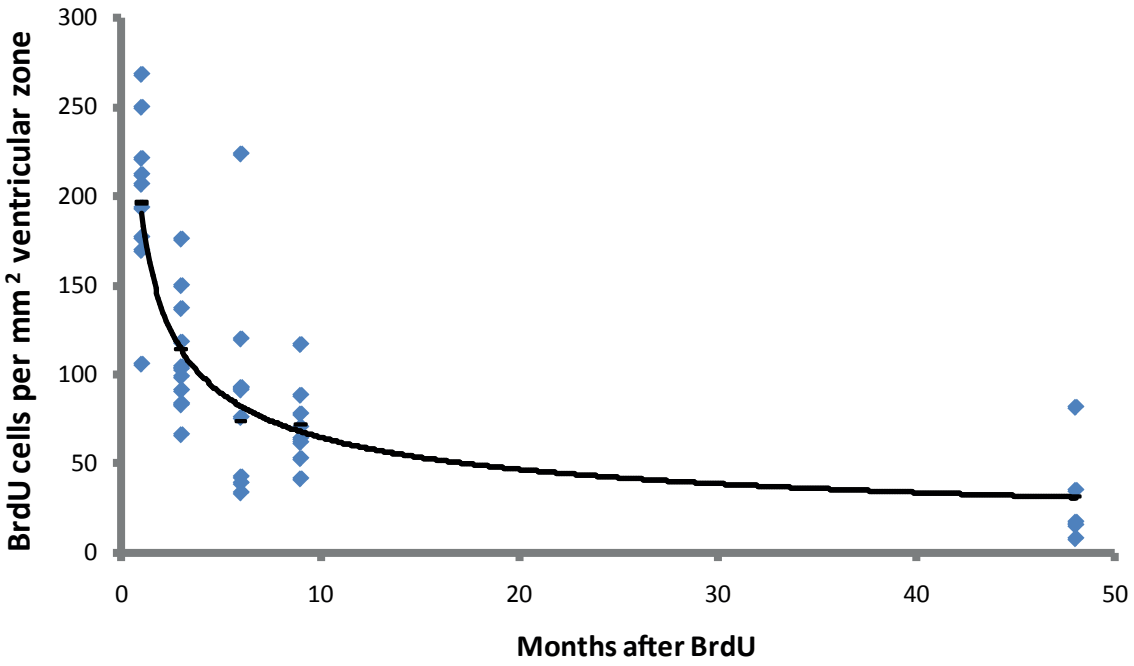
b. There is a non-linear decrease in the number of BrdU-positive ventricular zone cells with survival time after BrdU-injection that most likely represents continuing division of the BrdU-positive progenitors. Each data point represents the average of left and right ventricular zone counts for each bird. Group means are represented as black bars. The line represents the regression line through the group means and has the equation $y=190x^{-0.5}$ (R^2= 0.99). The sharpest decline occurs between the first and third month after BrdU treatment.

Figure 3.8:

a



b


BrdU cells per mm 2 ventricular zone
300
250
200
150
100
50
0
0
10
20
30
40
50
Months after BrdU

survival times of up to 4 years, it is unlikely that an influx of newly-labeled BrdU neurons would be significantly contributing to the total number of BrdU-positive neurons counted in HVC. We suggest that the more parsimonious interpretation of the data is that there is no loss of the originally labeled cohort between 1 month and 4 years.

Estimating cell death using pyknotic cell profiles

In the canary, there is a temporal correlation between peaks of cell death in HVC and peaks in the incorporation of new neurons suggesting that death of older neurons precedes the addition of new ones to HVC (Kirn et al. 1994). As a final comparison to the canary studies we looked for cell death in the adult zebra finch HVC. To assess cell death we stained brain sections from 3 adult zebra finches (kept under a constant photoperiod) and 3 canaries experiencing a January light-cycle, which is the time in the year when cell death is at its highest in the canary HVC (Kirn et al. 1994). When a cell undergoes apoptosis it goes through a very stereotyped chain of events including pyknosis, which is the condensation of chromatin within the cell nucleus that can be clearly seen using a nissl stain like cresyl violet. Pyknotic cells can be identified by a shrunken and sometimes invisible cytoplasm containing either a very densely stained nucleus or 2 or more dense nuclear inclusions (Figure 3.9a-d). Pyknotic profiles are usually scarce, even in the HVC of a January canary, so we sectioned the brains at 20 μm, stained every third section with cresyl violet and scanned the whole of HVC in all the stained slides for all 6 birds. Both left and right HVCs were examined for pyknotic cells in each bird

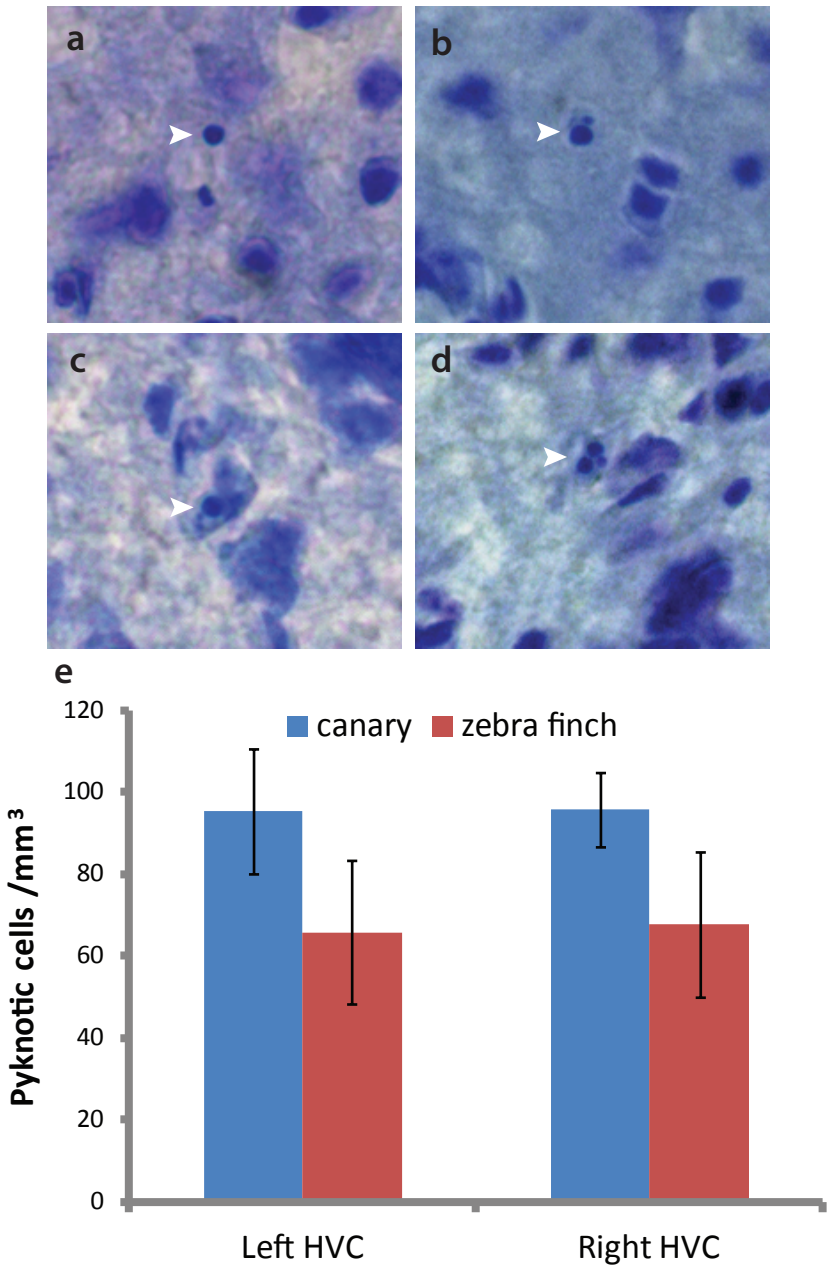


Figure 3.9: The number of pyknotic cell in the adult zebra finch HVC is not significantly different from the number in the HVC of spring canaries.

There were pyknotic profiles present in cresyl-stained HVC sections from adult zebra finches. Pyknotic profiles (arrowheads) were identified by either a single very densely stained nucleus (**a,c**) or two or more dense nuclear inclusions (**b,d**) in a shrunken cytoplasm (**b,c,d**). Sometimes the cytoplasm was totally invisible (**a**). There were no significant differences in the number of pyknotic profiles identified in the HVCs of January adult canaries or adult zebra finches (**e**; unpaired t-test with equal variance: $t(4)= 1.85$, $p= 0.14$). Three birds of each species were counted. Error bars represent SEM.

and the number of pyknotic cells was averaged between the 2 hemispheres.

There were several convincing pyknotic cell profiles in the HVC of adult zebra finches and while the average number of dying cells in HVC was lower than in the HVCs of January canaries, this difference did not reach significance (Figure 3.9e, unpaired t-test with equal variance: $t(4)= 1.85$, $p= 0.14$). Since we only counted dying cells in 3 birds of each species, the lack of significance in the data is not surprising. However, we were more interested in showing the presence of dying cells in the zebra finch HVC than in making a direct comparison to the level of cell death in the canary, so more birds were not added to the experiment. Given that cell death in January canaries is particularly elevated compared to other times of the year, it was surprising to see that cell death in the zebra finch HVC is of the same order of magnitude, especially considering that we have failed to find any evidence for loss of either retrogradely labeled HVC-RA or adult-born HVC neurons. There are 2 possible explanations for this discrepancy: the first is that these presumptive dying cell profiles may not be from neurons at all since it is virtually impossible to identify what cell type a pyknotic body used to be; the second is that if these pyknotic profiles are from dying neurons, they may be new neurons that have just reached HVC and have not yet survived the first month. In canaries there is a massive culling of over half of all new neurons reaching HVC between 2 weeks and 1 month after birth (Kirn et al. 1999). We have only looked at the survival of adult-born neurons in the zebra finch HVC from 1 month onward and we see no reduction in their numbers. It is quite possible that, as in the canary HVC, there is an initial culling of

new neurons during the first month and these early deaths are responsible for the pyknotic profiles we have seen here. Indeed, it could be that for both canaries and zebra finches, most of the pyknotic profiles seen are those of young neurons that failed to graduate into adulthood.

Comparing our results to the past literature.

Our observation that there is no loss of adult-born neurons between 1 month and the later survival times directly contradicts previously published data, also from zebra finches, showing a significant loss of adult-born neurons from HVC between 1 and 4 months (Wang et al. 1999). Although this study was looking at the effects of deafening on neuronal recruitment, the hearing control birds showed a 4-fold reduction in the number of ^{3}H-positive neurons surviving at 4 months after ^{3}H-thymidine injection compared to the number present after just 1 month. This dramatic loss of new neurons starkly contrasts with our failure to see any loss of neurons over a similar time period (and much beyond it). There are 3 notable differences between the 2 experimental procedures that could account for the conflicting results. The first is the choice of birth-date label; Wang et al. (1999) used ^{3}H-thymidine whereas we used BrdU. It is possible that incorporation of ^{3}H-thymidine into the DNA of neurons causes some toxicity that was responsible for the demise of many of the new neurons observed at 1 month by Wang et al. (1999) However, it seems unlikely that ^{3}H-thymidine toxicity could account for a loss of 3 quarters of the new neurons, especially given that the amount of ^{3}H-thymidine injected was similar to the amount previously given to canaries in which the full cohort of labeled neurons

persisted for 8 months (fall-born neurons, (Alvarez-Borda et al. 2004). If the dose of ^{3}H-thymidine used by Wang et al. (1999) was toxic to neurons, we would expect to have seen a similar loss of labeled neurons in the canary HVC. Of course there could always be a species difference in the neuronal sensitivity to tritium but given the dramatic loss of neurons in the zebra finches it is unlikely that ^{3}H-thymidine is responsible for the neuron loss observed in the Wang et al. (1999) study. The other 2 methodological differences between this paper and our experiment are the age of the birds used and the conditions in which they were housed. Bird age (Wang et al. 2002) and social environment (Lipkind et al. 2002) are both known to have an impact on the survival of new neurons in HVC so it is quite possible that these factors have contributed to the conflicting data reports. These issues will now be discussed in more detail.

Do age and social environment cause replacement of HVC-RA neurons in the adult zebra finch?

In Wang et al. (1999), the birds used were heterogeneous in age, ranging between 5 months and 3 years at the time of ^{3}H-thymidine injection, whereas the birds in our experiment were all between posthatch day 90 and 98 when they received the first day of BrdU. It is known that the overall number of neurons that are recruited to HVC and survive for at least 1 month decreases with age (Wang et al. 2002) but is it not known whether this difference results from reduced production of new cells by the stem cell population, from a reduction in survival of the neurons

once they reach HVC, or from a combination of both of these factors. In addition, it is possible that there are age differences in the survival of new neurons beyond 1 month which could account for the differences between our data and those of Wang et al. (1999). Perhaps neurons born early in adulthood and thus labeled with BrdU in our experiments are destined to survive indefinitely but for some reason neurons born in older birds and thus labeled with ^{3}H-thymidine by Wang et al. (1999) are destined to become only transient members of the HVC population. Since we do not understand the functional implications of long- versus short-term survival of new HVC neurons, or in fact the functional implications of new HVC neurons at all yet, it is difficult to predict either the cause or the result of a potential age-related decline in long-term survival. Nonetheless, we set out to see whether the ages of the birds used in the 2 experiments could account for the data conflicts by examining the survival of new neurons in the HVCs of older zebra finches.

Social environment is known to affect the recruitment of new neurons to the HVC of adult zebra finches: birds housed in a busy aviary with 45 others (both males and females) for the 40 days following ^{3}H-thymidine injections have 2.5 times more new neurons surviving in HVC than birds housed in the same sized aviary but with just 1 female companion (Lipkind et al. 2002). When Wang et al. (1999) saw a reduction in the survival of new neurons between 1 and 4 months, the birds were housed in cages with 3 other males. Our experimental birds, in which we saw complete survival of new neurons from 1 month onwards, were housed alone in single cages but kept on a rack in a busy room where they could see and hear many

other zebra finches. Each bird could interact with either 1 or 2 other males, depending on whether they were in the center or at the end of the rack, but only through the bars of the 2 cages (Figure 3.10a). In both experiments, the number of other birds that each male could interact with was roughly similar (1-2 versus 3) but in Wang et al. (1999) the birds could physically interact with one another whereas in our experiment they were prevented from physical contact by the bars of their cage. It is possible that physical interaction is important in establishing normal social relationships and so perhaps the differences in the survival of new neurons between these 2 experiments can be explained by the different housing conditions the birds experienced. Another important consideration is that in Wang et al. (1999) two control birds were always housed with two deafened males, since the object of the research was to look at the effect of deafening on neuronal recruitment to HVC. It is possible that deafened birds are abnormal in their social behavior and that an altered social structure amongst the 4 males in each cage resulted in short survival of new HVC neurons in the hearing control birds.

To determine whether age of the birds was responsible for the differences in neuron survival observed between Wang et al. (1999) and our data we injected twelve birds aged between 2 and 2.25 years with BrdU for 3 consecutive days. We split the old birds into 2 groups and allowed them to survive for either 1 or 3 months. Longer survival times were not possible due to the time constraints of the experiment and 1 and 3 months, as opposed to the 1 and 4 months used in Wang et al. (1999), were chosen to allow a direct comparison to the 1 and 3 month survival

Figure 3.10:

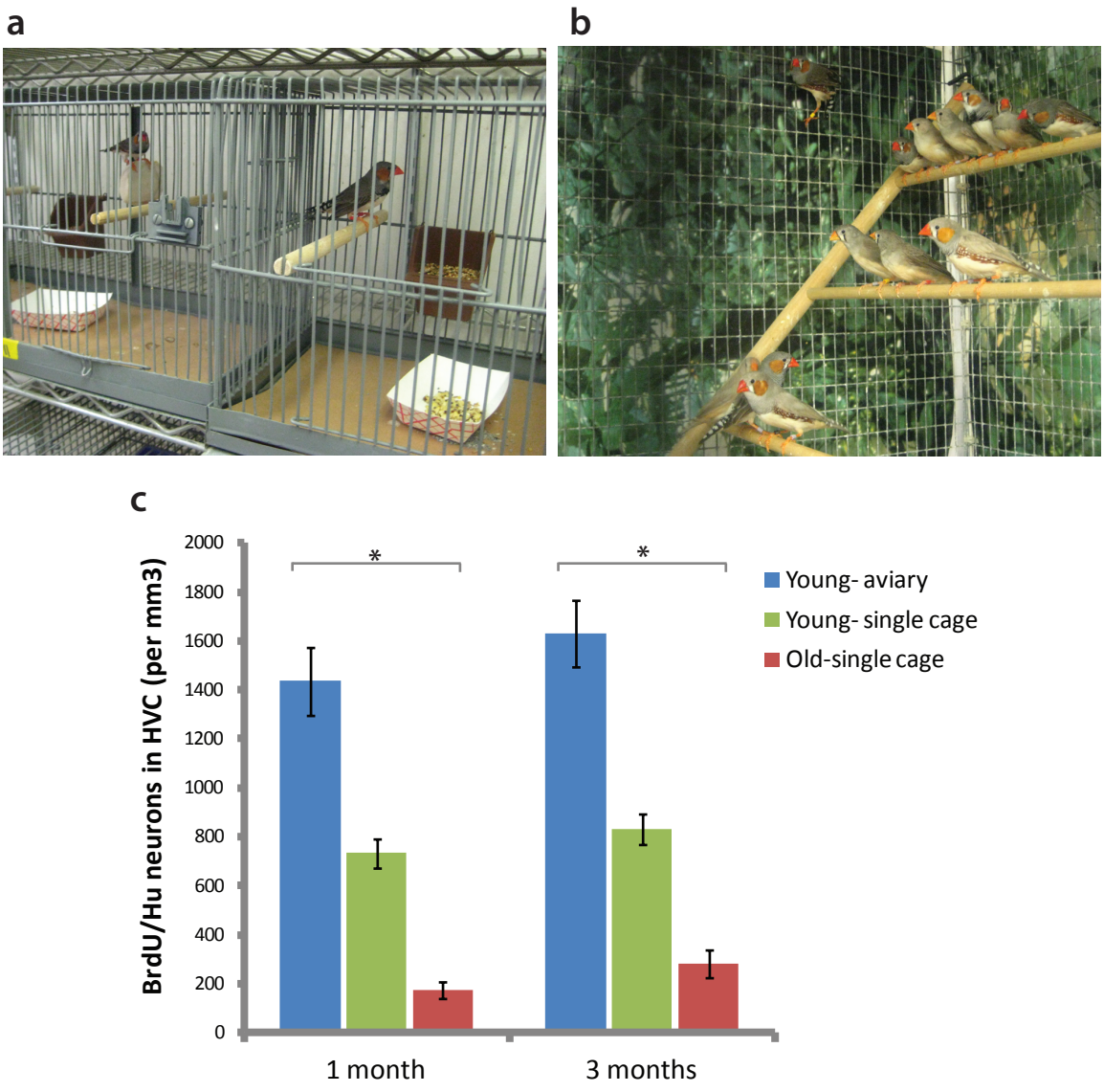
Age and social environment affect the recruitment of new neurons to adult HVC but not the survival of new neurons between 1 and 3 months.

a. Housing conditions for the birds housed in single cages. Birds were housed one per cage and the cages were placed end to end on a rack in a room with many other males also in single cages.

b. Birds in the more social environmental setting were housed in a large aviary with 29 other adult zebra finches.

c. The graph shows the number of BrdU-positive neurons surviving either 1 or 3 months in HVC in birds in 3 different conditions. 'Young-single cage' birds were 90 days of age at the time of BrdU injections and were housed in single cages after BrdU (**a**). 'Young-aviary' birds were 90 days of age at the time of BrdU injections and were then housed in a busy aviary setting (**b**). 'Old-single cage' birds were two years of age at the time of BrdU injections and were then housed in single cages (**a**). Aviary conditions caused the number of BrdU-positive neurons in HVC to double in young birds. Ageing (from 90 days to 2 years) caused a 4-fold reduction in the number of BrdU-positive neurons in the HVC of birds housed singly. The effects of social environment and age were both highly significant (*= $p < 0.0001$). In all three experimental conditions, there was no significant difference in the number of BrdU-positive neurons between 1 or 3 months survival time suggesting no loss of the new neurons over this time period.

Figure 3.10:


a
b
c
BrdU/Hu neurons in HVC (per mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
1 month
3 months
Young- aviary
Young- single cage
Old-single cage

groups of young birds from our previous experiment. Except for the age of the birds at the time of injection, the protocol used for BrdU injections (spacing of injections, dosage etc) was identical to the one used for the young birds to enable a direct comparison of new neuron numbers between the 2 age groups.

In order to test a possible effect of social environment on the persistence of new neurons in HVC, we treated 14 birds aged between 90 and 99 days of age with 3 consecutive days of BrdU injections and then moved them into a busy social aviary containing 30 birds in total (15 males and 15 females, Figure 3.10b). Half the birds were sacrificed after 1 month and the other half after 3 months so that the number of BrdU-positive neurons in HVC could be directly compared to those from the birds housed in single cages in our original experiment. It is important to note that all 14 birds were not injected together. Instead, the two groups of 7 birds were staggered to ensure that each bird experienced the same set of 14 other males in the aviary for the duration of the experiment. If all birds had been treated together, the 3 month survival group would have experienced the removal of the 1 month group and their replacement by new males for the remaining 2 months. Since this changeover would have required new relationships to be established and thus would count as a second change in the social environment, it could have had an additional effect on neuron survival that would confuse and interpretation of the final neuron counts. Accordingly, the 2 survival groups were run sequentially so all birds experienced the same set of 29 other birds for the duration of the experiment.

For both new sets of experimental birds (old, singly housed and young, socially housed), brains were sectioned and the sections were stained with antibodies for BrdU and Hu to allow counts of BrdU-positive neurons at the 2 different survival times within each condition. BrdU/Hu double positive neurons were counted in every third section for HVC and averaged across left and right HVCs within each bird. These counts were then compared to the 1 and 3 month survival data from the young, singly housed birds in the original experiment. Both age and social environment had a robust effect on the number of BrdU/Hu double positive neurons in HVC whereas there was no effect of survival time within each group and no additive effect between group and survival time (Figure 3.10c; unweighted-means two-way ANOVA: effect of group, $F(2, 46)=112.17$, $p<0.0001$; effect of survival time, $F(1, 46)=3.27$, $p=0.078$; interaction effect, $F(2,46)=0.21$, $p=0.81$). This means that while both age and social environment influence the overall recruitment of new neurons to HVC, they do not affect the subsequent survival, at least during the span between the end of the first and third month after BrdU injection. In fact, in all 3 conditions there was a non-significant trend towards more new neurons at 3 months than 1 month, suggesting that during that time the labeled ventricular zone neuronal progenitors may have produced more labeled neurons. Thus, in both young and older adults and in birds housed either singly or in groups, new neurons that survive the first month in HVC will go on to survive for at least 3 months. Despite testing different ages and environmental conditions, we have still failed to find any evidence of neuron turnover in the adult zebra finch HVC.

In the 2 year old birds, the number of new neurons recruited to HVC within the first month was a quarter of the number recruited in 90 day olds (Mean ± SEM: 175 ± 32 neurons per mm^3 for old birds, 735 ± 60 neurons per mm^3 for young birds), which is similar to the age-related decline described previously for the zebra finch HVC (Wang et al. 2002). In the socially housed young birds, the number of neurons recruited in the first month was twice that of the young birds housed in single cages (Mean ± SEM: 1436 ± 138 neurons per mm^3 for socially-housed, 735 ± 60 neurons per mm^3 for singly-housed) which is again similar to the previous reports showing an effect of a complex social environment on HVC neuronal recruitment (Lipkind et al. 2002). Thus, our data showing the effects of age and social environment on neuronal recruitment closely replicate the reports from others. Where our data differs is that we see no evidence of loss once the new neurons are recruited, at least during the survival span from 1 to 3 months after treatment with birth-date marker, which is compatible with the net increase in HVC neuron numbers we see after sexual maturity.

The experiments described above have been unable to explain the discrepancy between our data and those of Wang et al. (1999), already discussed. It is noteworthy that we have independently replicated the observation that adult-born neurons persist for 3 months in 3 independent groups of birds. Thus, under a range of conditions, new neurons that survive in HVC for 1 month will go on to survive for at least 2 more months. The first experiment described in this chapter suggested that the number of neurons that survive 1 month in HVC will still be present 4 years

later. We do not know whether this long-term persistence would also apply for neurons added to the HVC of older birds, or for the extra neurons recruited to the HVC of young socially housed adults because we did not follow the survival of BrdU-positive neurons beyond 3 months in these groups. However, taken together, our data suggest that adult-born neurons entering the zebra finch HVC are less transient than those recruited to the HVC of canaries.

As with HVC, neuronal recruitment to an auditory area of the zebra finch brain, the caudal nidopallium (NC), is also stimulated two-fold by a more complex social environment. Forty days after birthdate labeling, twice as many labeled new neurons are present in the NC of birds that were housed in a complex social setting compared to birds housed alone (Barnea et al. 2006). However, when birds were killed at 5 months after injections of the birthdate marker, the number of labeled new neurons in NC was the same between the 2 housing conditions, suggesting that the extra neurons recruited in the complex setting were lost in the period of time between 40 days and 5 months after their birth. For the NC, a more complex social environment stimulates the recruitment of new neurons but the extra neurons are only temporarily added to the brain area. In this study by Barnea et al. (2006), a third group of birds that were housed in the complex social environment and killed 2 months after injection of the birthdate marker had fewer labeled neurons in NC than the birds that were killed after 40 days. Thus, by 2 months there had already been some attrition of the additional neurons recruited into the NC. These data contrast to our observations in HVC in which the extra neurons recruited in a complex social

environment persist for at least 3 months. Apparently, neuronal recruitment can proceed via different strategies within different areas of the same brain. The nature of the interaction between social environment and neuronal recruitment depends on the brain area in question and probably depends on the function of the area in question and how it is engaged under different social conditions.

Do age and social environment affect the production or survival of new neurons?

Moving zebra finches to a more complex social environment stimulates survival of new neurons in HVC but we do not know whether the environmental change acts to increase the production of new neurons at the ventricular zone (VZ), the number of neurons successfully migrating into HVC or the survival of the neurons once they reach their destination. Similarly, we do not know whether the decline in neuronal recruitment that occurs with ageing is because neurogenesis in the ventricular zone is lower or whether conditions that favour neuron survival in HVC decline with age. In an attempt to shed some light on the issue, we counted the number of BrdU-positive VZ cells in the new experimental birds, both old and socially-housed, and compared the number of actively-dividing progenitors to the number observed in the young, singly housed birds from the original experiment. At 1 month, there was a significant reduction in the number of labeled progenitors in the older birds compared to both young groups but there was no difference between the singly and socially housed young birds (Figure 3.11; Mean ± SEM: young, singly

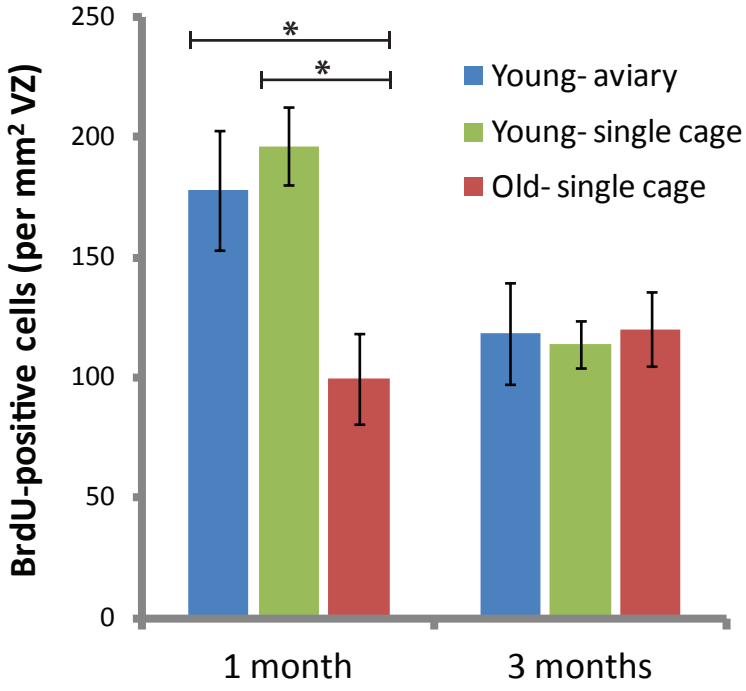


Figure 3.11: Age but not social environment affects division of ventricular zone progenitor cells in the adult zebra finch.

There is a significant difference between the number of BrdU-positive ventricular zone cells 1 month after BrdU injection in 2 year old birds compared to 90 day olds. While 90 day olds show a significant reduction in the number of labeled ventricular zone cells between 1 and 3 month survival times after BrdU, 2 year old birds do not show the same reduction, suggesting that the progenitors are dividing less often in the older birds. Social environment has no affect on the number of BrdU-positive ventricular zone cells as there is no difference between the young birds housed in single cages or a busy aviary at either of the survival times. *= $p < 0.01$.

housed = 196 ± 16 cells; young, socially housed = 178 ± 25 cells; old, singly housed = 100 ± 18; two-way ANOVA followed by Tukey HSD post-hoc analysis: $p<0.01$ for older birds compared to either of the young groups at 1 month, all other comparisons NS). After 3 months, all groups had the same number of labeled progenitor cells with both young groups showing a decrease in the extent of ventricular labeling between 1 and 3 month survival but in the older birds there was no difference between the 2 survival times. (Mean ± SEM: young, singly housed= 114 ± 10 cells; young, socially housed= 119 ± 21 cells; old, singly housed= 120 ±15).

Old and young birds in these experiments were treated with the same dosage and regime of BrdU. It may be parsimonious to assume that the access of BrdU to the brain and the amount of time it takes to be cleared from the blood are similar regardless of age. If so, then our observations at one month survival suggest that between 90 days and 2 years of age there was a 50% reduction in the number of neuronal progenitor cells still dividing in the ventricular zone above HVC, which then could account, in part at least, for the age-dependent drop in recruitment of new neurons counted at one month survivals. The age-dependent decrease, at any one time, in the number of active neuronal progenitors above HVC could be the result of the older birds having a smaller population of stem cells that are still capable of cell division, or it could be that older birds have the same number of active progenitors but that as a population these progenitors divide less often in the brains of older birds, or a combination of both of these factors could be responsible. If we are correct in our assumption that the gradual loss of ventricular zone labeling with

survival time in our 90 day old birds (Figure 3.8b) is the result of continued cell division and the gradual dilution of BrdU from the stem cell DNA, then the fact that there was no loss of BrdU-positive VZ between 1 and 3 months in the 2 year old birds suggests that their neuronal progenitors were dividing less often than those of younger birds. Of course, both variables could be at work: fewer progenitors and less frequent division of progenitors.

Similar age-related reductions in stem cell proliferation have been reported for the mammalian sub-ventricular zone (Maslov et al. 2004) and dentate gyrus (Georg & Gage 1996) but this is the first time a reduction of proliferation with age has been described in the songbird brain. Until these data, it was possible that the reduction in neuronal recruitment with age was the result of changing conditions in HVC reducing survival of new neurons as reported by Wang et al. (2002). Or course, our data do not exclude the possibility than in the older birds, compared to the younger ones, fewer of the newly produced neurons survive during the first month after being born, since our count starts after then.

In contrast to the effects of age on progenitor proliferation, a more complex social environment does not appear to have an effect on the division of stem cells. There is no difference in the number of labeled VZ cells at either survival time between the singly and socially housed young adults; the number of dividing progenitors and the rate at which they are dividing appears to be the same in both

social environments. This is not an unexpected result given that the birds in this and previous experiments (Lipkind et al. 2002; Adar et al. 2008) were not moved to a new social environment until after the end of the birthdate labeling injections, thus suggesting that the stimulatory effect occurs somewhere after cell division in the process of neurogenesis. Moreover, for an increase in social complexity to promote neuronal recruitment to HVC it must occur within the first month after the birth of a new neuron: moving birds to a social aviary setting either 1 or 3 months after birthdate-labeling has no effect on the number of neurons surviving in HVC 40 days later (Adar et al. 2008). A more complex social environment seems to act somewhere after the division of progenitors in the ventricular zone but within the first month after birth to improve neuron survival in HVC. From work in the canary, we know that adult-born neurons take between 1 and 2 weeks to reach HVC and begin the maturation process (Kirn et al. 1999). Between 2 weeks and 1 month after birth there is substantial culling of the neurons, with only half of the original cohort surviving to 1 month (Kirn et al. 1999; Alvarez-Borda et al. 2004). Assuming the timing of cellular events are similar between the canary and the zebra finch, it seems likely that an increase in social complexity could stimulate the survival of some of the neurons that would normally be lost during the initial culling event in a more simple environment.

As a final analysis, we looked for a correlation between the number of labeled VZ cells present 1 month after BrdU injections and the number of BrdU/Hu neurons surviving in HVC after the same time period. If division of progenitors in the

ventricular zone is the limiting factor in the recruitment of new neurons to HVC, there should be a positive correlation between these 2 factors amongst individual birds. Taking all 3 experimental groups together (old; young, single cages; young, aviary) there is a positive correlation between the number of BrdU-positive VZ cells and the number of BrdU-positive neurons in HVC (R^2= 0.29, p= 0.01) but this is not surprising given that both variables are significantly lower in the older birds. The data are more revealing when the regression analysis is run for each group individually; the variables are only correlated within the young, aviary-housed birds and not the old or young, singly-housed birds (Figure 3.12; young, aviary-housed: R^2= 0.68, p= 0.02; young, singly-housed: R^2= 0.043, p= 0.59; old, singly-housed: R^2= 0.015, p= 0.81). These data add further support to the idea that enriching the social environment acts to promote survival within the first month after neuron birth. They suggest that in the singly-housed birds, neuronal death within the first month is important in determining the overall number of neurons that will survive in HVC because the same number are present at 1 month regardless of the amount of progenitor division occurring at the ventricular zone. However, in the socially-housed birds, neuronal death is less important because the number of neurons surviving at 1 month is largely dictated by the amount of dividing progenitor cells present in the bird. As more of the new neurons are surviving the initial culling period in a complex social environment, the number of neurons being produced by the ventricular zone in a given bird becomes more important in determining the final number that will be present in HVC after the first month.

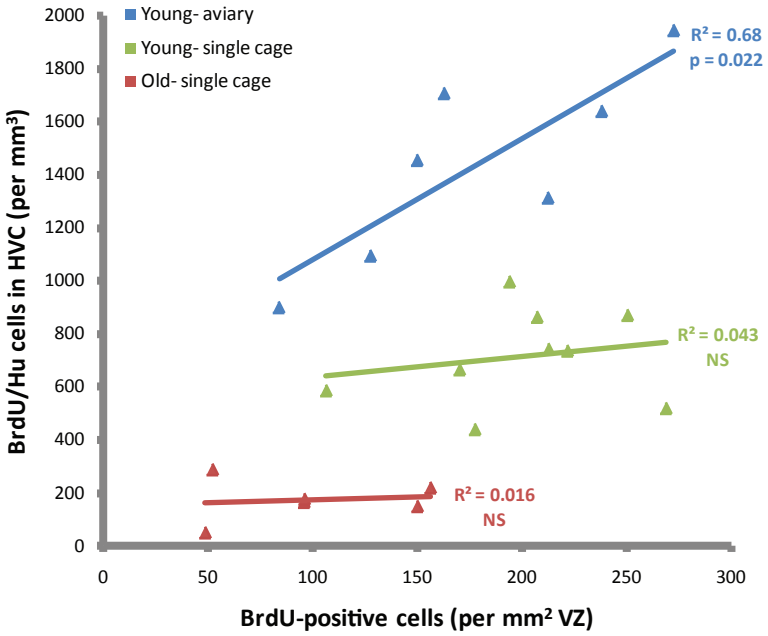


Figure 3.12: The number of new neurons in HVC is only correlated with the number of dividing progenitors in socially housed birds.

The number of BrdU-positive neurons in HVC is significantly correlated with the number of BrdU-positive cells in the ventricular zone in the young, aviary-housed birds (R^2= 0.68, p= 0.022) but not in the young or old groups housed in single cages, which would be expected if more of the new neurons survive in the socially housed birds (see Fig. 3.10).

Since we do not know the function of adult-born neurons in the zebra finch HVC, we cannot say why a more complex social environment would require more neurons to be recruited. However, as the functional role of HVC seems restricted to either song production or song perception, we can speculate that new neurons adding to HVC have a role in one of these behaviors. In the canary, singing stimulates the survival of adult-born neurons (Li et al. 2000), and thus the most obvious explanation for the increase in neuron survival in complex social environments is that the birds are singing more when they are surrounded by other birds. Anat Barnea and colleagues tested this hypothesis by measuring the amount of singing performed by male zebra finches either housed in an aviary environment with 45 other birds or housed with just 1 female. The birds housed in the busy aviary were actually engaged in singing significantly less of the time than birds housed with just one female (Adar et al. 2008). Therefore, in zebra finches it seems more likely that an increase in the amount of auditory processing of conspecific song rather than an increase in song production is the key feature of a busy social environment that promotes survival of new neurons in HVC. This is not to say, however, that an increase in singing in a fixed social environment would not also increase the survival of adult-born neurons in the zebra finch HVC as it does in the canary (Li et al. 2000).

Summing up.

We have shown that, in contrast to the canary, neuronal replacement is not a significant correlate of adult neurogenesis in the zebra finch HVC. Two independent

labeling methods, retrograde tracer labeling of HVC-RA neurons and birthdate labeling of adult-born neurons, have failed to reveal any significant losses of HVC neurons over the first 9 months of adulthood. In addition, neurons added to the HVC of young adults seem to persist for several years, perhaps indefinitely. These observations fit harmoniously with the data presented in Chapter 2 showing net addition of neurons to the adult zebra finch HVC. Given the notable differences in singing behavior between canaries and zebra finches and the known role of HVC in song, it is perhaps not surprising that the cellular events surrounding neuronal recruitment in adults are not equivalent in the 2 species. Neuronal replacement in the canary correlates with seasonal changes in song instability that are likely to be important for learning new syllables in these open-ended learners. Zebra finches master their song in the juvenile period and make no further significant changes to their song motif in adulthood. A gradual replacement of the HVC-RA neurons that encode the song pattern would seem counterintuitive in a species where the adult song remains fixed and stereotyped. Addition of new adult-born neurons to HVC in the zebra finch may provide alternative functions to the song system than the speculated role of neuronal replacement in learning in the canary. Perhaps in the zebra finch the extra HVC neurons are involved in keeping the song pattern stereotyped and stable in the face of aging. The potential relationship between adult song and the net addition of new neurons to the zebra finch HVC will be explored in Chapter 5. Lastly, although both age and social environment have dramatic effects on adult neurogenesis and the immediate survival of new neurons in HVC, neither seem to reduce the long-term survival of new neurons, at least up to 3 months. This

tells us that our failure to observe any loss of HVC neurons from the zebra finch HVC over longer periods of time is unlikely to be an artifact of the way the experiments were done but more likely a real feature of the neuronal events that occur in the adult brain of this species.

CHAPTER 4: WHAT KINDS OF NEURONS ARE ADDED TO THE ADULT ZEBRA FINCH HVC?

HVC contains three main neuron types

HVC contains three main types of neuron, each with different anatomical and physiological properties (Dutar et al. 1998). HVC-RA neurons send posterior projections down to nucleus RA and are relatively silent except during singing when they exhibit a single burst of activity during the same, brief 6 ms window during delivery of the song motif (Hahnloser et al. 2002). HVC-X neurons also increase their firing rate during singing and burst in a stereotyped manner but they fire more often with 0 to 4 bursts per song motif (Kozhevnikov & Fee 2007). They have a larger soma than the other HVC neurons and send anterior axonal projections down to Area X (Katz & Gurney 1981; Prather et al. 2008). The interneurons in HVC have a much higher spontaneous firing rate than the projection neurons and fire tonically during singing (Kozhevnikov & Fee 2007). Anatomically they can be defined as having no axons that pass outside the boundaries of HVC and can be identified by the expression of one or more of three calcium-binding proteins that are commonly found in fast-spiking neurons: parvalbumin, calbindin and calretinin. Antibody staining for these three proteins finds them absent from both classes of projection neuron whereas either 1, 2 or all 3 of them are expressed in combination in the interneuron population (Wild et al. 2005). Electrophysiological and histological data indicate that these three neuron types constitute the large majority of all the neurons in HVC, but the presence of additional, rarer neuron types cannot be ruled out. For example, it is known that there are a few neurons in HVC that send projections to

the avalanche nucleus of the caudal mesopallium (Nottebohm et al. 1982) but due to their scarcity their physiological properties and role in the song system are unknown.

HVC-RA neurons are produced in adulthood

Early work using horseradish peroxidase (HRP) labeling in combination with systemic injections of ^{3}H-thymidine suggested that the majority of new neurons added to the HVC of adult canaries were local interneurons. Firstly, injecting HRP into new neurons to fill the soma with dye did not reveal any labeled axons leaving the boundaries of HVC; their axonal arbors appeared to be completely contained within HVC (Paton & Nottebohm 1984). Because only a small number of cells were examined in this study and because of the possibility that the axon of a projection neuron leaves the plane of the tissue section being examined before it exits HVC, this evidence alone was not conclusive. However, then a second study reported that injecting HRP into nucleus RA or Area X failed to retrogradely label any new neurons in HVC (Paton et al. 1985). The authors of this latter study concluded that most neurons produced in the adult canary HVC were therefore interneurons. However, the validity of using HRP as a retrograde tracer was called into question when subsequent canary studies using fluorogold and latex microspheres reported that between 50% and 80% of new neurons in the adult HVC could be labeled from RA depending on the tracer used, the time of year in which the neurons were born and the amount of time allowed between neuron birth and retrograde tracer injection (Alvarez-Buylla et al. 1990; Kirn et al. 1991). Apparently the efficiency of HRP uptake by new RA-projecting neurons is much lower than their uptake of other

retrograde tracers. Subsequent work in both the canary and the zebra finch has shown significant labeling of new neurons with tracers injected into RA but no labeling with tracer from Area X and thus it seems that many of the neurons added to the adult HVC project to RA (Kirn et al. 1999; Kirn & Nottebohm 1993; Scharff et al. 2000; Scotto-Iomassese et al. 2007). Since the early studies, there have been no further experiments to determine whether HVC interneurons are also born in the adult canary HVC. It is therefore still not known whether new neurons that cannot be labeled from RA are interneurons; a type of HVC neuron that has not yet been identified; a subset of HVC-RA neurons that does not transport the tracers used; or the result of a failure of retrograde tracers delivered locally into RA to label every HVC-RA neuron present.

The types of neurons added to the adult zebra finch HVC has been directly investigated: Scotto-Lomassese et al. (2007) injected 4-month old males with BrdU to label new cells and killed them 1 month later in order to determine the phenotypes of the new neurons surviving in HVC (Scotto-Lomassese et al. 2007). A few days prior to sacrifice the birds received injections of fluorescent microspheres to RA and CTB to Area X to label the 2 populations of projection neurons. The authors found 42% of the month-old BrdU-positive neurons were retrogradely labeled from RA and none were labeled from Area X. They stained brain sections for 4 proteins found in interneurons and looked for their co-expression in BrdU-positive neurons. The interneuron markers used were GABA, a neurotransmitter expressed by inhibitory interneurons, and the 3 calcium-binding proteins parvalbumin, calretinin and

calbindin that have been shown to be present in HVC interneurons (Wild et al. 2005). No co-localization was observed between BrdU and any of the 4 marker proteins within HVC leading the authors to conclude that within 1 month of birth, no new neurons had become interneurons. It is possible, however, that 1 month is not enough for new neurons to fully mature and turn on the expression of these marker proteins. The authors argue against this possibility because they found a few examples of BrdU/GABA and BrdU/calbindin double-labeled neurons in the nidopallium and striatum of their experimental birds and thus claim that 1 month is a sufficient period for protein expression to occur. Of all the new neurons surviving 1 month in HVC in this study, 57% of them could not be identified as either RA-projecting, X-projecting or interneurons and so the results of Scotto-Lomassese et al. (2007) beg the question: what kind of neuron are they?

Can four-year survival birds resolve the issue?

In the canary, the number of new neurons that can be retrogradely labeled with tracer from RA increases from about 50% at 1 month after injection of the birthdate marker to about 80% after 8 months suggesting that it takes some time for new HVC-RA neurons to extend axons down to RA and become sufficiently connected to take up tracer. The same phenomenon could also be true in the zebra finch HVC; perhaps if Scotto-Lomassese et al. (2007) had waited longer than a month after the BrdU injections, more of the BrdU-positive neurons would have been labeled with tracer from RA. We tested this hypothesis directly in the 6 four-year

survival birds described in Chapter 3; these birds received 3 days of BrdU injections during posthatch days 91-93 and were sacrificed 4 years later. One week prior to sacrifice they were given bilateral injections of green fluorescent CTB to RA to label the HVC-RA neurons present. Both the BrdU-positive and BrdU/CTB double positive neurons in HVC were counted. We found that in these birds, 50% of the BrdU-positive neurons were also CTB-positive. Thus, even when adult-born neurons were allowed 4 years to establish all of their connections, only half of them could be labeled with tracer from RA.

To try and identify the remaining population of new neurons, we tested whether or not any of the 4 year old cells were expressing one or more of the 3 calcium-binding proteins previously shown to label HVC interneurons (Wild et al. 2005). If the lack of co-localization between the markers and BrdU in the Scotto-Lomassese et al. (2007) paper was due to 1 month being insufficient for expression of the proteins, then we would expect to see co-localization after 4 years of maturation. GABA was not used as a marker in these experiments because Scotto-Lomassese et al. (2007) found some expression of GABA in non-neuronal cells and there are also reports of GABA expression by glial cells in mammals (Blomqvist & Broman 1988; Reynolds & Herschkowitz 1987). Since HVC interneurons often express more than one of the three calcium-binding proteins parvalbumin, calretinin and calbindin (Wild et al. 2005), we combined the primary antibodies against all three proteins and used the antibody mix to label neurons expressing one or more of these markers. Scotto-Lomassese et al. (2007) show that this approach labels the

same total number of neurons as can be labeled by both GABA and Hu together (i.e. GABA-positive neurons) and thus it is likely that using a combination of antibodies for parvalbumin, calretinin and calbindin together identifies the entire interneuron population of HVC. We tested each antibody individually to ensure all three were working in our tissue and then proceeded to combine them and look for co-localization with BrdU in the 4 year old neurons (Figure 4.1).

Careful examination of the stained sections using confocal microscopy revealed a small number of ambiguously-labeled BrdU-positive interneurons. These cells appeared to be double-labeled; they had a clear cytoplasm that was positively stained for the interneuron antibodies and the cells contained a small BrdU-positive inclusion (Figure 4.2). However, the BrdU staining in these cells had labeled a region much smaller than a normal cell nucleus and therefore would not normally be counted as a BrdU-positive neuron. We wondered whether there might be a rare class of interneurons with abnormally small nuclei that is produced in adulthood and tested the possibility by staining adult zebra finch HVC tissue with each of the 3 calcium-binding proteins separately but in combination with a fluorescent DNA-binding stain (Hoescht 33258) that labels cell nuclei. Examination of parvalbumin-, calbindin- and calretinin-positive interneurons in confocal sections revealed all classes of interneurons had nuclei of the size normally encountered in interneurons and thus we ruled out the possibility of the double-labeled entities in the BrdU tissue as being true adult-born interneurons (Figure 4.3). Due to the small number of these aberrantly labeled interneurons, we did not attempt to systematically measure the

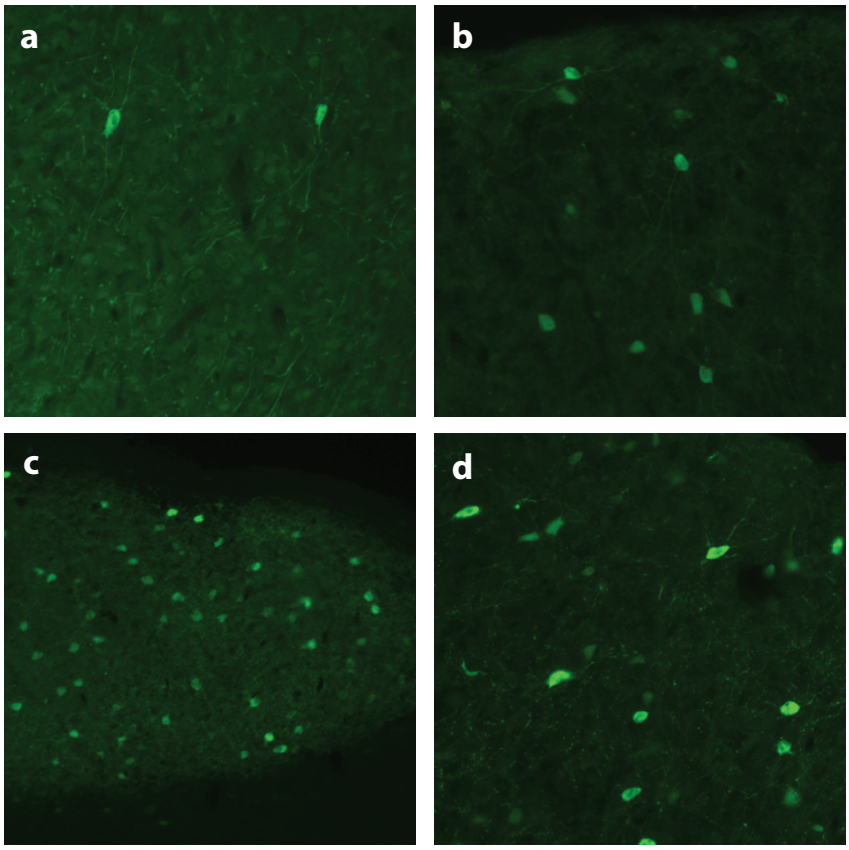


Figure 4.1: Antibody staining for three calcium-binding proteins that are expressed by interneurons.

Primary antibodies for calretinin (**a**), calbindin (**b**) and parvalbumin (**c**) were tested individually in adult zebra finch brain tissue and then combined in a single staining procedure (**d**) to act as a general stain for interneurons. Images **a**, **b** and **d** were taken at 20x magnification but image **c** was taken at 10x to show the increased parvalbumin staining in HVC relative to the surrounding telencephalon.

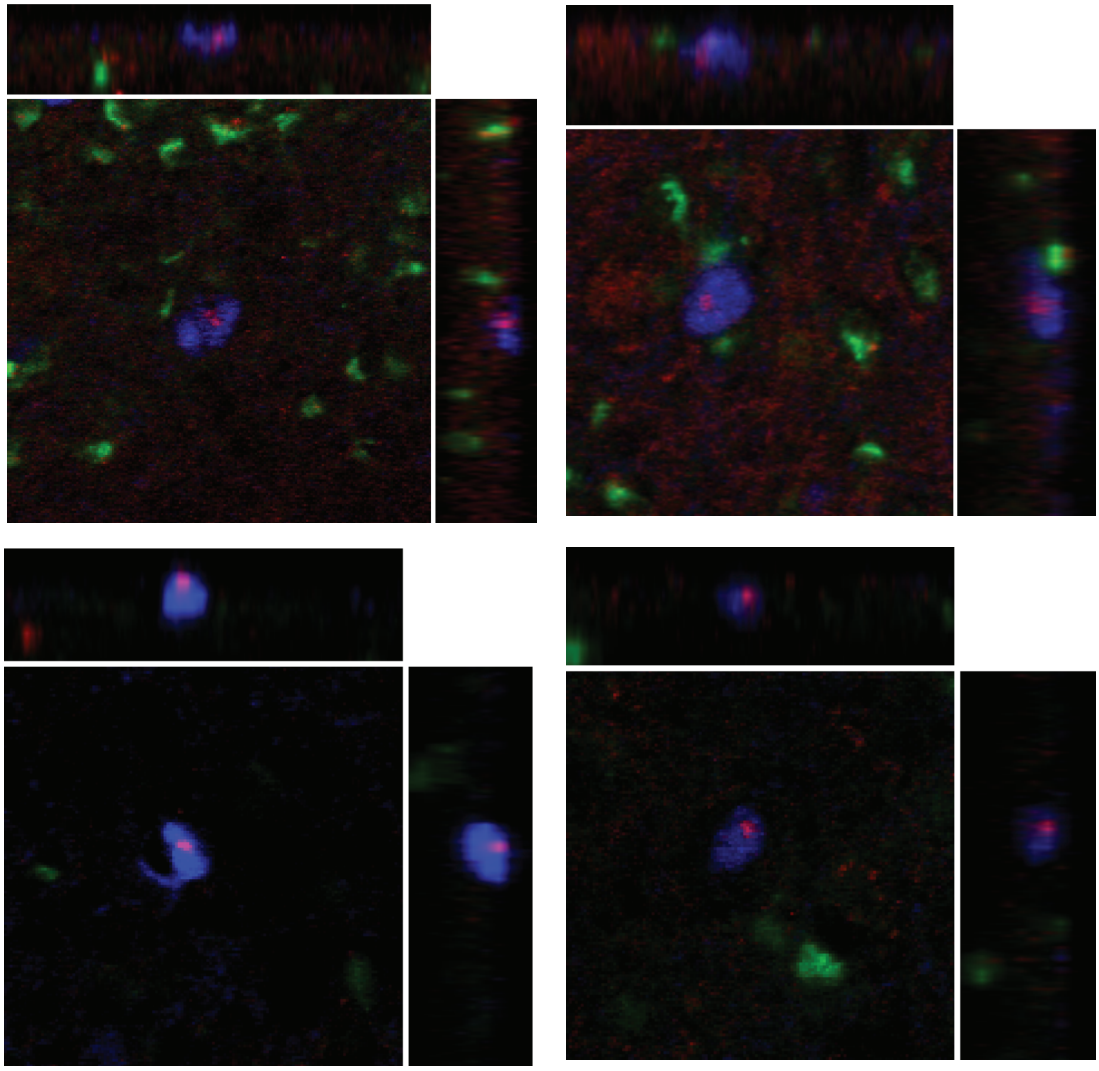


Figure 4.2: Examples of interneurons with small Brdu-positive foci.
Four examples of neurons that were positive for the mix of interneuron markers (calbindin + calretinin + parvalbumin) that also contained a small BrdU-positive inclusion that appeared too small to be the cell nucleus. Blue is the interneuron marker mix, red is BrdU and green is retrograde labeling by CTB from RA. Each image is a single optical section from a confocal stack and is presented with the two orthogonal views above and to the right.

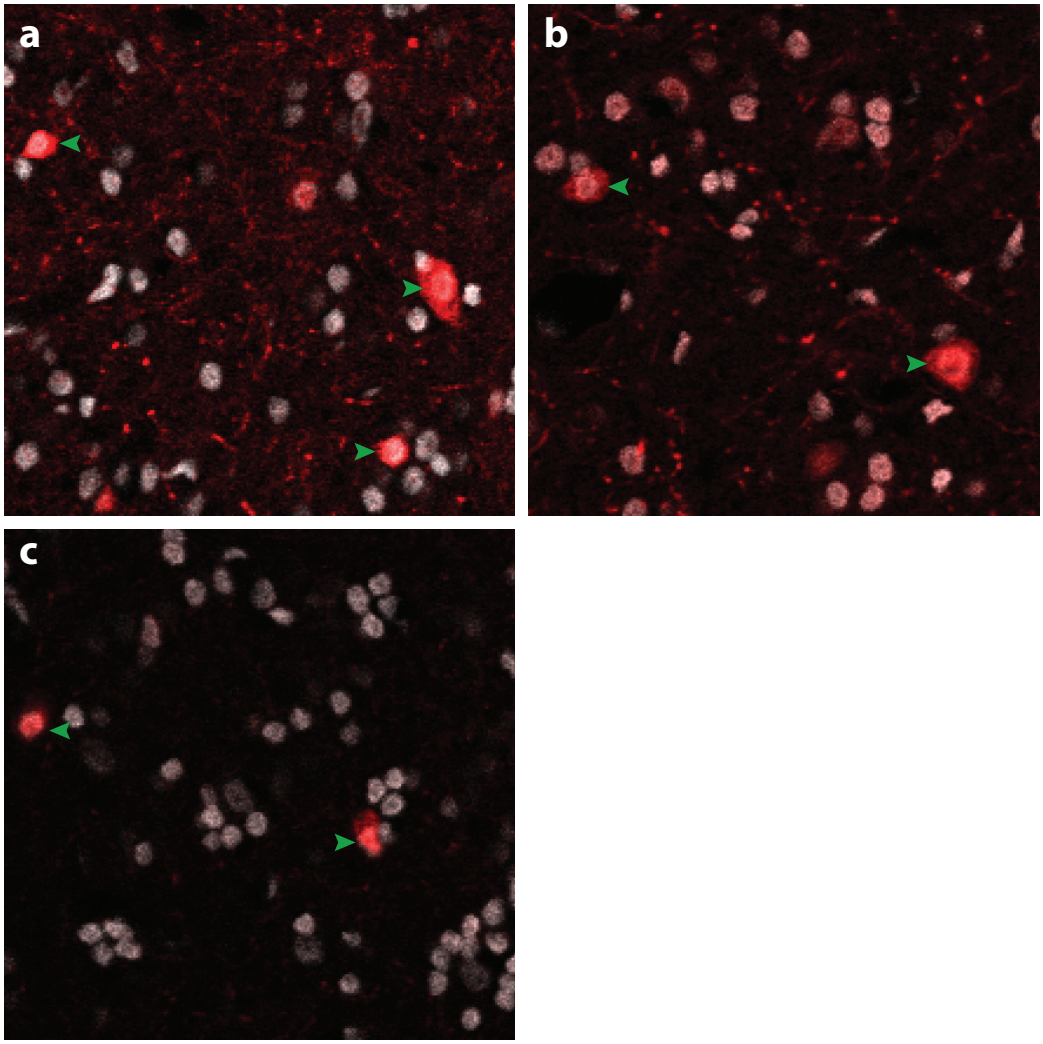


Figure 4.3: Interneurons have normal sized cell nuclei.
Calbindin-positive (**a**), calretinin-positive (**b**) and parvalbumin-positive (**c**) interneurons were found to have normal sized cell nuclei by staining with Hoescht, a nucleic acid stain. The interneuron markers are shown in red and the Hoescht-positive nuclei in white. Interneurons with their nucleus in the plane of the optical section are indicated with green arrowheads.

size of the BrdU-positive inclusions they contained. The inclusions were one order of magnitude smaller than the nuclei labeled by Hoescht so we deemed quantification to be unnecessary.

The significance of punctate BrdU inclusions in a small number of HVC interneurons is not known. Radiation can induce focal incorporation of BrdU in the DNA of human cancer cells in culture and these inclusions appear to represent repair foci (Kao et al. 2001). Thus, it is possible that the BrdU-positive nuclear inclusions we have observed in a small number of interneurons represent areas of DNA that were repaired during the time of BrdU injection. In the radiation-treated cancer cells, there were usually several BrdU-positive foci per nucleus whereas we only ever observed a single inclusion. Gamma irradiation of cells in culture is a harsh treatment that is likely to trigger an abnormally high amount of DNA damage compared to the naturally occurring damage in our tissue and thus could account for the observed differences in the number of repair foci. It is important to remember, however, that the BrdU was given to these birds 4 years prior to sacrifice so the BrdU-positive foci we saw were 4 years old. Experiments to follow the persistence of BrdU-positive repaired DNA over time have not been done so we cannot be sure that repair foci would still be visible after such a long survival time. It is also important to note that the BrdU-positive inclusions we saw were clearly distinguishable from true BrdU-positive nuclei and so we do not need to worry about them contaminating our counts of BrdU/Hu double-positive neurons in the other experiments described in this thesis. Apart from the small and rare labeled nuclear

foci, we encountered no interneurons with full BrdU-positive nuclei in any of the birds examined. Therefore our data support the findings of Scotto-Lomassese et al. (2007) that local interneurons in HVC are not produced in the brain of adult zebra finches.

Does the number of interneurons change with age?

Since we had already stained 4 year old birds for the calcium-binding interneuron markers, we decided to stain 2 additional age groups for the markers in order to see if the number of interneurons changes with age. Presumably, if interneurons are not produced in adulthood, the population should remain the same size across the adult lifespan (or reduce in numbers if some interneurons die). Using the optical fractionator method, we counted the number of neurons stained for either parvalbumin, calretinin or calbindin (using the mix of antibodies) in birds of 90 days, 2 years and 4 years of age (6 birds per group). Surprisingly, we found a significant increase in the total number and packing density of interneurons in HVC between 90 days of age and 2 years, but then no further increase between 2 years and 4 years (Figure 4.4; for density, one-way ANOVA: $F(2,17)= 12.12$, $p= 0.0007$; for total number, one-way ANOVA: $F(2,17)= 13.93$, $p= 0.0003$; posthoc Tukey HSD test reveals a significant difference between 90 days and the two older groups ($p< 0.01$) but not between 2 and 4 years).

An increase in the number of neurons expressing the calcium-binding proteins with age is unexpected given that we see no evidence of production of

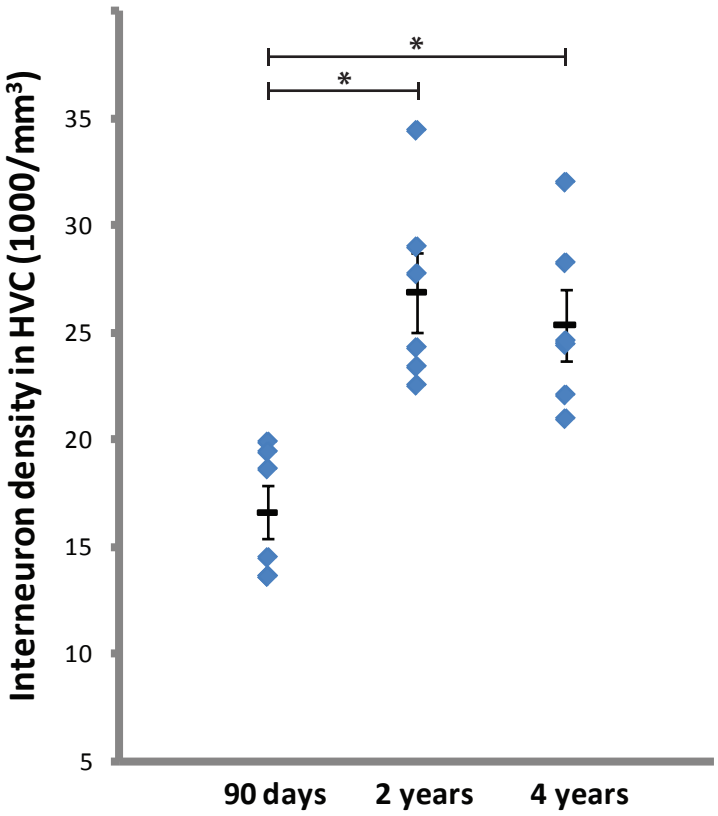


Figure 4.4: Interneuron density in HVC increases between 90 days and 2 years of age but not thereafter.

There is a significant increase in the density of interneurons in HVC between 90 days and two years but no further increase between 2 and 4 years of age. Interneurons were identified by staining with a mixture of antibodies to three calcium binding proteins (calbindin, calretinin and parvalbumin). Each data point represents an average of counts from the left and right HVC of one bird. Group means are represented as black bars with SEM error bars. *= $p<0.01$.

these neurons in adulthood. One possible explanation for the increase is that neurons already present in HVC at posthatch day 90 are not fully mature and thus have not begun to express the calcium-binding proteins. This scenario would be possible if new interneurons were produced towards the end of the juvenile period as it takes about 2 weeks for new neurons to migrate into HVC and begin the maturation process (Kirn et al. 1999). HVC interneurons are known to be produced in the brains of juvenile zebra finches. Scott and Lois (2006) infected the ventricular zone (VZ) of juveniles between 28 and 48 days of age with an oncoretroviral vector containing GFP, a construct that only infects cells during cell division, and found GFP-positive interneurons residing in HVC when they looked 35 days later. In these juveniles, the new HVC interneurons were produced from a different region of the VZ than the new HVC-RA neurons: interneurons were produced from the medial subpallial VZ whereas HVC-RA neurons were produced from the pallial VZ directly above HVC. New interneurons were also added to HVC in much lower numbers than new HVC-RA neurons. The authors did not perform the oncoretroviral vector injections in older juveniles so it is not known whether interneurons continue to be produced towards the end of the juvenile period. If they are still produced late in the juvenile period, and then take a week or more to fully mature in HVC, there could be a population of immature interneurons that would not have been counted at posthatch day 90 but would have been fully mature and therefore counted in the 2 year old birds. There is some evidence to support this explanation: Scotto-Lomassese et al. (2007) report that in 4 month old birds, the proportion of all HVC neurons that can be labeled with a mix of antibodies against the 3 calcium-binding

proteins is 8.9%. Similarly, we find 9.0% and 9.1% of all HVC neurons (as determined by counts of Hu-positive cells) express one or more of the marker proteins in 2 and 4 year old birds, respectively. However, in the 90 day olds only 6.5% of all HVC neurons can be labeled with the antibody mix suggesting there is an increase in expression of the marker proteins between 90 and 120 days of age. A direct test of this hypothesis would be to inject juveniles around posthatch day 75 with BrdU to label cells produced in the late juvenile period and then sacrifice the birds at posthatch day 120 to look for the presence of BrdU-positive nuclei in neurons expressing the calcium-binding proteins. This would show whether or not new interneurons are actually being produced at the end of the juvenile period, which is necessary if there is to be an immature population of interneurons present at posthatch day 90.

It is interesting that new interneurons are recruited to HVC in juvenile birds (Scott & Lois 2007) but that we find no evidence of their production in the adult HVC. It is possible that the different methods used to identify new neurons, oncoretroviral infection in the juvenile brain and BrdU in the adult brain, is the reason for the difference. Perhaps, for some reason, the dividing progenitors in the subpallial VZ region that generate new interneurons do not take up sufficient BrdU to produce visible BrdU-positive interneurons in the adult HVC, but in contrast, the progenitors above HVC do take up enough BrdU and thus we see new BrdU-positive HVC-RA neurons in adulthood. This scenario seems unlikely because BrdU-positive VZ cells can be observed in the subpallial region in adults treated with BrdU (personal

observations). As the subpallial VZ progenitors can take up enough BrdU to be visible, the most parsimonious explanation of our failure to find BrdU-positive interneurons in HVC is that they are no longer produced in adulthood. There could, however, be more than one type of progenitor present in the subpallial VZ region. Perhaps the interneurons are produced by a specific class of progenitors that do not get labeled with BrdU. Since oncoretroviral vectors are capable of labeling new interneurons in the young juvenile brain, similar vector injections should be performed into the subpallial VZ region of adult male zebra finches to see whether adult-born interneurons can be identified in HVC using this alternative labeling method. If adult-born interneurons are still not observed, it will be interesting to explore why this class of HVC neurons are generated during the juvenile learning period but not in the adult song system. It could be that the incorporation of new interneurons to HVC is important for vocal learning and that once their production comes to an end, no further learning is possible. This hypothesis could be explored by looking for the continued production of HVC interneurons using oncoretroviral injections in adult canaries, a species which is capable of vocal learning in adulthood.

The limitations of labeling with retrograde tracers.

As you can see, the question of which kinds of neurons are produced in the adult HVC still remains very much unresolved. We have confirmed previous reports that interneurons are not produced in adulthood and we have disproved the

hypothesis that waiting a sufficiently long amount of time after BrdU injection will allow all new neurons to be labeled by retrograde tracers from nucleus RA. If adult-born neurons cannot be retrogradely labeled after 4 years of survival in HVC, it is probably safe to conclude that they will never be labeled in this manner. Even after 4 years, only half of the neurons born at the beginning of adulthood take up CTB from RA. The remaining unlabeled 50% must be fully mature after such a long time but do not express any of calcium-binding proteins known to be present in HVC interneurons. Thus, half of the adult-born neurons that persist in the zebra finch HVC are of an unknown identity.

Given that the data described in Chapters 2 and 3 suggest adult-born neurons are accumulating in HVC, in a 4 year old bird the number of adult-born neurons of unknown identity would represent about 5-10% of the total HVC population (based on the rate of neuron addition published in Wang et al. (2002) and my counts of total HVC neurons in 4 year old birds). Thus, it is unlikely that these neurons belong to a new class of cell that has not yet been described in the song system. A more likely explanation is that limitations of the methods used to identify RA-projecting neurons results in an underestimation of the proportion of new neurons that send axons down to RA. There are several factors that could affect retrograde transport of tracers from RA. These include: whether the axon terminals come into contact with the tracer; how efficiently the tracer is taken up if contact is made; how much tracer must accumulate in the cell body before it is visible under the microscope; and whether or not some neurons are physiologically incapable of

taking up a particular tracer. None of these issues have been systematically explored in the song system. It is known from making small local injections of anterograde tracers into HVC that there is no obvious topography in the distribution of HVC-RA neurons within HVC (Yip et al. 2010). Additionally, HVC-RA axons terminals often traverse far across RA but branch very few times, typically once or twice only, so the area that they cover is very limited (Yip et al. 2010). Both these factors are important for considering the uptake of retrograde tracers from RA because some, like latex microspheres, do not diffuse far from the point of injection and the injection site seldom fills the whole of RA. Since axon terminals from HVC have very few branches, it is conceivable that a small local injection of a poorly-diffusing tracer would fail to reach several terminals. In theory this issue seems pertinent to efficiency of retrograde labeling but in reality it might be less important. Comparing the HVC-RA neuron counts made in Chapters 2 and 3 shows that for a given age, the same range of neurons are retrogradely labeled by both latex microspheres and CTB. In contrast to latex microspheres, CTB diffuses some distance from the site of injection. Inspection of RA after a successful injection of CTB usually shows that the entire nucleus is filled (personal observations). With a diffusible dye, one would expect many more HVC-RA neuron terminals to encounter the tracer and become retrogradely labeled than with a non-diffusible tracer like microspheres, but this is not what we observe in the zebra finch.

The efficiency with which HVC-RA neurons take up different retrograde tracers probably depends the most on which of the several possible mechanisms of

uptake is used by the particular tracer. For example, CTB uptake is mediated by a specific ganglioside receptor GM1 found in neuron membranes that is actively cycled into the neuron soma (van Heyningen 1974); DiI is a lipophilic dye that inserts into the plasma membrane and diffuses freely back along the axon (Godement et al. 1987); Fluorogold diffuses freely across the plasma membrane but then gets trapped in acidified endosomal compartments because it is weakly basic and becomes charged in the acidic environment (Wessendorf 1991). The mechanism of transport by latex microspheres has not been completely elucidated but appears to involve non-specific interactions with receptors clustered in lipid rafts (Katz & Iarovici 1990). It could be that CTB and latex microspheres label HVC-RA neurons with similar efficiencies because both rely on receptor-mediated uptake for retrograde transport. Perhaps there is a class of neurons that is added to HVC in adulthood, sends projections down to RA but then does not possess the necessary receptors to take up either CTB or latex microspheres and thus has not been identified in this work or by the Scotto-Lomassese et al (2007). It might be worthwhile to test some of the other known tracers with different modes of transport to see if a higher proportion of adult-born neurons can be labeled from RA. A positive result would add support to the hypothesis that the large number of unidentified new neurons in the zebra finch HVC represents an inefficiency of tracer uptake/transport by HVC-RA projection neurons rather than the presence of a new neuron type added in adulthood.

Another way to test for HVC-RA neuronal identity would be to bypass the use of retrograde tracers altogether and find marker proteins expressed by the HVC-RA

neuron population. Some potential candidate markers were identified in a microarray screen comparing the gene expression of HVC-RA and HVC-X neurons isolated by laser capture microdissection (Lombardino et al. 2006). Four genes were found to be expressed more highly in the RA-projecting neurons: regulator of G-protein signalling 4 (*RGS4*), Neuroserpin (*NSP*), Hippocalcin-like 1 (*HPCAL1*) and hemoglobin alpha 1 (*HBA1*). Of these, *RGS4* is a particularly promising candidate because it is very highly expressed in HVC and RA compared to the rest of the telencephalon, as shown by situ hybridization (Li et al. 2007). The suitability of these candidates to be HVC-RA neuron markers must be determined experimentally using in situ hydridisation: successful markers will show expression either exclusively in the HVC-RA neurons or at a level high enough above expression in the other cell types that HVC-RA neurons can be unambiguously distinguished. Ideally, expression of the gene's protein product will match the expression profile of the gene so that immunohistochemisty, rather than in situ hybridization, can be used to visualize the neurons. Since BrdU is identified using antibodies, it will be much easier to experimentally combine BrdU and antibody markers of HVC-RA neurons in order to test the neuronal phenotypes of adult-born neurons in the zebra finch HVC.

CHAPTER 5: THE RELATIONSHIP BETWEEN NEURON ADDITION AND SONG

The paradox in zebra finches.

The discovery that HVC-RA neurons are constantly adding to the adult zebra finch song system raises questions relating to their potential function in the song system. As discussed earlier, HVC-RA neurons are thought to have a premotor role in song production because they increase their firing during singing and burst at a very specific and stereotyped moment in the song motif (Hahnloser et al. 2002; Kozhevnikov & Fee 2007). This class of neurons is mostly added to the song circuit after hatching and during the juvenile song learning period (Goldman & Nottebohm 1983; Scott & Lois 2007; Alvarez-buylla & Theelen 1988; Ward et al. 1998). Their recruitment to HVC declines as the bird reaches sexual maturity and the critical period for song learning closes (Wilbrecht et al. 2006). Overlap between the developmental timing of HVC-RA neuron addition and song learning in the zebra finch, together with the seasonal correlations between HVC neuronal recruitment and new syllable acquisition in the canary (Kirn et al. 1994) lead us to suppose that adult-born neurons in HVC may be involved with the learning of new song elements. However, despite a decline in neuronal recruitment to the zebra finch HVC after sexual maturity, we have shown in the present thesis that the continued addition of new neurons causes a doubling of the HVC-RA neuron population over the first two years of adulthood, even though no further song imitation is possible in this species reaching sexual maturity. Thus, the potential role of adult-born neurons in song motor learning, at least in the zebra finch, is called into question. While it is exciting

to consider that new neurons could be responsible for the increases in vocal plasticity associated with song learning, it is important to remember that the original data suggesting such a role was entirely correlational. Therefore another factor, like hormone levels, which are known to vary developmentally and seasonally, could regulate both song learning and neuronal recruitment completely independently of one another.

A potential role for neuron addition in adult song stereotypy and stability.

The net addition of adult-born neurons to HVC, as opposed to a continuous replacement process, suggests an alternative role for adult neurogenesis in the zebra finch song system, especially when considering the reported age-related changes in adult song. Even though zebra finches do not learn new songs or syllables after sexual maturity, the song becomes progressively more stereotyped with age. Variability in the fundamental frequency of syllables is significantly lower in 4 year olds compared to 6 month olds (Kao & Brainard 2006) and the song motif is more consistently reproduced at 15 months of age than it was at 4 months (Pytte et al. 2007). In addition, song becomes more resilient to the effects of deafening as a bird gets older: the song of young adults begins to lose both temporal and acoustic structure within a week or two after bilateral cochlea removal, but the song of older birds can retain normal song structure for up to a year before changes begin to show (Nordeen & Nordeen 1992; Lombardino & Nottebohm 2000; Brainard & Doupe 2000). The transition from low to high song stability occurs around 6 months of age

(Lombardino & Nottebohm 2000) right at the time when we see the greatest rate of increase in the number of HVC-RA neurons. Thus, the net addition of new HVC-RA neurons to the adult zebra finch song system could be contributing to the improvements in song stereotypy and song stability that occur as a male ages.

Variability in vocal output could result from several sources, but one known source is the glutamatergic inputs to RA from lMAN that show variable firing patterns with respect to the song motif (Hessler & Doupe 1999; Olveczky et al. 2005). This variable input to the motor program is essential for juvenile song learning (Bottjer et al. 1984; Scharff & Nottebohm 1991) and as development progresses the number of lMAN-RA synapses decreases considerably (Herrmann & Bischof 1986). Stereotyped adult song emerges when the dominating source of synaptic inputs to RA shifts from the lMAN-RA neurons with their variable firing pattern to the HVC-RA neurons that exhibit highly stereotyped activity (Aronov et al. 2008). Despite the reduction in lMAN-RA synapses during song learning, lMAN still appears to contribute to the variability of adult song. As described in the introduction, song motifs sung while the bird is by itself and which are referred to as “undirected” are less consistent between renditions and contain noisier and more variable syllables than motifs “directed” toward a female (Roland 1980; Kao et al. 2005). Undirected singing is accompanied by a significant increase in the variable firing rate of lMAN compared to directed song and moreover, lesions or pharmacological inactivation of lMAN reduce the variability of undirected song to that of female-directed song (Kao et al. 2005; Kao & Brainard 2006; Stepanek & Doupe 2010). Interestingly, aging has

a similar effect to that of lMAN lesions on the variability of syllables: it reduces variability in the fundamental frequency of undirected syllables to that of female-directed syllables (Kao & Brainard 2006). However, this age-related reduction cannot be attributed to a concurrent age-related reduction in the variability of lMAN activity during undirected singing (Kao & Brainard 2006). Therefore another factor must be responsible for the improvements in undirected song stereotypy that occur with aging. Considering that the emergence of stereotypy during vocal learning coincides with a shift in the balance of lMAN and HVC inputs to RA, it is possible that the significant addition of new HVC-RA neurons to the adult song system acts to further increase HVC inputs relative to those from lMAN. This strengthening of HVC's dominance over the motor output of RA would act to further reduce the relative variable contributions from lMAN resulting in a more stereotyped and reproducible song motif.

It is not known what causes song to become more resilient to the deleterious effects of deafening with age. As discussed in the introduction, one suggestion is that experience in the form of vocal practice and not chronological age is actually the mediator of stability, with the song becoming more "ingrained" in the motor pathway each time the bird sings it (Lombardino & Nottebohm 2000). Another hypothesis is that adult neurogenesis in HVC is a source of vocal plasticity and therefore the age-related decline in neuronal recruitment to HVC results in stabilization of the motor program (Lombardino & Nottebohm 2000; Wang et al. 2002). This idea was largely based on the belief that new neurons were replacing older neurons, as they appear

to do in the canary HVC. Now that we know adult neurogenesis in the zebra finch results in net addition of HVC-RA neurons to the song system, a more straightforward explanation can be proposed: that an increase in the number of HVC-RA neurons results in stabilization of the motor program. If adult-born HVC-RA neurons participate in the song motor program, something that has not yet been directly tested, then their addition to HVC could increase the number of neurons active at any one moment in the song motif (because the song motif is not lengthening with age). Theoretically, an increase in the redundancy of HVC-RA neurons could allow the system to tolerate the loss of correct functioning of more neurons before there are noticeable effects on the vocal output. For example, if the loss of auditory feedback through deafening caused the HVC-RA neurons to gradually drift from their normal time-locked firing pattern, this drift would be more apparent in the song of younger birds where fewer neurons control each moment in the song. In older birds with twice the number of HVC-RA neurons encoding a moment in the song, more neurons could potentially lose their correct firing pattern before any change would appear in the song motif, and as a result the song of older birds would take longer to deteriorate after deafening.

Putting these ideas to the test.

The ideal experiment to test for a causal relationship between HVC-RA neuron addition and the stability and stereotypy of adult song would be to block the addition of new neurons to HVC in adult zebra finches and see whether the normal

age-related changes in song occur in the absence of net neuron addition. Because new neurons integrate over most of the telencephalon in adult song birds, including other areas in the song system, this approach would require the development of tools to specifically regulate the progenitors that provide neurons to HVC, otherwise we could cause non-specific effects that would confuse the interpretation of any resulting behavioral changes. Until such tools become available, we have to rely on correlative evidence to see whether neuronal addition and changes in adult song are directly related.

We attempted to explore the relationship between net neuronal addition of HVC-RA neurons and adult song by using manipulations known to interfere with both song stereotypy and stability. We subjected young adult males to either deafening or to the combination of lMAN electrolytic lesions followed by deafening (L+D), allowed them to reach 1 year of age and then counted the number of HVC-RA neurons to determine whether addition of new HVC-RA neurons had proceeded as it does in hearing control birds. As described in the introduction, deafening in young adults results in degradation of the spectral and temporal structure of song whereas prior lesions of lMAN completely block degradation and normal song structure is preserved (Brainard & Doupe 2000; Brainard & Doupe 2001; Scott et al. 2007). If addition of HVC-RA neurons and changes in song are causally related, one might expect such disruptions to affect the process of neuronal recruitment to HVC.

A timeline for the experimental procedure is shown in Figure 5.1. For the deaf group, ten males had their songs recorded starting at about 125 days of age and then their cochleae were removed bilaterally a few days later. The birds were between 127 and 136 days of age at the time of deafening. The next group, also 10 males, received the combination of lMAN lesions and deafening (L+D): their song was recorded, then bilateral electrolytic lesions of lMAN were performed between 115 and 122 days of age, after which their song was recorded again. Two weeks later both cochleae were removed so that these birds were approximately the same age at deafening as those of the previous group (128-134 days old). All 20 experimental birds were recorded continuously for the month after deafening to document any changes in their song and then again for a few days at 2 later time points, around 220 and 340 days of age. Eight control birds were housed under identical conditions as the birds in the deaf and L+D groups and recorded for the same periods to allow a direct comparison of song changes and neuron numbers between birds in the 3 groups. Just after the last recording session around posthatch day 340, all 28 birds received bilateral injections of CTB to RA to retrogradely label HVC-RA neurons present seven months after the time of deafening. According to the data presented in Chapter 2, we would expect the hearing controls to have added a significant number of new HVC-RA neurons over this seven month period. If deafening or the combination of lMAN lesions and deafening have an effect on the addition of neurons to HVC then we would expect there to be a difference in the total number of HVC-RA neurons between groups by the end of the experiment. Of course, total HVC-RA neuron numbers can be influenced by both addition and loss

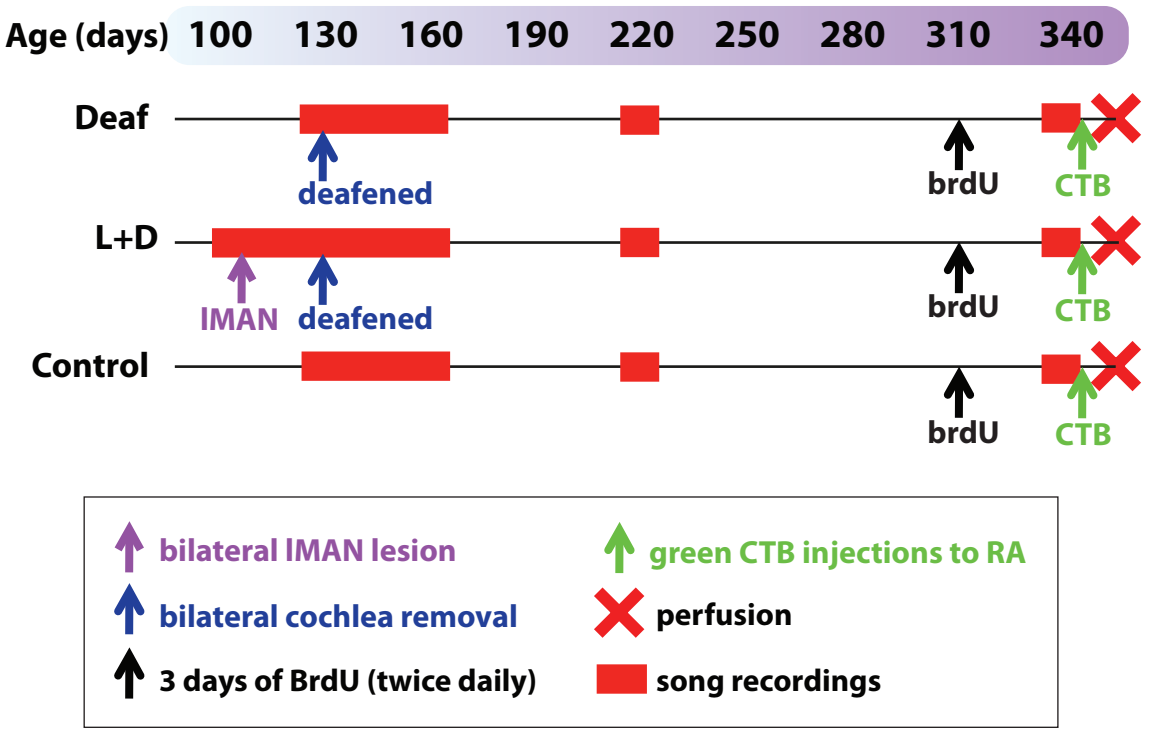


Figure 5.1: Experimental timeline to investigate the relationship between changes in song structure and addition of HVC-RA neurons.
Birds in the 'deaf' group (n=10) received bilateral cochlea removal at around 130 days of age (range: 127-136). They had their songs recorded a few days prior to surgery and then continuously for the month after deafening to document changes. Birds in the 'L+D' group (n=10) received bilateral electrolytic lesions of IMAN at around 118 days of age (range: 115-122) followed 2 weeks later by bilateral cochlea removal. Their songs were recorded before any surgical manipulation, in the two weeks between IMAN lesions and deafening and then continuously for the month after deafening. Birds in the 'control' group (n=8) were just recorded for a month starting around 130 days of age (range: 120-130). All 28 birds were recorded again at 220 and 340 days of age. Immediately after the final recording session, birds received bilateral injections of green CTB to RA to label the HVC-RA neurons present and were then sacrificed 6 days later. Counts of the number of HVC-RA neurons were made to determine whether 7 months of disrupted auditory feedback had affected the number of HVC-RA neurons. One month prior to sacrifice, birds received BrdU injections twice a day for 3 consecutive days to birthdate label new neurons. Counts of BrdU-positive neurons in HVC were made to determine whether the surgical manipulations had affected the rate of neuronal recruitment to HVC.

of neurons. Although in Chapter 3 we found no loss of HVC-RA neurons over a similar length of time in normal hearing birds, we cannot assume that one of the surgical manipulations performed here would not induce neuronal death. Thus, in addition to counts of total HVC-RA neurons, we also treated all 28 birds with BrdU injections 1 month prior to sacrifice to estimate the rate of neuronal recruitment in birds of the control, deaf and L+D groups. If the total HVC-RA neuron numbers are the same between the experimental groups, these counts of new neurons surviving for 1 month in HVC could provide additional information about the dynamics of neuronal recruitment after the different surgical manipulations.

The brains of all 28 birds were sectioned through both HVC and lMAN. Every third HVC section was stained for BrdU and the neuronal marker Hu to facilitate identification of neurons born 1 month prior to sacrifice. The stained slides were imaged using 3-colour confocal microscopy to capture HVC-RA neurons retrogradely labeled with CTB as well as the BrdU and Hu staining. These confocal stacks were used to count the following: the number of HVC-RA neurons; the number of BrdU/Hu double positive new neurons; the number of triple-labeled BrdU/Hu/CTB new HVC-RA neurons; the number of BrdU-positive ventricular zone cells lining the ventricle above HVC. Additionally, the same slides were imaged on an inverted fluorescent microscope at lower magnification to measure the volume of HVC. Every other section through lMAN was stained with cresyl violet and imaged using brightfield microscopy to determine how successful the lMAN lesions were. LMAN volumes were measured in the control and deaf birds and for L+D birds, the

volume of any remaining lMAN was measured. For all counts and volume measurements, both left and right hemispheres were analyzed and then combined to give an average value for each bird.

Not all L+D birds had complete bilateral lesions of lMAN. For birds with incomplete lesions, the volume of the remaining segment of lMAN was measured and compared to the average lMAN volume of the 8 control birds. Two L+D were excluded from further analysis because 60% of the normal lMAN volume of both hemispheres was still intact. The 8 L+D birds that were included in the final analysis had a mean value of 17% (range 0 -43%) of lMAN remaining (Figure 5.2). The volume of lMAN remaining in the L+D birds was significantly lower than the average lMAN volume for both other groups (one-way ANOVA: $F(2,25)= 28.86$, $p< 0.00001$; Tukey post-hoc analysis: $p < 0.00001$ for L+D compared to both control and deaf groups). LMAN volume in the deafened birds was slightly lower than that of the control group but the difference was not significant (Tukey post-hoc analysis: $p= 0.99$).

The song changes resulting from deafening, or from the combination of prior lMAN lesion and deafening, in adult zebra finches have been analyzed and described in detail in several previous studies (Nordeen & Nordeen 1992; Lombardino & Nottebohm 2000; Brainard & Doupe 2000; Brainard & Doupe 2001). Accordingly, we did not undertake a rigorous analysis of the nature of changes in the

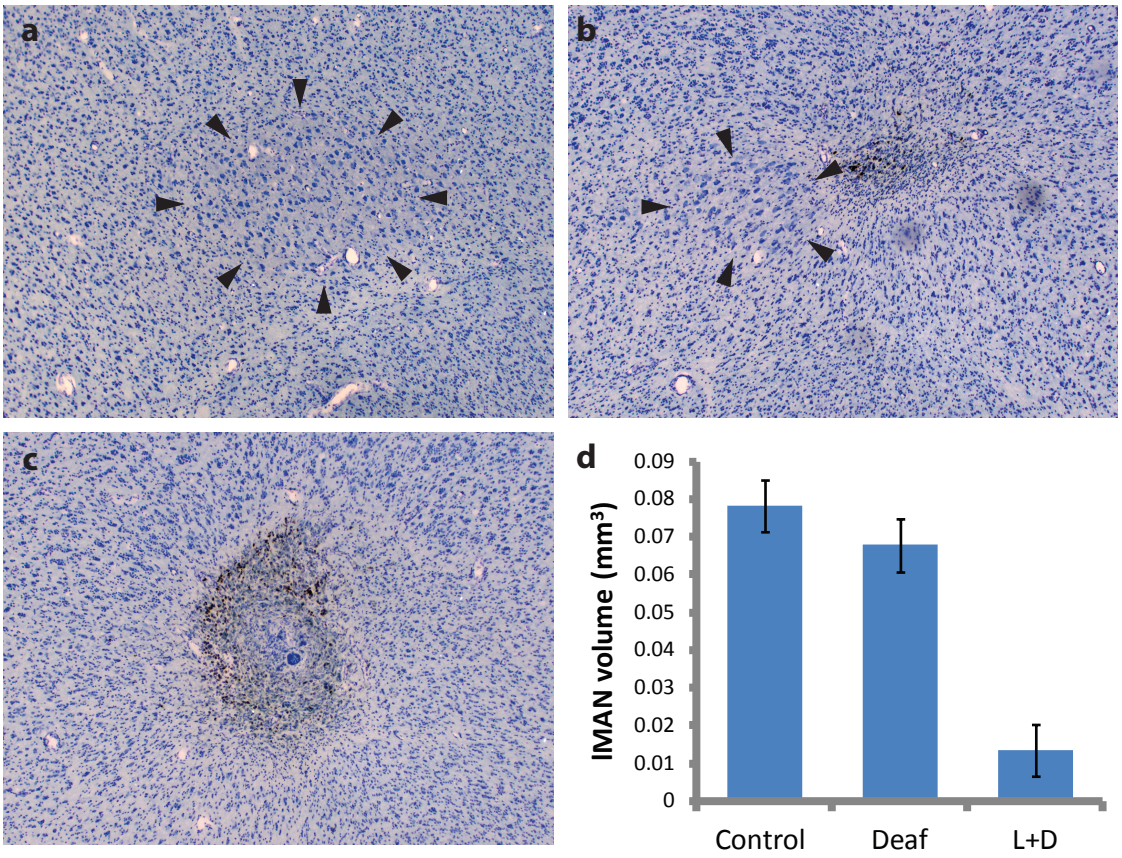


Figure 5.2: Quantifying the electrolytic lesions of lMAN in the L+D birds. Brightfield images of lMAN tissue stained with cresyl violet showing (**a**) an intact lMAN from a control bird, (**b**) a partial lMAN lesion that spared 21% of the nucleus (based on the average lMAN volume from the control birds) and (**c**) a complete lesion of lMAN. The boundaries of lMAN were identified using the large magnocellular neurons that make up the lMAN core. Arrowheads mark the edges of lMAN in a and b. (**d**) lMAN volume was significantly reduced in the L+D group compared to both the control and deaf groups ($p < 0.00001$). Comparing the average lMAN volumes from the control and L+D groups, the L+D birds had an average of 17% of lMAN remaining. There was no significant difference in lMAN volume between the deaf and control birds ($p = 0.99$). Error bars represent SEM.

song of our experimental birds, but some analysis was necessary to ensure that our surgical manipulations had replicated the previous observations. We examined the songs of all three groups produced before any surgical manipulation (around posthatch day 130 for the control and deaf birds and around posthatch day 118 for the L+D birds) and at the final recording session (around posthatch day 340 for all groups). In addition for the L+D group, we also looked at the songs produced on a day between the lMAN lesion and deafening surgeries.

Inspection of the song files showed that all 3 groups exhibited the expected patterns of song changes (Figure 5.3). At 340 days of age the songs of the control birds were highly similar to the songs recorded at day 130, with at most a subtle change in one or two notes of the motif. An example of such a change is shown in the top two panels of Figure 5.3 where there was a change in the harmonic structure of the note underlined in red. In comparison, the songs of all 10 deafened birds were noticeably different between the pre-surgery recording at day 130 and the final recording at day 340. The deafened birds showed a progressive deterioration of both spectral and temporal features of the song motif but the extent to which these features degraded was variable between individuals, as has been previously described (Nordeen & Nordeen 1992; Lombardino & Nottebohm 2000). By the final recording session, all 10 birds had severe degradation of their original pre-surgery motif. In the 2 examples presented in Figure 5.3, the deaf bird #1 showed the most modest changes of the group. There is spectral degradation of most of the original syllables but some are still recognizable and the song still retains some temporal

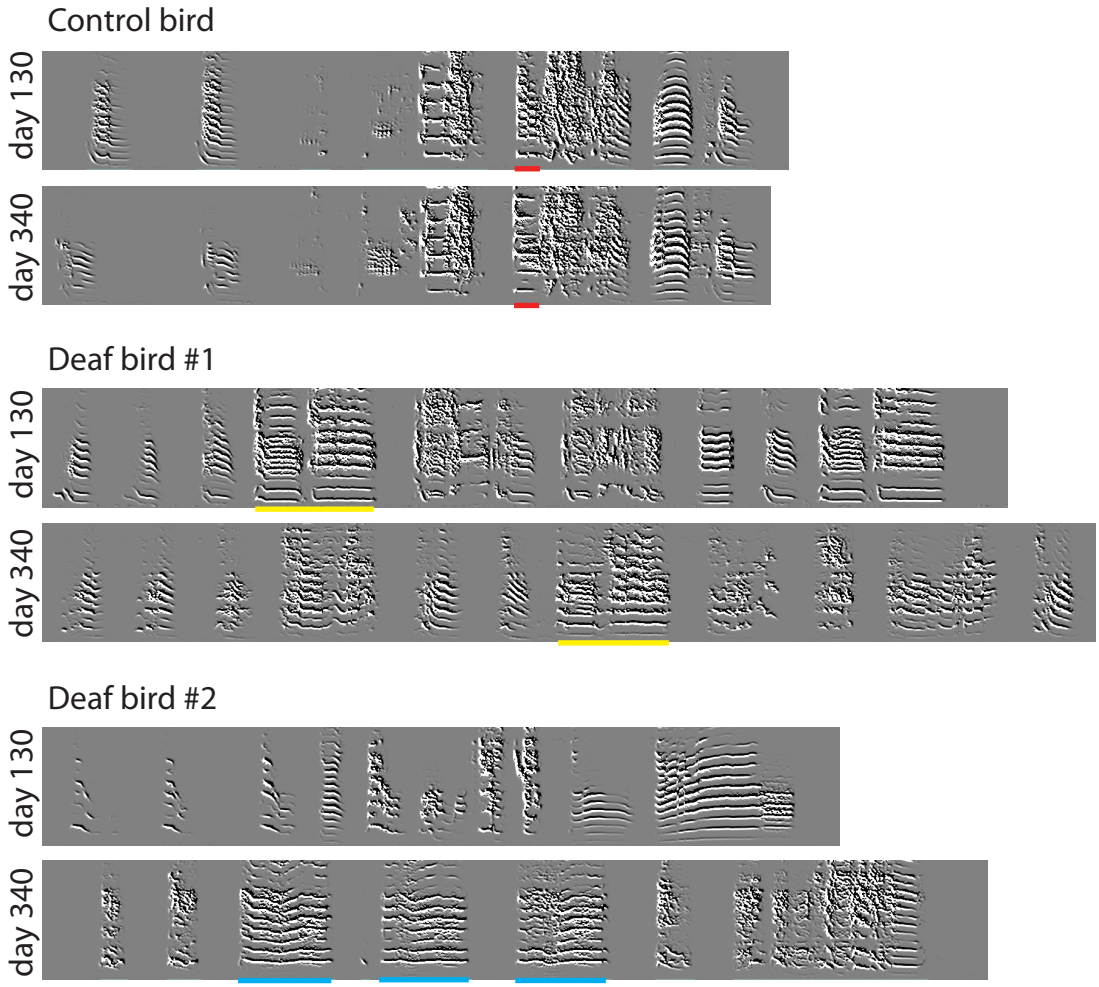
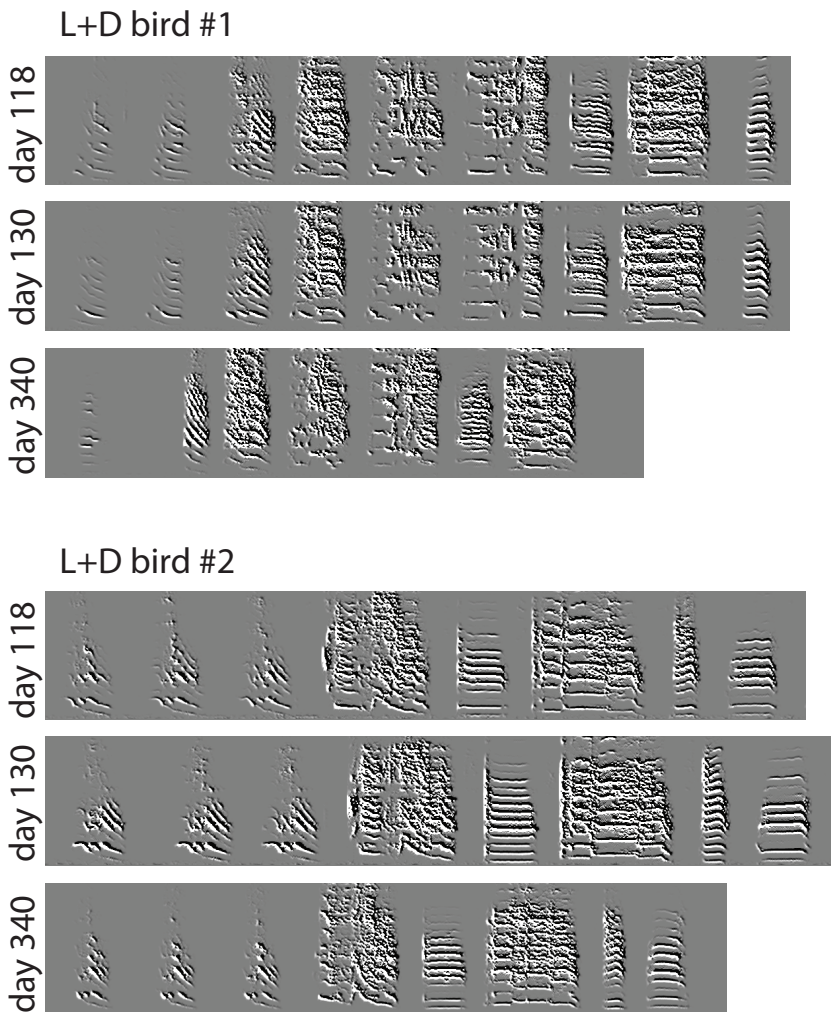


Figure 5.3: Song examples from control, deaf and L+D birds produced before surgery and at the final recording session replicate the behavioral changes seen in earlier studies.

Songs from the control birds only showed very minor changes between 130 and 340 days of age. The example here showed a change in the harmonic structure of the first part of one of its syllables (underlined in red) but all other syllables retained good spectral structure and the overall motif structure was unchanged. In contrast, the song of all ten deaf birds showed significant degradation of both spectral and temporal structure. Most of the syllables produced by deaf bird #1 were lost by day 340 but one was still recognizable (underlined in yellow). The song of deaf bird #2 showed disruption of the temporal structure: many of the short syllables present in the pre-surgery song were absent by day 340 and abnormally long syllables had appeared. The majority of syllables produced by this bird on day 340 resembled unstructured and 'wavering' harmonic stacks (underlined in blue). **Continued on the next page...**



...Figure 5.3 continued from previous page:
Lesioning lMAN prior to deafening prevented most of the spectral degradation of syllables. For the L+D birds, songs produced on day 118 represent their pre-surgery motifs. Songs from day 130 represent songs produced between lMAN lesion and deafening. Songs from day 340 represent the song at the end of the experiment. lMAN lesions by themselves had no overt effect on the song structure (compare songs from day 118 and day 130). By day 340, most of the birds had preserved the original syllable order and motif structure but all showed an increase in song tempo resulting in an overall shortening of the motif. The syllables produced by L+D bird #1 showed some spectral changes between day 130 and 340 which were more pronounced than was typically observed for control birds but all syllables were still recognizable as versions of the original pre-surgery syllables, something that was not observed in the deaf group. Therefore, lMAN lesions prior to deafening prevent much of the song deterioration that results from deafening alone.

structure. The second example, deaf bird #2, shows more pronounced changes in the temporal structure as all the short duration syllables have disappeared. Most of the syllables produced by this bird at the final time point lacked any fine spectral structure and just resembled “wavering” harmonic stacks (underlined in blue in Figure 5.3), similar to those described by Brainard and Doupe (2001).

In contrast to the deafened group and consistent with earlier studies, changes in the song motifs of the L+D group between 130 and 340 days of age were much less marked. As can be seen from the 2 examples in Figure 5.3, most of the pre-surgery syllables were present in the final recordings and showed preserved spectral morphology, with the occasional subtle change similar to those observed in the hearing controls. However, there was one striking difference between the songs recorded from the L+D birds compared to the controls: the motif tempo increased significantly between posthatch day 130 and 340 in all 8 birds. An increase in the tempo of undirected song after lMAN lesions in both hearing and deaf birds has been previously reported (Williams & Mehta 1999; Kao & Brainard 2006; Brainard & Doupe 2001).

To quantify the deterioration of song after deafening we used Sound Analysis Pro software (Tchernichovski et al. 2000) to compare song motifs recorded at several time points after surgery to the original pre-surgery motif. The software breaks down the motifs into their component syllables and compares each syllable

using a range of acoustic features including pitch, frequency, duration, pitch modulation and entropy. Each pre-surgery syllable is matched to the most similar syllable in the post-surgery motif and the syllable pair is scored on how much of their length can be matched and how accurate the match is. The software averages across all syllables in the motif and calculates a composite 'similarity score', taking into account the order in which the matched syllables appear in the post-surgery motif.

For the deafened birds, song motifs produced at posthatch day 140, 160, 220 and 340 (which corresponds to 10, 30, 90 and 210 days after deafening) were compared to the original pre-surgery motif. For each time point, 25 post-surgery motifs were compared to a canonical pre-surgery motif which was selected by screening through several pre-surgery song files and finding a motif that represented the most consistently sung pattern of syllables. Within each bird and time point, an average similarity score was calculated using all the individual motif comparisons, and this score was then normalized to the average score generated when 25 pre-surgery motifs were compared to the canonical pre-surgery motif. This normalization step was included because the consistency with which the pre-surgery song was produced varied between individuals. The sound analysis data confirms that all deafened birds showed deterioration of their song motif and that the extent and rate of that degradation was variable between individuals (Figure 5.4).

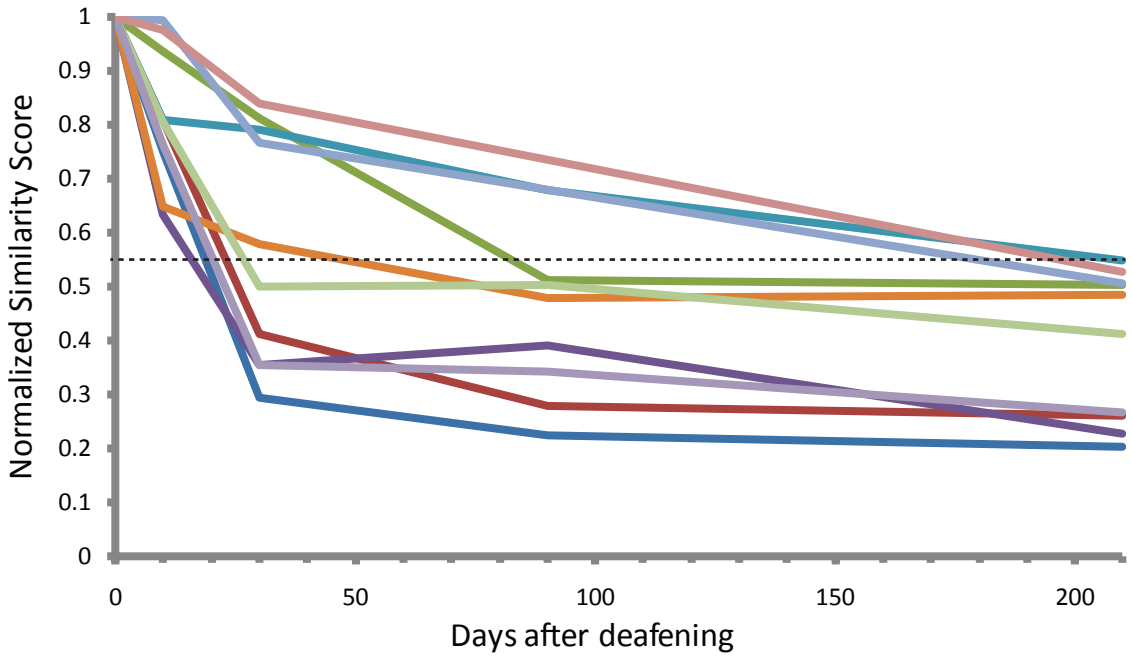


Figure 5.4: Birds deafened at 130 days of age show progressive and variable loss of their adult song motif.

For each bird and each time point after deafening, 25 song motifs produced on a given day were compared to a canonical pre-surgery motif from day 130 using Sound Analysis Pro software (Tchernichovski et al. 2000). A similarity score was generated for each comparison that reflects how similar the two motifs are (see methods). Twenty-five pre-surgery motifs from day 130 were also compared to the canonical pre-surgery motif and averaged to get an indication of the baseline motif variability for each bird. At each time point after deafening (10, 30, 90 and 220 days) the average similarity score was divided by the baseline similarity score to generate a normalized similarity score. All ten birds showed a gradual and progressive decrease in the normalized similarity score indicating that the song motif gradually deviated from the pre-surgery motif. The rate and extent of song disruption was variable between birds. The point at which the normalized similarity score fell below 0.7 was used as an estimation of the rate of song degradation (dashed line; see Figure 5.9).

Due to the shortening of syllables between posthatch day 130 and 340 in the L+D birds, computational sound analysis struggled to identify syllables in the post-surgery recordings that were, by eye, clearly recognizable as versions of the original syllables. To get a more accurate indication of syllable similarity, we asked human observers to judge the syllables, as had been done in several of the previous studies analyzing the changes to song after the combination of IMAN lesions and deafening (Brainard & Doupe 2000; Brainard & Doupe 2001; Scott et al. 2007). We asked 2 individuals who were blind to the experimental design but familiar with looking at zebra finch song spectrograms to visually score syllables from day 130 and day 340 in all 3 experimental groups. Syllables were cropped out of song spectrograms for assessment so that no sequence information was available. Scorers were asked to match each unique pre-surgery syllable from day 130 to a syllable produced on day 340 and rate how similar they were on a scale of 0-3, with a score of 0 meaning there was no matching syllable and a score of 3 indicating there was a well matched syllable. The score for each syllable was then averaged to give a syllable similarity index (SSI) for each bird. In addition to the 26 experimental birds, scorers were also asked to score the similarity of two groups of pre-surgery syllables from the same bird but taken from different song bouts in order to determine the maximum score possible. Because even completely unrelated zebra finch syllables tend to share some spectral characteristics, we also asked scorers to rate some syllable sets containing the pre-surgery syllables of two different birds to get a baseline SSI for comparing completely unrelated songs.

Visual song scoring revealed that syllables of control birds had not changed significantly between posthatch day 130 and day 340 as the SSI did not differ from the maximum index value obtained when syllables from within posthatch day 130 were scored (Figure 5.5). The deaf birds had significantly lower SSIs than both the control and L+D groups (one-way ANOVA: $F(2,25)= 65.81$, $p< 0.0001$; followed by Tukey post-hoc analysis: $p< 0.0001$ for deaf compared to both other groups) and scores were not significantly different from the baseline generated by comparing unrelated songs. The mean SSI of the L+D group was significantly higher than that of the deaf group indicating that the lMAN lesions had prevented the full effects of deafening on song deterioration. However, in contrast to the previous reports of lMAN lesions and deafening (Brainard & Doupe 2000), our L+D birds did show a significant decrease in SSI compared to the control birds (Tukey post-hoc: $p= 0.023$). From examining the song files, it appeared that this decrease in similarity was due to the decrease in syllable duration making it harder to match syllables, rather than more profound degradation of syllables in the L+D group. It is possible that because the lMAN lesions were not complete in all birds, the decrease in SSI relative to controls may have resulted from an incomplete rescue of the effects of deafening. However, this interpretation seems less likely because we find no correlation between the percentage of lMAN remaining in our L+D birds and their SSI scores ($R^2 = 0.332$, $p= 0.135$).

Once we were confident that we had replicated the previously reported behavioral phenotypes with our surgical manipulations, we then looked to see

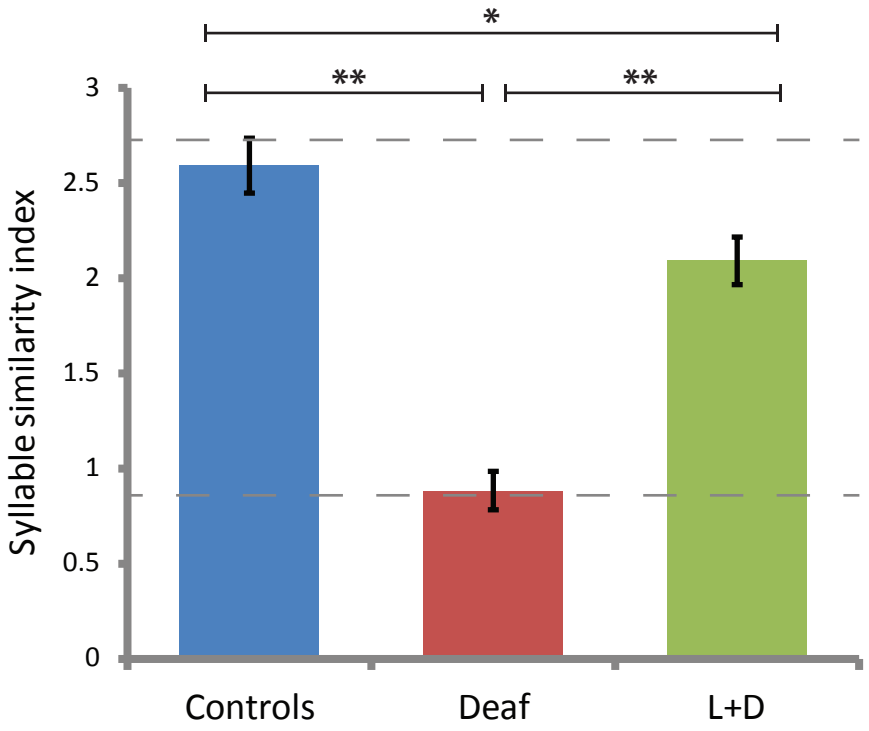


Figure 5.5: Prior lesioning of lMAN partially prevents the loss of syllables that result from deafening at 130 days of age.

For all experimental birds, syllables produced at 130 days of age were compared to the syllables produced at 340 days of age by 2 blind observers. Each syllable was rated on a scale of 0-3, with 0 indicating no matching syllable and 3 indicating a good match. The average of all syllable scores was taken to generate a syllable similarity index (SSI) for each bird. To determine the possible range of SSI values, syllables from unrelated birds were compared to generate a baseline and the syllables from 2 different songs produced on day 130 by the same bird were compared to generate the maximum SSI. This range is indicated by the grey dashed lines on the graph. The average SSI from the control birds was not significantly different from the maximum SSI ($p < 0.01$). Deaf birds had a significantly lower SSI than either the controls or the L+D group and the deaf SSI was not significantly different from the baseline SSI ($p < 0.01$). The average SSI from the L+D birds was significantly higher than that of the deafened birds but was also significantly lower than the control SSI. Error bars represent SEM.

*= p= 0.023; **= p< 0.0001

whether deafening or the combination of lMAN lesions and deafening in early adulthood had any effect on the net addition of HVC-RA neurons that normally occurs in hearing birds (Chapter 2). We counted the number of retrogradely labeled HVC-RA neurons present in all three groups of birds at 340 days of age and found no differences in the total number or density of HVC-RA neurons between the three groups (Figure 5.6; for total number, one-way ANOVA: $F(2,23)= 0.37$, $p= 0.70$; for density, one-way ANOVA: $F(2,23)= 0.76$, $p= 0.48$). Additionally, all 3 groups have a number and density of HVC-RA neurons that is within the range of values observed for birds of a similar age in the original experiment in Chapter 2. Therefore, it appears that removal of auditory feedback has no effect on the normal addition of HVC-RA neurons to the adult zebra finch HVC between 130 days and 1 year of age, regardless of whether the song structure deteriorates or is preserved by prior lMAN lesion. Apparently, auditory information is not necessary for HVC to acquire the full count of HVC-RA neurons expected from the age of the individual bird.

In contrast to the group similarities in HVC-RA numbers, there was a big difference in the number of BrdU-positive neurons surviving in HVC in the L+D birds compared to the control and deaf groups, which were not different from each other (Figure 5.7; one-way ANOVA: $F(2,25)= 6.424$, $p= 0.006$; Tukey post-hoc analysis: control vs L+D, $p= 0.018$; deaf vs L+D, $p= 0.009$; control vs deaf, $p= 0.99$). The L+D birds had over twice as many new neurons surviving for 1 month in HVC than the control or deafened birds (Means ± SEM: L+D = 132 ± 26; control = 59 ± 9; deaf = 59 ± 11). To determine whether these extra neurons were projecting to RA, we also

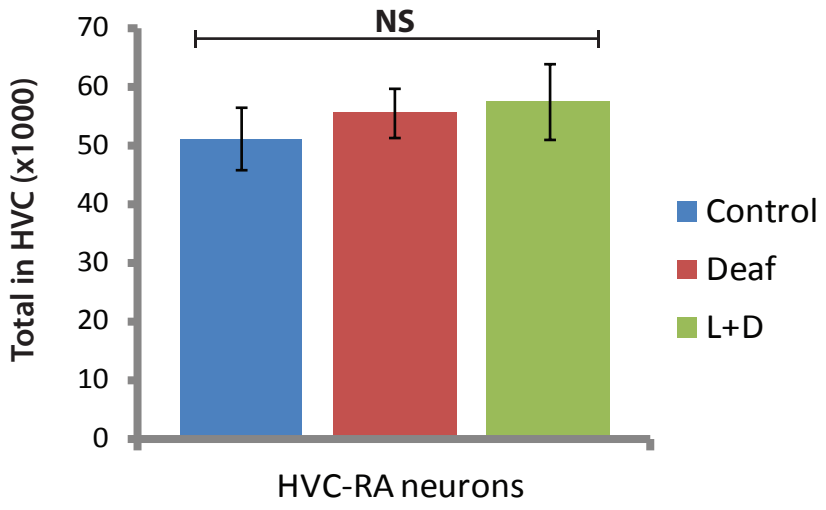


Figure 5.6: Net addition of HVC-RA neurons in adulthood continues in the absence of auditory feedback and occurs independently of changes to the adult song structure.

The mean number of HVC-RA neurons (average of left and right) labeled with CTB from RA on day 340 did not differ between hearing controls, birds that were deafened on day 130 or birds that received lMAN lesions prior to deafening on day 130.

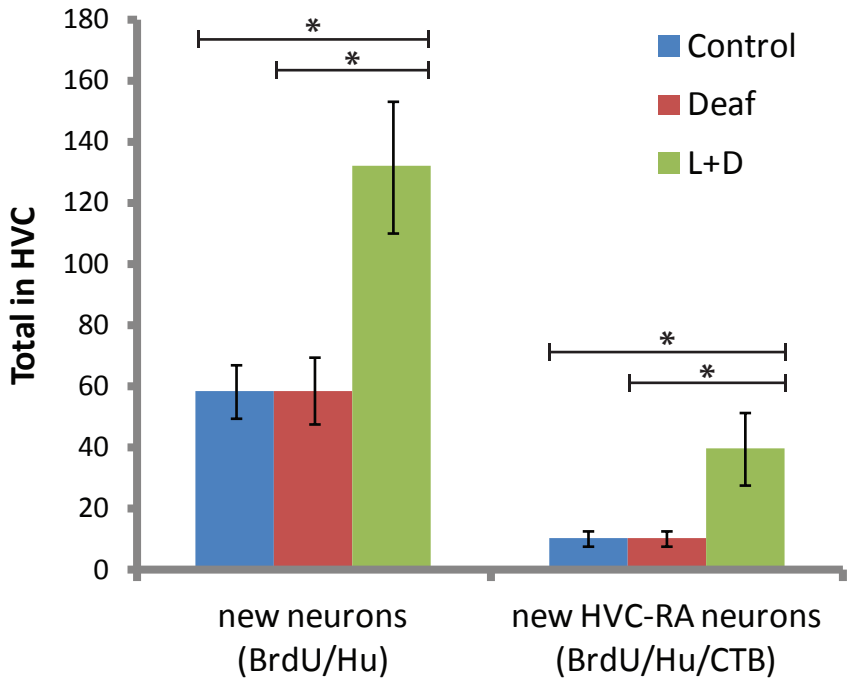


Figure 5.7: The combination of lMAN lesions and deafening causes an increase in the recruitment of BrdU-positive neurons to HVC.

The mean number of BrdU-positive neurons (average of left and right) present in HVC 1 month after BrdU injection was twice as high in L+D birds as in control or deaf birds. Many of the extra new neurons were HVC-RA neurons as the number of BrdU-positive neurons that were also retrogradely-labeled from RA was 4 times higher in the L+D birds than the 2 other groups. BrdU injections were given 6 months after deafening and birds were sacrificed 1 month after BrdU injections. Error bars represent SEM. *= $p < 0.01$

counted the number of BrdU/Hu/CTB triple-labeled neurons in HVC and found there was an even greater 4-fold increase in the L+D birds relative to the other groups (Means ± SEM: L+D = 40 ± 13; controls = 10 ± 2; deaf = 10 ± 3; one-way ANOVA: $F(2,22)= 8.30$, $p= 0.002$; Tukey post-hoc: control vs L+D, $p= 0.005$; deaf vs L+D, $p= 0.004$; control vs deaf, $p= 0.98$). Of the extra neurons added to HVC in the L+D birds, it seems a greater proportion than normal became HVC-RA projecting neurons. Finally, we counted the number of BrdU-positive cells lining the ventricle above HVC and found no differences between the three groups (one-way ANOVA: $F(2,27)= 1.40$, $p = 0.27$) suggesting that the increase in BrdU-positive neurons in the L+D group is due to an increase in survival of new neurons within the first month after neuron birth rather than an increase in the production of new neurons by the ventricular zone progenitors.

Interpreting the results.

There are two main conclusions to be drawn from these data. The first is that the survival of new HVC-RA neurons in adulthood does not require auditory information, since net addition of new neurons proceeds whether hearing is intact or not. This finding seems counterintuitive considering that the HVC-RA neurons are thought to control song production and the maintenance of adult song requires intact hearing. However, it is in accordance with previous observations that deafening does not affect either the long-term (4 month) survival of neurons in the adult HVC (Wang et al. 1999) or the 1-month survival of new neurons in the HVC of juveniles

aged 65 days (Wilbrecht et al. 2002). In fact, the developmental increases in HVC neuron number and HVC volume proceed as normal in deafened juveniles even though the young birds are incapable of learning a song (Burek et al. 1991). There is no doubt that auditory information is essential for both juvenile song learning and adult song maintenance, but apparently it is not important for the recruitment and survival of HVC-RA neurons during either of these periods. This does not negate the role of HVC-RA neurons in song production, because auditory information could still be essential for dictating the formation of appropriate synaptic contacts within RA or with other HVC neurons, but it does suggest that the program of neuronal addition to HVC is more autonomous than we might have expected.

Our second conclusion relates to effect of the combination of lMAN lesion and deafening on neuronal recruitment to HVC. This combination of surgeries stimulates the 1-month survival of adult-born neurons, especially new HVC-RA neurons, without causing a net increase in the overall HVC-RA neuron population. Therefore, this combination of surgeries must also induce death of some of the HVC-RA neurons; lMAN lesion and deafening in adulthood promotes the turnover of HVC-RA neurons. We do not know which HVC-RA neurons are being lost in this context; it could be that the newly recruited neurons are short-lived and constantly being replaced by a new cohort of incoming neurons, or, it could be that older HVC-RA neurons are gradually lost and numerically replaced by new cells in which case we would expect the learned song to deteriorate severely, which is not what happens. We know from the counts of BrdU-labeled progenitors in the ventricular zone that the

increase in BrdU-positive neurons in the L+D group comes from an increased survival of new neurons within the first month, rather than stimulation of neuron production, but we do not know how long these additional neurons would survive beyond the first month. As adult song is stabilized by the combination of lMAN lesions and deafening, we speculate that it is more likely that the new neurons are short-lived and constantly being turned over rather than there being a gradual loss of the older HVC-RA neuron, which presumably encode the learned song pattern. This hypothesis can be tested directly by measuring the survival of new HVC neurons in L+D birds at longer time intervals after BrdU injection.

There has been a previous study looking at the effects of lMAN lesions and deafening on neuronal recruitment in the adult zebra finch HVC (Scott et al. 2007). Deafened and L+D birds were given ^{3}H-thymidine injections 2-3 days after deafening and sacrificed 4 months later to assess the number of new neurons surviving in HVC. Although there was a trend towards increased neuronal recruitment in the L+D birds, this difference did not reach significance. Because of the differences in the timing of birthdate labeling relative to deafening between that study and ours, the results cannot be compared directly. The fact that there was no significant increase in neuronal survival in L+D birds after 4 months (Scott et al. 2007) when there was a significant 2-fold increase after 1 month (our data) could suggest that the additional HVC-RA neurons that we counted are short-lived and die somewhere between 1 and 4 months after birth. However, an alternative interpretation is that the combination of surgeries does not immediately stimulate the survival of new neurons

in HVC and so the timing of the ^{3}H-thymidine injections only a few days after deafening in the Scott et al. (2007) study did not reveal the effect that we saw by looking at the survival of neurons born 6 months after deafening. As mentioned above, the only way to distinguish between these 2 scenarios would be repeat our experimental protocol of giving BrdU after 6 months but then increase the time between BrdU and sacrifice to 4 months or longer to see if the additional neurons are still present in HVC at longer survival times.

If the combination of lMAN lesions and deafening does cause an increase in the turnover of HVC-RA neurons, an explanation for this effect is not obvious. It is possible that lMAN lesions alone (without subsequent deafening) are sufficient to cause the increase in turnover that we observed in our L+D birds and this is something that should be tested directly. If lMAN lesions induce HVC-RA neuron turnover in hearing birds, one could envisage a scenario where lMAN activity is required for the formation of strong synapses between HVC-RA neurons and their downstream targets, and without the formation of strong connections the survival of new HVC-RA neurons is compromised. Although there is no direct evidence to show lMAN activity modulates the strength of HVC-RA synapses, the system is set up for such a possibility: lMAN-RA terminals innervate the same RA premotor neurons as the HVC-RA neurons (Canady et al. 1988) and lMAN-RA neurons signal almost exclusively through NMDA receptors (Mooney & Konishi 1991), key mediators of long-term potentiation (reviewed in Bliss and Collingridge (1993)). If this scenario were accurate, it could also explain why lesioning of lMAN prior to deafening

prevents degradation of the adult song pattern. Although new neurons do not need auditory input to integrate into HVC or send axonal projections to RA they may still require auditory information to make the appropriate synaptic connections with neurons in RA. Without auditory instructions to guide them, as in the deafened birds, new HVC-RA neurons could make synaptic connections haphazardly and this accumulation of random connections could disrupt the motor pattern in RA. Lesioning lMAN prior to deafening would preclude the formation of disruptive synaptic connections in RA by new HVC-RA neurons, leading to both reduced survival of the new neurons and maintenance of the adult song pattern. If these speculations were realistic, lMAN lesions alone would have to increase HVC-RA neuron turnover, and it would also have to be the newly recruited HVC-RA neurons that are being replaced rather than older HVC-RA neurons. Both of these predictions can be tested with modifications of the original experimental protocol. In addition, lMAN-RA neurons release neurotrophic factors like brain-derived neurotrophic factor (BDNF) into RA (Johnson et al. 1997) which can promote the survival of neurons via retrograde transport from their axon terminals (reviewed in Zweifel et al. (2005)). Lesions of lMAN could therefore reduce the survival of newly recruited HVC-RA neurons through a more general loss of neurotrophic support in RA.

Are HVC-RA numbers related to measures of song stereotypy or stability?

Our attempts to uncouple HVC-RA neuron addition and changes in song stereotypy and stability have failed. New neuron addition occurs regardless of the

state of adult song. However, causation does not need to go both ways; net neuronal addition could influence song structure without manipulations that change the song necessarily having an impact on the addition of neurons to HVC. As an alternative strategy, we wondered whether we could use natural variation in the number of HVC-RA neurons within our experimental birds to find correlational evidence for the involvement of net neuronal addition in the stereotypy and stability of adult song. Firstly, we used the control birds to look for a relationship between HVC-RA neuron number and the normal improvements in song stereotypy that occur with age. Secondly, we used the deafened birds to look for a relationship between the number of HVC-RA neurons and the rate at which song deteriorates after removal of auditory feedback.

For each control bird we had recordings of their song from 130 to 340 days of age and a count of the number of HVC-RA neurons present at posthatch day 340. We hypothesized that if the net addition of HVC-RA neurons in adulthood is involved in the increase in song stereotypy that has been previously documented (Kao & Brainard 2006; Pytte et al. 2007) then there might be a correlation between the degree of improvement in song stereotypy and the number of HVC-RA neurons within this group of 8 birds. To assess changes in song stereotypy over the 7 month period, we used Sound Analysis Pro software to quantify the similarity of motifs produced within a single day. For songs produced on day 130 and 340 independently, we selected 5 song bouts at random and compared each motif in the bout to every other motif in the same bout. These pair-wise comparisons were run

for all 5 song bouts to generate a total of 60 to 100 motif similarity scores, depending on the number of motifs contained in each song bout. The scores were averaged for each bird to produce a mean motif similarity score for the 2 time points.

Seven of the 8 control birds showed an improvement in motif similarity between posthatch day 130 and day 340 and although the changes in stereotypy were subtle, as a group this improvement was significant (Figure 5.8a; Mean ± SEM: posthatch day 130 = 67.5 ± 6.3; posthatch day 340 = 76.6 ± 4.1; paired samples *t*-test: $t(7)= 3.217$, $p= 0.015$). We subtracted the mean similarity score of motifs produced on posthatch day 130 from the score of motifs produced on posthatch day 340 to quantify how much the stereotypy of each song had improved over the 7 months. This 'shift in similarity score' was then correlated to the density of HVC-RA neurons present in each bird at posthatch day 340. There was a weak correlation between the 2 variables but it did not reach significance (Figure 5.8b; $R^2= 0.44$, $p= 0.075$). There was no correlation at all between the shift in similarity score and the total number of HVC-RA neurons present at day 340 ($R^2= 0.087$, $p= 0.47$).

The caveat of this approach is that we are assuming that the number of HVC-RA neurons present at the end of the experiment relates to the number of neurons that were added over 7 months. We are assuming that those individuals with more HVC-RA neurons at day 340 have more because they have experienced a greater rate of neuronal addition, rather than the alternative explanation which is that they

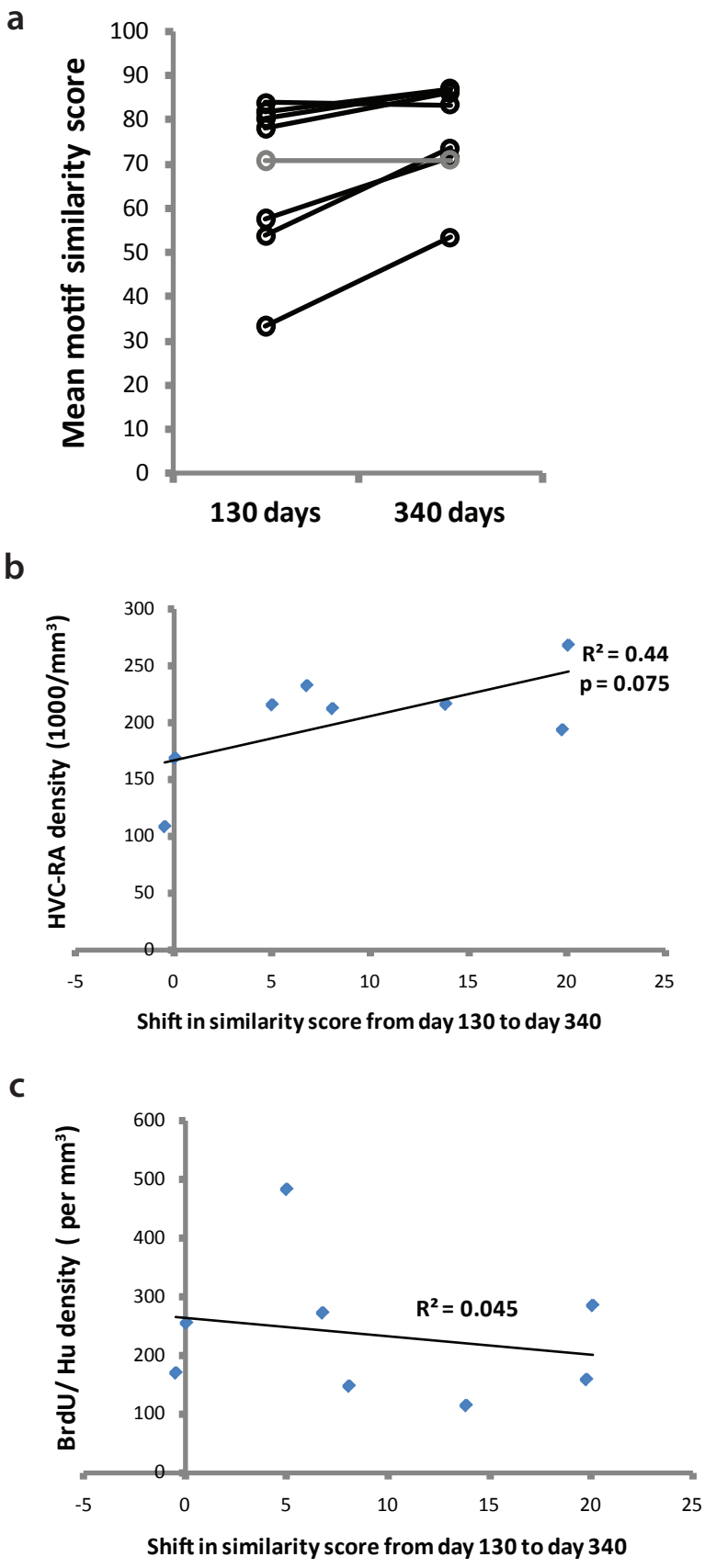
Figure 5.8: There was no relationship between the improvements in song stereotypy that occurred in the hearing controls and either the number of retrogradely-labeled HVC-RA neurons or the number of BrdU-positive neurons recruited into HVC.

a. To measure motif stereotypy in the control birds, song motifs from 5 songs produced within a given day, either day 130 or day 340, were compared to each other using Sound Analysis Pro software (Tchernichovski et al. 2000). An average motif similarity score was calculated for each bird and each day. Seven of the 8 controls showed an improvement in the stereotypy of their motif between day 130 and day 340 (black). One bird showed a slight decrease in motif stereotypy between the 2 time points (grey). As a group, the difference was significant (paired samples t-test $p= 0.015$).

b. The mean similarity score of motifs produced on day 130 was subtracted from the score of motifs produced on day 340 to get a 'shift' in similarity score that indicated how much the song stereotypy had improved over the course of the experiment. There was no significant correlation between the shift in the motif similarity score and the density of retrogradely-labeled HVC-RA neurons present at day 340 amongst the 8 control birds.

c. There was also no significant correlation between the shift in the motif similarity score and the density of BrdU-positive neurons in HVC when BrdU was given at 310 days of age and birds were sacrificed 1 month later.

Figure 5.8:


a
Mean motif similarity score
100
90
80
70
60
50
40
30
20
10
0
130 days
340 days
b
HVC-RA density (1000/mm³)
300
250
200
150
100
50
0
R² = 0.44
p = 0.075
-5
0
5
10
15
20
25
Shift in similarity score from day 130 to day 340
c
BrdU/ Hu density (per mm³)
600
500
400
300
200
100
0
R² = 0.045
-5
0
5
10
15
20
25
Shift in similarity score from day 130 to day 340

already had more HVC-RA neurons at the start of adulthood. Our data may be confounded by natural variation in the number of HVC-RA neurons present at the start of adulthood, before any adult-born neurons have added to the system. To test for a relationship between neuronal addition and song stereotypy another way, we looked for a correlation between the improvements in motif similarity and the number of BrdU-positive neurons that have survived a month in the HVCs of our control birds. We showed in Chapter 3 that for hearing birds housed in the same conditions as the controls, new neurons that survive 1 month in HVC will go on to survive at least 8 more months. Thus, we can assume that the number of neurons surviving for 1 month in the control birds is indicative of the rate of increase of the HVC-RA population they experienced over the course of the experiment. If the net addition of HVC-RA neurons is involved in improving the stereotypy of adult song, we would expect the number of BrdU-positive neurons to be correlated with the shift in similarity score within the 8 birds. However, there was no correlation between the density of BrdU/Hu neurons surviving 1 month in HVC and the shift in similarity score in our control birds (Figure 5.8c, R^2= 0.045, p= 0.62). Taken together, we find no evidence that the net addition of new neurons to the adult zebra finch HVC has an impact on the stereotypy of adult song. However, due to low number of birds included in this analysis and the relatively low improvements in song stereotypy they showed over the course of the experiment, we would not rule out a role for new neurons in adult song stereotypy based on these data alone.

To test for a relationship between the net addition of HVC-RA neurons and

age-related improvements in the stability of adult song, we looked to see whether the rate or extent of song degradation after deafening in our 10 deafened birds was related to the number of HVC-RA neurons they had. Presumably, if more HVC-RA neurons result in a song that is more resilient to the effects of deafening, these 2 variables will be positively correlated between individuals. To measure the rate of deterioration of adult song, we used the similarity scores generated using Sound Analysis Pro software to determine how many days after deafening it took for the normalized similarity score to fall below 0.7 (Figure 5.3b). To assess the overall extent of song deterioration, we used the normalized similarity score of the final recordings made on day 340. Neither the rate nor the extent of song deterioration after deafening showed a correlation to the number of HVC-RA neurons present in the deafened birds in this experiment (Figure 5.9). Thus, we find no evidence that the net addition of HVC-RA neurons to the adult zebra finch HVC is related to the stability of adult song after deafening.

Summing up.

The role of adult-born neurons in the zebra finch HVC still seems as unclear as ever. New neurons are adding to HVC across adulthood in this species and many of them become HVC-RA neurons. This neuron class is believed to be responsible for encoding the temporal pattern of song (Hahnloser et al .2002; Fee et al. 2004) and yet net addition of HVC-RA neurons in adulthood seems to occur independently of changes in the adult song. Deafened birds, that show extensive deterioration of

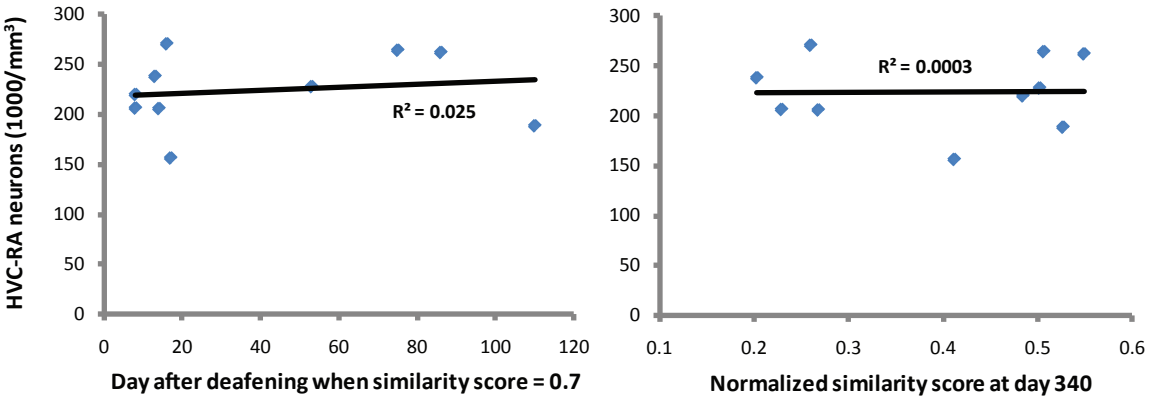


Figure 5.9: There was no relationship between the rate and extent of song degradation after deafening at day 130 and the number of retrogradely-labeled HVC-RA neurons present at day 340.

The rate of song degradation after deafening was determined by the number of days after deafening it took for the normalized similarity score of song to fall below 0.7 (see figure 5.3b and methods for details on how the normalized similarity score was calculated). The extent of song degradation was determined by the final normalized similarity score of song produced on day 340. There was no correlation between either measure and the number of retrogradely-labeled HVC-RA neurons present at day 340 amongst the ten deaf birds. Therefore, the net addition of HVC-RA neurons in adulthood does not appear to be related to the stability of song after deafening.

both the spectral and temporal structure of song, experience the same amount of net HVC-RA neuron addition as both hearing controls and L+D birds that retain relatively normal song characteristics. Moreover, the net addition of HVC-RA neurons does not seem to be related to changes in the song of hearing controls over a 7 month period or to the stability of song after deafening. Auditory information is essential for initial song learning in the juvenile and maintenance of that song in the adult, and yet addition of new neurons to HVC can occur without any auditory input. Although our experiments are indirect and definitely not exhaustive, evidence to suggest that the addition of new neurons to the HVC of adult zebra finches has any bearing on song structure or singing behavior is proving hard to find.

Three studies have suggested that not all HVC-RA neurons are active during singing and it could be that many of the new HVC-RA neurons added to the adult brain fall into this category. Electrophysiological recordings of the HVC-RA neurons, identified by antidromic stimulation of RA, found only about half (8/18) fired during singing in awake birds, with the remainder either active during calls only (3/18) or silent during both calls and song (7/18) (Fee et al. 2004). An alternative way to assess neuron activity, albeit less directly, is to look at the expression of immediate-early genes: a class of genes, typically transcription factors, that are rapidly and transiently induced after neural activity (Sheng & Greenberg 1990). Looking for the expression of one such gene, ZENK, in male zebra finches revealed around 60% of the HVC-RA neurons labeled with a retrograde tracer from RA induced ZENK expression directly after singing (Jarvis et al. 1998). A third study found expression

of *c-fos*, another immediate-early gene, was activated in HVC-RA neurons but not HVC-X neurons after singing. The authors did not quantify the percentage of HVC-RA neurons that were activated but Figure 5b of the manuscript clearly shows several HVC-RA neurons that were retrogradely labeled with a tracer injected to RA but that did not turn on expression of the *c-fos* gene after singing (Kimpo & Doupe 1997). Thus, three independent studies using two alternative methods to assess neuronal activity have found a significant proportion of HVC-RA neurons that are not activated during singing. Experiments have been done in canaries to show that new neurons added to the adult HVC can respond to auditory stimulation (Paton & Nottebohm 1984) but no studies have looked at motor-driven activity during singing in adult-born neurons. Given that such a large proportion of HVC-RA neurons do not appear to be song-activated, it is quite possible that all HVC-RA neurons added after sexual maturity are also inactive during song production. This would explain how there can be significant net addition of new neurons across adulthood without any dramatic changes to the adult song. Due to the technical difficulty of intracellular recordings in awake, singing zebra finches and the low proportion of neurons that can be labeled with a few days of birthdate marker injections, it would be very challenging to obtain direct electrophysical recordings from adult-born neurons of a known age. However, it would be relatively easy to label adult-born neurons with BrdU or ^{3}H-thymidine, inject a retrograde tracer into RA to label the HVC-RA neurons and then look for the co-expression of ZENK by these neurons after singing. This experiment would contribute significantly to our understanding of the role adult neurogenesis in the zebra finch HVC.

CHAPTER 6: DISCUSSION

To start: a note on scrupulous data interpretation.

Throughout this thesis I have tried to address how important it is to consider the limitations of an experimental technique when interpreting data. No method is perfect; each comes with an underlying set of assumptions or restrictions for its use. The user must be aware of the limitations and keep them in mind while trying to evaluate the experimental outcomes. Labeling neurons with birthdate markers, retrograde tracers or antibodies and using those labels to make inferences about the age or phenotype of a neuron population is no exception. The central finding of this thesis, that there is net addition of HVC-RA neurons in the adult zebra finch brain, is based on the observation that the number of HVC neurons that could be retrogradely labeled with a particular tracer (CTB) from an injection into RA increased as the bird's age at the time of injection increased. This observation alone does not tell us that there are more HVC-RA neurons in older birds, just that the number that can be retrogradely labeled with CTB increased with age. One could imagine several reasons why the ability of projection neurons to take up tracer might increase with age, without there actually being any more neurons in older birds.

When there are multiple interpretations of a data set, the best approach is to apply a different experimental technique to the problem, preferably one that has a different underlying set of assumptions. In this case, we stained HVC with an

antibody that has been shown to specifically label mature neurons (anti-Hu C/D; Barami et al. 1995) and found that the number of Hu-positive neurons in HVC also increases with age. Again, there could be more than one interpretation of these data but when considered with our initial result, and the published observations that neurons recruited to the zebra finch HVC in adulthood can be retrogradely labeled with a tracer from RA (Nordeen & Nordeen 1988), the most parsimonious explanation is that the total number of HVC-RA projection neurons is increasing with age and that this increase is also reflected in the total HVC neuron population.

Although the age-related increases in either the total HVC neurons or specifically the HVC-RA neurons, had not been observed in adult zebra finches in a previous study (Wang et al. 2002), we are confident that the net addition of HVC-RA neurons with age is a real phenomenon, at least in the male zebra finches of our breeding colony at the Rockefeller University Field Research Center. In the experiments described in Chapter 3, HVC-RA neurons were retrogradely labeled with microspheres in birds that were 95 days of age at the time of tracer injection. In Chapter 5, HVC-RA neurons were retrogradely labeled with CTB in birds that were 1 year of age at the time of tracer injection. The number and density of retrogradely labeled neurons in the 1 year olds was almost double that of the 95 day olds and the numbers in both experiments were completely consistent with the number and density of labeled neurons reported in Chapter 2 for birds of the same age. These additional experiments, while designed to test other hypotheses, provide an independent verification that the HVC-RA neurons significantly increase in number

as zebra finches get older. There can always be an alternative way to explain a set of results, but the more independent pieces of data, ideally generated using more than one experimental method, that point to the same interpretation, the more confident one can be that the phenomenon being described is real.

The use of birthdate markers to identify neurons of a certain age also has limitations. In Chapter 3, BrdU injections were given to birds at 92-94 days of age and the survival of BrdU-positive neurons was followed for up to 4 years. The assumption of this method is that a BrdU-positive neuron identified later will represent a neuron born somewhere between day 92 and 94, but as already discussed, this assumption could be incorrect if BrdU-positive progenitor cells continue to produce labeled daughter neurons after day 94. In this experiment, we found no loss of BrdU-positive neurons in HVC between 1 month and 4 years after BrdU injection. The fact that we cannot be sure of the exact birth date of each labeled neuron makes interpretation of the results difficult. Although these data seem to suggest that none of the originally labeled cohort died between 1 month and 4 years, the same data would also result if the rates of continued progenitor division and death of the originally labeled cohort of neurons were balanced. There is no way to distinguish between the 2 interpretations using just these two data points. However, we would argue that because the pool of labeled progenitors drops off so dramatically between 1 and 3 months, but much less so thereafter, it is likely that numbers of BrdU-labeled neurons surviving longer than 3 months are mostly those originally labeled. Since there is a net accumulation of HVC-RA neurons with age,

the simplest explanation is that most of the BrdU-positive neurons that are present after one month in HVC are still present up to 4 years later. Thus, although when taken individually the pieces of data in this thesis could have alternative interpretations, when considered together as a whole, the most parsimonious explanation is one of net addition and long-term survival.

With these considerations in mind, I will now attempt to discuss the wider implications of the data presented in this thesis. To put the results into a broader context will inevitably involve making certain assumptions for which rigorous supporting data may not exist. I will try to be clear when I am directly reporting on my experimental data and when I am drawing more speculative conclusions.

Net addition verses numerical replacement

There are several ways in which the recruitment of new neurons to a brain area could proceed, and each one is likely to have different functional consequences for the brain region involved. Recruitment could be part of a continuous replacement process where, for each new neuron coming in, an existing neuron dies. The new neuron does not need to physically replace the dying cell, for example by occupying its place in an existing circuit, but it can numerically replace it so that the overall number of neurons does not change. If replacement occurs, it could be that all neurons of the population eventually die and are replaced, or some could be more susceptible to replacement than others. For example, there could be an older

"privileged" cohort that persists while the more recently recruited neurons are constantly recycled. This latter case would mean new neurons only become transient members of the neuron population while the older neurons would be permanent. The converse, of course, could be possible too, the older neurons being at greater risk of dying than the younger ones. Alternatively, new neurons could add to a brain area without any accompanying cell death at all. This would result in a continued growth in neuron number and the new neurons would become permanent members of the neuron population.

Of course, these are extreme cases and they are not mutually exclusive. Neuronal recruitment could be accompanied by a mixture of numerical replacement and net addition. For example, there could be two types of neurons recruited; one class that survives for the long-term and another that is more transient. In this case, the short-lived neurons would be constantly replaced while the long-term neurons would add to the population. How the total number of neurons would change in this scenario would depend on the relative rates of recruitment of the two classes of neurons. As long as the rate of recruitment exceeds the rate of cell death over a period, a net increase in numbers will ensue.

In the canary HVC, the main mode of neuronal recruitment is numerical replacement. Cell death accompanies recruitment (Kirn & Nottebohm 1993; Kirn et al. 1994; Nottebohm et al. 1994) and despite a continuous influx of adult-born

neurons, the total number of HVC neurons does not change, at least over 8 months (Kirn et al. 1991). Some of the neurons recruited to the adult HVC, particularly those born in the spring, are short-lived and have almost all disappeared after 8 months (Alvarez-Borda et al. 2004). But there are also some neurons, many of those born in the fall, that persist for at least 8 months. It is not known whether neurons recruited to HVC in the fall would be culled during the surge in cell death that occurs in the following spring or whether they would survive until the next fall and beyond. It is also not known whether the total number of HVC neurons in canaries is gradually increasing from year to year. We can be sure that numerical replacement occurs in the adult canary HVC but we do not know whether neurons are recruited exclusively as part of a replacement process or whether some neurons, perhaps those born in the fall, are recruited for the long-term and contribute to a gradual net increase in HVC neurons from year to year.

In the body of work presented in this thesis, we have found that, in contrast to the canary, neuronal recruitment to the zebra finch HVC predominantly occurs via a process of net neuronal addition. New neurons born in adulthood migrate into HVC and at least 50% of them become HVC-RA neurons, as determined by their ability to transport a retrograde tracer from RA. This neuron addition results in a significant increase the total number of HVC-RA projection neurons with age and because HVC does not increase in volume to accommodate the extra neurons, it also results in a significant increase in the packing density of the HVC-RA neurons. In contrast, the

total number and packing density of the HVC-X projection neurons, which are not produced in adulthood, does not change with age.

The increase in HVC-RA neurons is dramatic: between 90 days and 2 years of age, their numbers double. Thereafter, the rate of increase slows dramatically, which is in line with the prior observation that the rate of neuronal recruitment to HVC slows with age (Wang et al. 2002). We have also provided evidence that adult-born neurons survive for very long periods in the zebra finch HVC. Treating 92-94 day old male zebra finches with systematic injections of BrdU and then counting the number of BrdU-positive neurons present in HVC after 30, 90, 180 and 270 days, and also 4 years, revealed no reduction in the number of BrdU-positive neurons over this time span, at least not under the laboratory conditions in which these birds were housed.

From these data we can conclude that at least one mode of neuronal recruitment is at work in the adult zebra finch HVC that has not yet been documented in the brains of other songbirds: net neuron addition. New neurons added to the HVC of young adults that survive the first month apparently persist for several years and cause a net increase in the neuron population size. As described above, a net increase in total neuron numbers could result even if some neuronal replacement were occurring, as long as the rate of addition exceeded the rate of death. However, by retrogradely-labeling the HVC-RA neurons present at 95 days of

age and following their survival, we found no evidence of their loss over the next 9 months. Thus, there appears to be no replacement of either the neurons born around 90 days of age and recruited to HVC over the next month, or of the HVC-RA neurons that can be retrogradely labeled at 95 days of age. For both cases, taken together, the most likely explanation is that new neurons were added to HVC without any accompanying loss of the older HVC-RA neurons. Additionally, most of the new neurons that survived the first month (because we have no data about events before then) would probably survive for many months thereafter; we have no evidence for any turnover of these neurons from one month on. All of these observations are congruent with the steady increase in the population of HVC-RA neurons observed between 90 days and 2 years of age.

The events of neuronal replacement that we describe for the zebra finch HVC starkly contrast to the events that have been described in the canary HVC. A comparison between canaries and zebra finches is relevant to this discussion because the large body of work describing neuronal replacement in the canary had, until now, guided our thoughts on the events in other songbird species, including the zebra finch. There are numerous published reports looking at some aspect of neuronal recruitment in the HVC of adult zebra finches (i.e. Lipkind et al. 2002; Adar et al. 2008; Pytte et al. 2007; Scott et al. 2007; Wang et al. 2002; Wang et al. 1999; Scotto-lomassese et al. 2007) and most of them have discussed their results with the preconception that neurons were being recruited as part of a neuronal replacement process. Only one study tested this preconception directly by

selectively killing some of the HVC-RA neurons in adults and reporting an increase in the incorporation of adult-born HVC-RA neurons relative to controls in which no neurons were killed or in adult birds where HVC-X neurons were killed instead (Scharff et al. 2000). However, although this report suggested that neuronal replacement of the HVC-RA neurons can be induced in the zebra finch, it did not address whether replacement occurs in intact zebra finches. The fact that our investigation has revealed so many stark contrasts between the canary and zebra finch song system highlights the caution that must be used when extrapolating scientific findings between species, even ones as closely related as the canary and the zebra finch. This has implications for the studies of adult neurogenesis in rodents that try to make inferences about the process in humans.

The main reason for comparing HVC neuronal recruitment in canaries and zebra finches is their differing breeding strategies and singing behavior. Zebra finches are opportunistic breeders and sensitive period learners that do not modify their songs after sexual maturity (Zann 1996). This means that at any time of the year a male must be ready to use its song to court a female and accordingly their songs remains stereotyped all year round. Canaries on the other hand, are both seasonal breeders and open-ended learners, who continue to modify their song and add new syllables each year (Nottebohm et al. 1986). A third species, the white-crowned sparrow, has yet another lifestyle. They are seasonal breeders but unlike the canary they are sensitive period learners that do not add new song elements after sexual maturity. White-crowned sparrows show seasonal changes in the size

of HVC and number of neurons it contains (Smith et al. 1995), which are likely to be the result of seasonal changes in neuronal replacement. Although neuronal replacement has not been directly shown in this species, moving males to non-breeding conditions (by castration and putting them on a short-day photoperiod) causes a rapid loss of 26% of HVC neurons over 4 days (Thompson et al. 2007) indicating that, as in the canary, there is seasonal regulation of cell death in HVC. Moreover, inhibition of apoptosis by infusion of a caspase-3 inhibitor into HVC prevents the seasonal induction of cell death (Thompson & Brenowitz 2008) and reduces the subsequent recruitment of new neurons to HVC by 48% (Thompson & Brenowitz 2009). Thus, as has been described for the canary (Kirn et al. 1994), cell death and neuronal recruitment seem to be temporally related and are likely to result in neuronal replacement in the HVC of white-crowned sparrows.

The temporal correlation between peaks in neuronal replacement and peaks in new syllable learning in the canary led to the hypothesis that neuronal replacement was involved in the process of vocal learning in adulthood (Kirn et al. 1994). White-crowned sparrows seem to contradict this idea because they show seasonal changes in neuronal recruitment as adults but do not learn new song elements after sexual maturity. However, although the white-crowned sparrow does not learn new song elements each year, their adult songs do show seasonal changes in stereotypy (Smith et al. 1995). In both canaries and white-crowned sparrows, adult song gets more variable and noisy outside of the breeding season when it is not needed for courtship (Smith et al. 1995; Nottebohm et al. 1986).

Therefore, it is possible that neuronal replacement in HVC induces periods of vocal plasticity and that these periods are necessary but not sufficient for vocal learning to occur. This hypothesis has been proposed previously (Brenowitz et al. 1991) but was weakened by the observations in zebra finches where neuronal replacement was believed to occur in the adult HVC despite the song remaining stable and stereotyped across adulthood. Here we have resolved the paradox: it appears that zebra finches do not replace HVC neurons in adulthood, or at least not to any degree that can be seen using similar techniques to those that revealed replacement in the canary HVC. New neurons are recruited to HVC in the absence of any appreciable cell loss to cause net neuronal addition and perhaps, as a result, the adult song remains stereotyped all year round. In contrast, seasonal breeding species recruit new neurons via a process of neuronal replacement and this turnover of HVC neurons is correlated to periods of vocal plasticity that may facilitate new song learning in some species.

What is the significance of net neuronal addition to HVC?

The addition of HVC-RA neurons in the zebra finch results in a significant increase in the packing density of HVC-RA neurons with age. This increase in density is most likely achieved through a combination of the neurons getting smaller and closer in space because we observed a statistically significant reduction in both the nuclear size of HVC-RA neurons and in the distance between HVC-RA nuclei with age. During development of the mammalian cortex, there is a large decrease in

the density of neurons as they rapidly increase the size of their dendritic trees (Eayrs & Goodhead 1959; Schade & van Groenigen 1961; Ptacek & Fagan-Dublin 1974). The expanding dendritic arbors form an excess of synaptic connections that are later refined by elimination of redundant or weak synapses (Purves & Lichtman 1980). Based on these developmental events, it follows that an increase in the packing density of the HVC-RA neurons may be associated with smaller or less branched dendritic arbors. The size and complexity of a neuron's dendritic tree typically scales with the number of synaptic inputs it receives (Lichtmann 1977; Purves & Hume 1981; Snider 1987) and so perhaps the increase in packing density of HVC-RA neurons with age indicates that their connections within HVC are getting more refined. Perhaps, as in early cortex development, weak or redundant inputs to HVC-RA neurons are gradually eliminated in adulthood which results in smaller or less branched dendritic trees and a concurrent increase in the HVC-RA packing density with age. Retrogradely labeling HVC-RA neurons with CTB or latex microspheres, as we have done in this thesis, provides no information about dendritic morphology because both tracers are localized to the cell body. There are, however, other tracers that fill the entire soma that could be used to observe dendritic morphology of the HVC-RA neurons in adult zebra finches of different ages. Biotinylated dextran amines are tracers that can be transported retrogradely from the axon terminal but fill the whole neuron, including the dendrites, in a Golgi-like manner (Jiang et al. 1993). These dextran amines are less efficient retrograde tracers than CTB and microspheres so small injections to the target nucleus can result in the labeling of just a small number of neurons (Jiang et al. 1993). Thus, this class of tracers could

be injected into RA in adults of different ages to label a sparse number of HVC-RA neurons. The size and complexity of their dendritic arbors could then be measured to see if the age-related increase in HVC-RA neuron packing density is accompanied by an age-related reduction in the size or branch number of HVC-RA neuronal dendrites.

As already discussed, the HVC-RA neurons are thought to dictate the temporal pattern for learned song, due to their precise and stereotyped firing patterns during singing (Hahnloser et al. 2002; Kozhevnikov & Fee 2007). Accordingly, we would expect a doubling in the number of HVC-RA neurons between 90 days and 2 years of age to have an impact on the motor output of the song system. However, the assumption that the HVC-RA neurons recruited during adulthood would show the same activity patterns during singing as the HVC-RA neurons that were recruited during the juvenile period has not been tested. The age of the HVC-RA neurons from which electrophysical recordings were made during singing was not known (Hahnloser et al. 2002; Kozhevnikov & Fee 2007). Since there is evidence to suggest that a large proportion of the HVC-RA neurons are not active during singing, it is possible that adult-born HVC-RA neurons do not participate in song (discussed in Chapter 5; Fee et al. 2004; Jarvis et al. 1998; Kimpo & Doupe 1997). But, if we assume the newly recruited HVC-RA neurons are active during singing, then it follows that a doubling in their number would have implications for the adult song.

During song development, the balance of synaptic inputs to RA shifts from being dominated by those from lMAN to those from HVC (Herrmann & Bischof 1986; Aronov et al. 2008). This shift results in a gradual increase in the stereotypy of vocalizations because the inputs from HVC are temporally precise compared to the variable inputs from lMAN (Hahnloser et al. 2002; Kozhevnikov & Fee 2007; Hessler & Doupe 1999; Olveczky et al. 2005). A doubling of HVC-RA neurons over the first 2 years of adulthood would dilute out the influence of variable lMAN inputs on motor output even further and thus could result in an increase in the stereotypy of the adult song with age. Importantly, this hypothesis predicts that only the stereotypy of undirected song would improve because lMAN activity is much reduced during directed singing and therefore does not significantly contribute variability to the vocal output in the presence of a female (Kao & Brainard 2006). These predictions are supported: the stereotypy of adult undirected song increases as birds get older (Kao & Brainard 2006; Pytte et al. 2007) but there is no reported change with age in the stereotypy of directed song in adulthood (Lombardino & Nottebohm 2000; Kao & Brainard 2006).

To explore whether the net addition of HVC-RA neurons in adulthood is involved in the improvement in stereotypy of undirected song, we looked for a correlation between the number of HVC-RA neurons present in birds at 340 days of age and the extent to which the stereotypy of their song, gauged by the similarity between successive motifs, had improved between recordings made at 130 and 340 days of age. This is far from an ideal experiment: we have no information about the

number of HVC-RA neurons that each bird had at 130 days. We are using the number of neurons present at day 340 to get an idea of how many neurons have been added since day 130 when in reality it will reflect individual variability in the number of neurons present at 130 days and variability in the number of neurons added since then. But still, because we had the data available we went ahead and ran the regression analysis. We found a weak correlation between the extent to which a bird's song had improved between day 130 and day 340 and the density of HVC-RA neurons present on day 340 but the relationship did not reach significance.

Due to the pitfalls of this approach, we do not take our inability to find a significant relationship between HVC-RA neurons and song stereotypy as strong evidence that one does not exist. Other experiments are needed to thoroughly test the hypothesis that the addition of HVC-RA neurons is involved in the refinement of adult song. First, it must be shown that adult-born HVC-RA neurons are active during singing, a prerequisite for them having a premotor involvement in song. As discussed in Chapter 5, this can be done by looking for the expression of immediate-early genes in birthdate labeled HVC-RA neurons after periods of singing. Second, a better experimental design to test for a correlation between neuron addition and song stereotypy is needed that uses a larger sample size and more rigorous song analysis methods. For example, the number of HVC-RA neurons present at 90 days of age could be retrogradely labeled with microspheres and then a second tracer could be used to label the HVC-RA neurons present at 1 year. Any neurons labeled with the second tracer but not the microspheres were presumably recruited to HVC

after 90 days of age. The number of single-labeled neurons could then be correlated to how much the song stereotypy improved over the same time period. Interpretation of the retrogradely labeled neuron counts would, however, be limited by the same caveats that we encountered in our experiments in Chapter 3; a residual deposit of microspheres in RA could be taken up by HVC-RA neurons recruited after the initial injection, which would result in an under-estimation of the number of HVC-RA neurons that had been added between 90 days and 1 year of age. In addition, preliminary experiments would be required to ensure that the presence of microspheres in RA did not reduce the efficiency of uptake of the second tracer, which could further hinder interpretation of the final neuron counts.

Clearly, due to the limitations discussed above, a better way to explore this hypothesis would be to look for a causal relationship between net HVC-RA neuron addition and the increase in stereotypy of adult song. Reducing the number of dividing progenitors in the ventricular zone above HVC, either genetically or chemically, would presumably also reduce the number of HVC-RA neurons recruited in the adult. This would first have to be tested directly, as it is possible that even a significant reduction in the production of new neurons could be compensated for by an increase in the number of neurons that survive the initial culling period in HVC. However, if the net addition of HVC-RA neurons could be reduced for a long enough period of time using a progenitor ablation strategy, and if the hypothesis were correct, then we would expect to see a retardation in the normal improvements in song that occur with age in the treated birds relative to untreated controls.

Addition of HVC-RA neurons occurs in the absence of auditory feedback

The dynamics of HVC-RA neuron addition in adulthood does not appear to require auditory feedback. Birds deafened at 130 days of age show the same rate of neuronal recruitment, as determined by the number of BrdU-positive neurons surviving for 1 month in HVC, and the same overall increase in the number of retrogradely labeled HVC-RA neurons with age as hearing controls. Therefore, the incorporation of new HVC-RA neurons and their subsequent survival does not seem to require any auditory instruction. These results are in agreement with a previous study that reported no change in the 4-month survival of adult-born neurons in HVC after deafening (Wang et al. 1999). At first glance, these data would appear to weaken the argument for the adult-born HVC-RA neurons having any role in the refinement of adult song. But, deafening during the juvenile period also has no effect on the developmental recruitment of HVC neurons (Wilbrecht et al. 2002) or the normal developmental increases in HVC-RA neuron numbers (Burek et al. 1991) even though it does prohibit juveniles from successfully imitating an adult song. Thus, adult-born HVC-RA neurons, just like those born during juvenile development, could still be involved in the song motor program, even though their recruitment and survival is not affected by the removal of auditory feedback. As discussed in Chapter 5, it is possible that both juvenile-born and adult-born HVC-RA neurons can integrate into HVC perfectly well in the absence of hearing but that they require auditory instruction to make appropriate synaptic connections in RA. Perhaps, as first proposed by Nordeen and Nordeen (1992), it is the accumulation of new

'unguided' HVC-RA neurons in the song system that results in the progressive disruption of the song motif after deafening in adults.

It is also important to consider that deafening is a very unlikely event in nature. If the net addition of HVC-RA neurons in the zebra finch conveys an advantage to the bird, by enabling refinement of the song or otherwise, it does not follow that this program of addition would be dependent on hearing and consequently disrupted if the bird could no longer hear himself or others. Since deafening does not occur in nature, there need not be any provisions in place to deal with it.

Is neuronal turnover induced by lesions of lMAN?

There was no suggestion of neuronal replacement occurring in the adult zebra finch HVC under any of the conditions we tested except one: the combination of lMAN lesions and deafening (L+D). We found no evidence for turnover of adult-born neurons in HVC, at least over a period of 3 months, in young adults (90 days), in older adults (2 years) or in young adults housed in a busy social aviary (see Chapter 3). There was no loss of neurons labeled with BrdU and counted at 1 and 3 month survival times in any of these 3 conditions. However, when we treated birds with the combination of lMAN lesions and deafening as part of the experiment described in Chapter 5, there was a 2-fold increase in the number of new BrdU-positive HVC-RA neurons despite the total number of HVC-RA neurons not being

different from the number in control birds, suggesting some neuronal replacement had occurred in their HVC-RA population.

The L+D birds received lMAN lesions at 118 days of age followed 2 weeks later by deafening and were then sacrificed at 345 days of age. One month prior to sacrifice, the birds were given BrdU injections for 3 days to birthdate label adult-born neurons that were then counted 1 month later. In addition, 5 days prior to sacrifice, the birds received bilateral injections of a retrograde tracer, CTB, into RA to label the HVC-RA neuron population. By 340 days of age, the L+D birds had the same number of retrogradely labeled HVC neurons as both the control birds and as birds that were deafened without prior lMAN lesions. All 3 three groups had HVC-RA neuron densities that were characteristic of birds their age showing that the net addition of HVC-RA neurons had proceeded as normal in both deafened and L+D birds. However, counts of BrdU-positive neurons present in HVC 1 month after BrdU injection revealed that twice as many adult-born neurons had survived in the L+D birds relative to the other 2 groups and that many of them had been retrogradely labeled from RA. These data do not directly show replacement of the HVC-RA neurons in the L+D birds but they had twice the number of new HVC-RA neurons added over a month without an increase in the total number of HVC-RA neurons added over a 7 month period. The simplest explanation of these events is that most of the newly recruited HVC-RA neurons are only surviving in HVC for short periods and thus not contributing to the total HVC-RA population over the longer term. The other possible interpretations of these data are discussed in Chapter 5. To

determine whether our interpretation outlined above is correct, we could directly test the survival of BrdU-labeled neurons in the HVC of adult L+D birds by increasing the survival time between the BrdU injections and the time to sacrifice. If our interpretation is correct and the combination of lMAN lesions and deafening in adulthood results in turnover of the adult-born HVC-RA cohort then the number of BrdU-positive neurons surviving in the HVC of L+D birds should decrease as survival time increases.

Another key follow-up experiment is to find out whether lMAN lesions alone also cause the increase in new neuron survival that we saw in the L+D birds or whether the effect is specific to the combination of surgeries. We propose that lMAN lesions on their own would also result in an increase in the number of BrdU-positive HVC neurons that can be counted after 1 month. In the discussion in Chapter 5, we speculated that lMAN activity could be necessary for the formation of strong synapses between the incoming HVC-RA neurons and their target neurons in RA. The terminals of lMAN neurons innervate the same RA neurons as the HVC-RA neurons (Canady et al. 1988) and lMAN activity in RA is facilitated exclusively through NMDA receptors (Mooney & Konishi 1991). NMDA is a well-known mediator of long-term potentiation and synaptic strengthening (reviewed in Bliss and Collingridge 1993) and so it is possible that lesioning lMAN removes NMDA receptor activation in RA, which in turn compromises the formation of strong synapses between the HVC-RA neurons and their downstream premotor targets in RA. For the existing of HVC-RA neurons that have already formed stable synapses with

appropriate targets in RA, this may not be a problem, but for adult-born neurons recruited after lMAN lesion the inability to form strong connections could result in a reduction in their survival and thus lead to an increase in turnover in the adult-born population. This hypothesis could be tested by infusing a selective NMDA inhibitor like AP-5 (Collingridge et al. 1983) into RA and looking to see whether the survival of brithdate-labeled neurons in HVC is affected by the absence of NMDA signaling.

If this hypothesis were to stand up to testing, it might also explain the behavioral changes that result from deafening, or the combination of lMAN lesions and deafening, in adulthood. After deafening alone, there are severe disruptions in the temporal and spectral structure of song but prior lesions of lMAN prevent most of this degradation from occurring (Brainard & Doupe 2000; Brainard & Doupe 2001; Scott et al. 2007; and replicated in this thesis). As suggested in the above section, the song deterioration after deafening could result from the integration of HVC-RA neurons into the vocal pathway without auditory guidance that make inappropriate connections with the premotor neurons in RA. It could be that the accumulation of random connections over time, as more and more new HVC-RA neurons are recruited, results in the observed progressive changes in song structure after deafening. When lMAN lesions are performed prior to cochleae removal, the lack of NMDA signaling in RA would prohibit new HVC-RA neurons from making strong connections at all, and thus there would be no accumulation of aberrant synapses in the vocal motor pathway. This would result in the adult song motif persisting after

deafening, which it does, and would also cause a reduction in the long-term survival of new HVC-RA neurons, which can be tested.

A possible role for neuron addition in social behavior

Zebra finches are highly social and often live in large colonies that can contain up to 1000 other individuals (Zann 1996). Each male learns his song through imitation of an adult tutor, but the process is seldom perfect and so each male will often have his own unique song motif. Thus, in large colonies the ability to recognize and discriminate other males based on their song is not a trivial task. The song system ~~is~~ has been implicated in conspecific song discrimination because neurons in several of the song system nuclei show robust auditory responses that are selective for zebra finch song (Doupe 1997), including in the HVC-RA projection neurons (Mooney 2000). In this thesis we have replicated previous studies showing that a more complex social environment stimulates the recruitment of neurons to HVC (Lipkind et al, 2002; Adar, Nottebohm & Barnea 2008). We found that housing young birds in an aviary with 29 other finches, as opposed to alone in a single cage, caused a 2-fold increase in the number of BrdU-positive neurons in HVC at 1 and 3 months after BrdU injection. This effect is not the result of increased proliferation because there was no difference between groups in the number of BrdU-positive ventricular zone cells. Instead, the social environment appears to act to promote the survival of incoming new neurons within the first month after their birth.

Many aspects of the bird's environment are different when living in an aviary setting compared to a single cage. In the aviary the birds have more space to move around in; they are exposed to females which may induce hormonal changes; they have direct physical contact with both other males and females; they experience a richer auditory environment; and they may also sing more. To tease apart which of the factors are important for increasing new neuron survival, Adar et al (2008) housed birds in the same sized aviary, either with one other female or 45 other conspecifics of both sexes. In this case, the space to move around, exposure to females and physical contact were eliminated as contributing variables. They measured how much the birds sang in each context and found that, unexpectedly, singing rates were much lower in the busy social setting than when housed with just a female (Adar, Lotem, & Barnea 2008). They concluded that an increase in auditory information load was most likely to be the key mediator in the increased survival of adult-born neurons that results from a complex social environment (Lipkind et al. 2002; Adar, Lotem, & Barnea 2008). In these studies, retrograde tracers were not used to identify the new neurons as RA-projecting but we have shown that many of the extra neurons recruited to HVC in an aviary setting are retrogradely labeled HVC-RA neurons. In a range of brain systems that receive new neurons in adulthood, use of the system can stimulate recruitment and survival. This appears to be true for singing and the canary HVC (Li et al. 2000); food caching and the hippocampus of black-capped chickadees (Barnea & Nottebohm 1994); olfaction and the olfactory bulb in mice (Petreanu & Alvarez-Buylla 2002) and associative learning and the hippocampus in rats (Gould et al. 1999). Since a busy social

environment seems to upregulate HVC-RA neuron survival via an increase in auditory processing, it follows that adult-born HVC-RA neurons might have a role in conspecific song perception. If this were the case, a net increase in the number of HVC-RA neurons with age could reflect an increase in the demand for song discrimination. As a male zebra finch proceeds through life, the number of unique male songs that he has encountered will accumulate. Perhaps, in order to remember the songs of old acquaintances and still be able to identify novel songs when new males are encountered, a male needs an ever increasing cohort of HVC-RA neurons for optimal discriminatory performance.

The idea that adult-born HVC-RA neurons are involved in conspecific song recognition is highly speculative but there are some basic experiments that can be done to begin to test the hypothesis. The first would be to deafen birds and place them in a social aviary setting to see if the removal of auditory input attenuates the effect of social complexity on HVC neuron survival. A positive result would be informative but a negative result would be difficult to interpret because, as mentioned above, deafening is an unnatural phenomenon. It is possible that a complex social environment could stimulate the survival of new neurons in anticipation of an increased auditory load, even if the increase in auditory input is never experienced. The alternative approach to separating the increase in auditory processing from other features of a social environment would be to see if the playback of many different conspecific songs, in the absence of other live birds, is enough to stimulate survival of adult-born HVC-RA neurons. Again, in this case a

positive result would be illuminating, but a negative result should be interpreted with care because the exposure of a bird to multiple songs in the absence of other social interactions is an unnatural situation. If exposure to many new songs via playback was not enough to stimulate HVC-RA neuron survival, it would not negate a potential role for new HVC-RA neurons in song perception in a real complex social environment. Finally, in situ histochemistry to look for the expression of immediate-early genes could be combined with birthdate labeling and either playback of multiple songs or immersion in a socially complex environment. If adult-born HVC-RA neurons were activated by conspecific song, perhaps the number or extent of IEG expression would correlate with the number of novel songs they were exposed to or the richness of the social environment.

Final conclusions

To sum up, we have observed that recruitment of neurons to the adult male zebra finch HVC results in a net increase in the total number of HVC neurons. This net increase is particularly rapid between 3 month and 2 years of age. Half of the new neurons project to RA and can be labeled with retrograde tracers. The identity of the other half is not known; they do not seem to be interneurons because they do not express any of the protein markers typically associated with HVC interneurons. There is no significant loss of the existing retrogradely labeled HVC-RA population, at least not during the first 9 months of adulthood, when the persistence of this population was tracked. As the number of neurons in adult HVC increases, so does

their packing density because HVC volume remains constant. In line with this net increase in HVC neurons, we have shown that BrdU-labeled adult-born neurons recruited into HVC at sexual maturity (90 days of age) and counted 1 month later are still present, in very similar numbers, 4 years later.

The purpose and functional implications of net neuronal addition to the adult zebra finch HVC, with concomitant increase in packing density, remain unknown. What is clear, however, is that the consequences of neuronal recruitment in the HVC of adult zebra finches are markedly different from those observed in adult canaries. The reasons for this disparity may relate to differences in adult vocal plasticity, breeding strategies or social behaviors between the two species. Exploring which of the differences in the lifestyle of zebra finches and canaries matter to HVC neuronal recruitment could help shed light on the functional role of adult-born neurons in the avian song system. The contrasting modes of neuronal recruitment in canaries and zebra finches highlights the peril of using observations from one species to “model” the “same” phenomenon in another species that is more difficult to study – as is often done when using rodents as models for humans. In addition, it should be clear that we are still far from having a satisfactory understanding of the function of neurogenesis in the brain of adult warm-blooded vertebrates. Hopefully, further studies that take into account the different life styles and needs of different species used in research will help us understand why some neurons, in some parts of the brain, continue to be produced in adulthood.

MATERIALS AND METHODS.

General methods for all experiments:

Animals

For the experiments described in chapters 2-5, 151 male adult zebra finches were used from our breeding colony at the Rockefeller University Field Research Center in Millbrook, NY. The care of all animals used in our experiments followed the standards set by the American Association of Laboratory Animal Care and the Rockefeller University Animal Use and Care Committee. Unless stated otherwise, birds were housed alone in single cages for the duration of the experiment on a 14/10 light/dark cycle with food and water provided ad libitum. Prior to the start of each experiment, birds were either housed with their parents and siblings in breeding cages (if $\leq$ 90 posthatch days at the experiment start) or in larger stock cages containing other males (if > 90 posthatch days at the experiment start). For the older birds, a detailed history is not available. It is most likely that they were used for breeding as younger adults and then retired into male stock cages for the rest of the time.

Retrograde tracer injection surgeries

For all retrograde tracer injection surgeries, animals were food and water deprived for 1 hour prior to being injected with the anesthetic Nembutol (pentobarbital sodium, 60 mg/kg; Ovation Pharmaceuticals, Deerfield, IL) into the breast muscle at a dose

of 5.7 microlitres per gram of body weight. Once under the anesthetic, birds were placed in a specially-built stereotaxic device for surgery and the feathers were plucked from the back of their heads. The scalp was dissected along the midline, nucleus RA or Area X were located using stereotactic co-ordinates and a small craniotomy was made above the target nucleus in both hemispheres. A small slit was made in the dura with a 30 guage hypodermic needle to facilitate entry of the 30u glass micropipette containing the retrograde tracer into the brain. The micropipette was then lowered to the target depth and the tracer was injected slowly into the target nucleus using pressure. Four injection sites were made per nucleus with 40 nL of tracer injected per site. The pipette was then slowly retracted and used to inject into the other hemisphere is the same manner. For birds receiving tracer into RA and Area X, two separate micropipettes were loaded with the two tracers and the pipettes were switched over between the last RA and the first Area X injection site. To finish the surgery, skull windows were replaced, the scalp was closed with cyanoacrylate and antibiotic (Neosporin ointment) was applied to the area. Post-surgery birds were kept warm under a heat lamp until they had recovered enough to perch. Two kinds of retrograde tracers were used in this thesis: the cholera Toxin B subunit (CTB) conjugates to fluorescent dyes (Molecular Probes, Eugene, OR) and red fluorescent latex microspheres (Lumafluor, Naples, FL). CTB was used as the retrograde tracer in most experiments because it diffuses from the injection site to fill the target nucleus and thus gives a bright retrograde signal in neurons throughout HVC. Red latex microspheres were used in the first experiment in Chapter 3 to allow direct comparison to a similar experiment conducted in the

canary (Kirn & Nottebohm 1993) and because microsphere labeling has been shown to stably persist in neurons for up to a year in vivo (Katz et al. 1984).

BrdU injections

BrdU (5-bromo-2-deoxyuridine) was used to birthdate label new neurons; BrdU is a synthetic nucleoside that gets incorporated into DNA in place of thymidine during DNA synthesized and can be detected by a specific antibody. It can cross the blood-brain barrier and thus can be administered systemically to identify neurons produced by cell divisions occurring at the time of injection. Adult male zebra finches were injected twice-daily with 100 µl of 10 mg/ml BrdU (dissolved in sterile saline) for three consecutive days. Injections per performed into the breast muscle at 8 am and 8 pm on the injection days. Birds were housed alone in single cages for the three days and the remainder of the experiment, unless otherwise stated.

Perfusions and brain processing

On the appropriate sacrifice date, birds were deeply anaesthetized with Nembutal and perfused intracardially with 0.9% saline containing Heparin (1:1000) to remove blood cells followed by 4% paraformaldehyde in 25 mM phosphate buffer as a fixative. The brains were removed, postfixed in 4% paraformaldehyde for 1 hour and cryoprotected through ascending concentrations of sucrose in PBS (5, 15 and 30%) for 2 days at 4°C. Brains were frozen in Neg-50 frozen section medium (Richard Allan Scientific**,** Kalamazoo, MI) and coronally sectioned at on a cryostat. Unless

otherwise noted, sections encompassing HVC were collected at 40 µm in three series of slides, with each series containing every third section. For lMAN, sections were collected at 20 µm in two series of alternate sections. Slides were stored at -80°C until further use.

BrdU/Hu immunohistochemisty

To identify BrdU-positive neurons in HVC, we looked for the presence of a BrdU-positive nucleus in a cytoplasm that was positive for the expression of Hu protein, a marker of the mature neurons (Barami et al. 1995). One series of HVC slides, containing every third section through the nucleus, was immunostained with antibodies against the neuronal marker HuC/D (16A11 primary made in mouse, Molecular Probes) and BrdU (rat monoclonal primary, Axyll Corp., Westbury, NY) as follows: on day 1, sections were air dried for 30 mins, rehydrated in TBS (tris-bufffered saline, pH 7.5) for 5 minutes, incubated in 60% formamide at 55ºC for 15 minutes, washed in TBS for 10 minutes, incubated in 2N HCl at 37ºC for 30 minutes, incubated in sodium borate solution (0.1 M NaB_4O_7 pH8.5) for 10 mins, washed 3 times in TBST (TBS containing 0.1% Triton X) for 5 minutes each, blocked with 10% normal goat serum in TBST for 30 minutes at room temperature and then incubated with the anti-Hu primary at 1:150 in TBST with 10% goat serum for 48 hours at 4ºC. On day 3, sections were washed 3 times in TBST for 10 minutes each and incubated with Alexa-647 conjugated goat anti-mouse secondary antibodies (Molecular Probes) at 1:500 in TBST for 2 hours at room temperate. Sections were washed 3 times in TBS for 10 minutes each, blocked with 10% normal goat serum in TBST for

30 minutes at room temperature and incubated with rat anti-BrdU at 1:500 in TBST with 10% goat serum overnight at 4ºC. On day 4, sections were washed 3 times in TBS for 10 minutes each and incubated with either Alexa-488 or Alexa-555 conjugated goat anti-rat secondary (Molecular Probes) at 1:500 in TBST for 2 hours at room temperate. Sections were finally washed 3 times in TBS for 10 minutes each, rinsed in dH_20 and coverslipped with an aqueous mounting media.

Image collection

In order to count the different types of neurons in HVC, confocal Z-stacks were collected of every third HVC section using a Plan-apochromat 20x/0.75 NA objective lens on a Zeiss inverted LSM 510 META laser scanning confocal microscope with a 2 µm optical section thickness. Either two or three color stacks were collected depending on the particular experiment. To measure the volume of HVC, 10x fluorescence images of every third section through HVC were collected with an Olympus IX70 inverted microscope and a 10x/0.3 NA objective lens.

Cell counts using the optical fractionator method

Counts of HVC-RA and HVC-X projection neurons, interneurons and Hu-positive neurons were all performed using the optical fractionator method (West & Gundersen 1990). Confocal stacks were imported into StereoInvestigator software with the Image Stack Module (MBF Biosciences, Williston, VT) and the optical disector probe was used to systematically sample within the X/Y dimensions of each

stack. The StereoInvestigator optical fractionator procedure with confocal stacks works as follows: a grid of squares is laid down at random on the top of the stack and a counting frame is placed at every grid intersection falling within the region of interest, in this case HVC. The square counting frames are converted into 3D counting boxes by defining the disector height, which is the height of the counting box in the Z-plane. This produces a series of 3D counting boxes placed evenly throughout the confocal Z-stack and all the cells contained within one of the boxes, but not touching any exclusion edges, are counted. A guard volume is applied to the top and bottom of the Z-stack and any cell nucleus in focus within the guard volume is excluded to ensure split cells are not counted. A disector height is selected to ensure that the counting volume plus the two guard zones are contained within the thickness of the confocal z-stack, which in turn is determined by the final thickness of the tissue section after processing. For the neuron counts in this thesis, a guard volume of 3 μm was used but the disector height was modified depending on the neuron type being counted. For counts of Hu-positive neurons and interneurons, the antibody staining procedures resulted in more tissue shrinking than for visualization of the projection neurons and thus a shorter disector had to be used. The grid and counting frame dimensions were chosen to give a count of about 400 cells per HVC for both RA-projecting and Hu-positive neurons and about 150 cells per HVC for X-projecting neurons and interneurons, which are much scarcer. The final grid, counting frame and disector dimensions used varied for each neuron type and are listed below in the methods for each individual chapter. Once the optimal dimensions

were determined for a given neuron type they were fixed for all birds in the experiment.

During the optical fractionator procedure, neurons were counted if they were in focus within one of the counting boxes without touching any of the exclusion edges, and if they met the morphological criteria defined for each neuron type, as described in the individual chapter methods below. In all cases, every third 40 µm section across the rostral-caudal axis of HVC was used for counting, which resulted in 8-10 sections per HVC. StereoInvestigator software sums the neuron counts from all the sections and uses it to estimate the total number of neurons present in the region of interest by multiplying it by the proportion of the region's volume that was sampled. The proportion of the total region sampled is determined by the fraction of sections that are sampled, the fraction of the total area that is sampled and the fraction of the section thickness that is sampled. The total number of neurons is determined by:

$$Total\ number = n \times \left(\frac{1}{SSF} \times \frac{1}{TSF} \times \frac{1}{ASF}\right)$$

n = the number of neurons counted in all the disectors across all the sections.

$\frac{1}{SSF}$ = Section Sampling Fraction: the fraction of all sections through HVC in which counts were made, in this case 1/3.

$\frac{1}{TSF}$ = Thickness Sampling Fraction: the fraction of the processed tissue thickness in which cells were counted that is calculated by disector height/final section thickness.

$\frac{1}{ASF}$ = Area Sampling Fraction: the fraction of the area in which counts were made that is calculated by counting frame area/grid square area.

Typically when using the optical fractionator method on real sections, as opposed to confocal stacks acquired previously, the entire region of interest is available to be sampled and so the final data refers to the total number of neurons present in that region. In these experiments, the confocal stack X/Y dimensions did not cover the entire cross-sectional area of HVC so total numbers refer to the number of neurons present in the volume of HVC that was included in the series of stacks. This volume was calculated by summing the cross-sectional area of HVC contained within each stack and multiplying it by the section thickness and sampling interval (40 µm x 3). The total neuron counts from StereoInvestigator were divided by the sampled volume to produce a final density measurement for each neuron type in HVC. Total numbers of each neuron type in HVC could then be calculated by dividing the density value by the volume of the entire HVC measured from 10x images (see below). For most birds, the left and right HVC were analyzed independently and then averaged but for some birds only one HVC was available due to an incomplete backfill with retrograde tracer or to bad tissue processing. In these cases, the data from a single HVC is presented as we found no systematic differences between left and right HVC.

BrdU/Hu neuron counts

To count BrdU/Hu double-positive neurons, a modified version of the optical fractionator method was used called the 'rare event protocol'. Instead of laying down a sampling grid onto the confocal Z-stacks, this method involved scanning the entire area of the stack for positive neurons and so the ASF becomes 1. Thus, to calculate the total number of BrdU/Hu neurons, the sum of counts made across every third section is only divided by 1/SSF x 1/TSF. The TSF is still calculated because this method includes a guard volume to avoid counting split cells and thus the entire height of the Z-stack is not available for counting. As for the normal optical fractionator method described above, the total number of BrdU/Hu positive neurons calculated in this manner refers to the number present in the volume of HVC that was included in the series of stacks. This was volume was calculated as above and used to estimate the density of BrdU/Hu neurons in the entire HVC. Total BrdU/Hu numbers were calculated by dividing the density values by the volume of HVC, as measured in 10x images (see below).

HVC volume measurements

Total HVC volume was estimated by tracing the nucleus perimeter in 10x images and calculating the cross-sectional area using ImageJ software (NIH, Bethesda,MD). In birds with retrograde tracer injections, the HVC perimeter was defined by the CTB backfill from RA. In birds without retrograde tracer injections, for example in the groups where just BrdU/Hu neurons were counted, the HVC perimeter was defined by the presence of large HVC-X cell nuclei stained with Hu. For both methods, the

cross-sectional area was measured for every third section along the rostral-caudal axis and the total summed area was multiplied by the section thickness and sampling interval (40 μm x 3) to get an estimate of HVC volume. These HVC volumes were used to convert neuron densities into total numbers of each neuron type present in every HVC.

Error of cell counts

To determine the precision of the cell counting procedure, the Gunderson coefficient of error (CE) was calculated for each HVC in the sample. This error coefficient compares counts from adjacent to non-adjacent sections as a way to measure the homogeneity of the neuron distribution across the region of interest. Sources of variability in the counts, like an uneven neuron distribution or uneven section thickness, cause greater differences between the counts made in adjacent sections than would be expected in homogeneous or sections with even thickness. The similarity of counts in adjacent sections is reflected in the Gunderson CE. Previous analyses in biological structures have indicated that a CE of 0.1 or lower is adequate to find a real difference in the number of objects counted between two different samples (Gundersen & Jensen 1987; West 1993) and thus we modified our disector settings to ensure CE values were lower than 0.1 in all experiments.

BrdU-positive ventricular zone counts

BrdU-positive cells in the ventricular zone above HVC were counted in every third 40 µm sections using the same confocal stacks that were taken for the counts of BrdU-positive HVC neurons. BrdU-positive ventricular zone cells were identified by the presence of a BrdU-positive nucleus that was negative for Hu staining and was touching the ventral ventricular surface. All positive cells in the 40 µm thick Z plane were counted. The length of the ventricular zone present in each confocal stack was traced in StereoInvestigator software (MBF Biosciences, Williston, VT).Total counts were converted to counts per mm^2 of ventricular zone by dividing them by the total ventricular zone that had been available to count, which was determined by summing the ventricular zone lengths from every counted section and multiplying it by the section thickness (40 µm). For each bird, the counts from the left and right ventricular zones were averaged.

Statistics

All statistical tests were performed using SPSS 16.0 (SPSS Inc., Chicago IL).

Specific methods for Chapter 2:

Animals and surgery

34 birds aged between 90 days and 11 years of ages received bilateral retrograde tracer injections of cholera toxin B (CTB, 1% solution in sterile PBS) conjugated to AlexaFluor dyes (Molecular Probes, Eugene, OR). Half the birds received injections to both nucleus RA and Area X in the same surgery: green Alexa-488-CTB to RA and red Alexa-594-CTB to Area X. These birds revealed a relationship between RA-projecting neurons but not X-projecting neurons with age and thus a second group of birds only received injections of green Alexa-488-CTB to nucleus RA to add more time points to the HVC-RA analysis. After surgery, 6 days were allowed for retrograde transport of the CTB before the birds were sacrificed by intracardial perfusion.

Staining and immunohistochemistry

Sections containing HVC were divided into 3 series containing every third section. One series was used for counting the two types of projection neurons, identified by retrograde labeling with Alexa-CTB. Slides were thawed, air dried for 30 minutes and counterstained with 1 μM ToPro-3 iodide (Molecular Probes) for 20 seconds to identify cell nuclei. After the confocal imaging of these slides, the same set was used to take 10x images for the HVC volume analysis. The second series of HVC slides was stained with an antibody against the neuronal marker HuC/D (16A11 primary, Molecular Probes) and used to count total neuron numbers. Sections were

incubated in citrate buffer (0.01M sodium citrate plus 0.01M citric acid) at 37°C for one hour followed by 0.125% pepsin in 0.1N HCl for 3 minutes to increase antibody penetration. They were blocked in 10% normal goat serum in 0.3% Triton X-100 and sequentially incubated with mouse anti-Hu primary (16A11, Molecular Probes) at 1:150 in blocking solution for 48 hours at 4°C and Alexa Fluor 647 goat anti-mouse secondary IgG (Molecular Probes) at 1:500 for 2 hours at room temperature. The third series of HVC slides was retained at -80°C and some birds were used for the interneuron experiments described in Chapter 4.

Image collection and neuron counts

For counts of the projection neurons, 3-color Z-stacks were collected containing the green CTB backfill from RA, the red CTB backfill from Area X and the far-red nuclear stain. HVC-RA and HVC-X projection neurons were identified by the presence of either green or red CTB, respectively, rimming a clear nucleus stained with ToPro-3 with a diameter greater than 4 μm. If a continuous area of CTB was touching two nuclei, only one nucleus was counted as a positive backfilled cell. For total neuron counts, 2-channel confocal Z-stacks were collected containing far-red Hu staining and the green CTB backfill from RA, which was used to define the boundaries of HVC during counting. Hu-positive neurons were identified as having clear Hu staining in a circular pattern typically with a dim region the center to mark the nucleus. All three neuron types were counted using the optical fractionator method (described above) using the following disector probe dimensions: for HVC-RA and

HVC-X neurons, grid square = 150 μm, counting frame = 50 μm and disector height = 20 μm; for Hu-positive neurons, grid square = 100 μm, counting frame = 25 μm and disector height = 16 μm.

To assess HVC-RA nuclear diameters and intranuclear distances, the confocal stacks from three HVC sections were selected at random for both HVCs in each bird and a single image from the center of each stack was analyzed using ImageJ software (NIH, Bethesda,MD). To measure nuclear diameters, 20 CTB-labeled HVC-RA neurons were chosen for each HVC (spread across the three images) and the diameter across the widest dimension of the ToPro-positive nucleus was measured. Although the neurons measured were selected at random, an effort was made to pick neurons that looked like they had been transected through the center of the nucleus and thus usually the larger neurons in the image were used. The 20 diameters from the left and right HVCs were combined to find the average nuclear diameter for each bird. There was no difference between the mean HVC-RA nuclear diameters from the left and right hemispheres. Intranuclear distances between HVC-RA neurons were measured in the three same images for each HVC. Another set of 20 CTB-labeled HVC-RA neurons were selected at random and the distance from the center of the ToPro-positive nucleus to the center of the nuclei of its three nearest CTB-labeled HVC-RA neuron neighbours was measured. All the intranuclear distances from left and right HVCs were averaged for each bird.

Cell profiler workflow

The same single images used for analysis of the nuclear diameters and intranuclear distances in ImageJ were used for the automated analysis using CellProfiler software (Carpenter et al. 2006). A workflow was developed to identify and measure the nuclear areas of all HVC-RA neurons in the image, defined by retrograde labeling with green CTB. Images were loaded into CellProfiler and 200 pixels were cropped from all four edges to ensure the image was centered within HVC. The cropping was checked by eye and for a few images there were non-HVC areas still present so they were cropped by hand. The cropped images were split into the 3 colour channels and each converted to grayscale. A threshold was applied to the blue ToPro channel to separate true nuclei from background using the robust background global thresholding method with a threshold range of 0.1-1. Nuclei were then identified as primary objects if they were within the range of 7-14 pixels in diameter and were not touching the borders of the image, in order to exclude areas of erroneous staining, small nuclear fragments and partially cropped nuclei. HVC-RA nuclei were then identified as secondary objects in the green CTB channel using the primary nuclei in the blue channel as the input and using the propagation method to extend the nuclear outlines to include any consecutive areas of green signal. These secondary objects were defined as the original nucleus plus any green CTB around it. Both primary and secondary object characteristics were exported to excel and sorted by the integrated intensity of CTB in the green channel. Secondary objects with an integrated intensity of less than 5 and greater than 50 in the green channel were deleted as an intensity of less than 5 does not appear by eye to be a CTB-

positive neuron and neurons with greater than 50 tended to be a cross-section right through the bulk of dye with only a fragment of nucleus present in the image. When CellProfiler identifies primary and secondary objects it assigns an ID number to each object and since the secondary objects were identified by propagating out from the primary nuclei, each identified HVC-RA neuron could be matched to its nucleus in the blue channel. This is important because often the green CTB signal will partly obscure some of the cell nucleus and thus measuring nuclei size in the composite RGB image may give an underestimate of true nucleus size. The area of each primary nuclei associated with a secondary object (and thus identified as a CTB-labeled cell) was extracted from the CellProfiler output and averaged to find the mean HVC-RA neuron nucleus area for each HVC. The sum of all the HVC-RA neuron nucleus areas was calculated and used to find the fraction of the total image area containing HVC-RA nuclei. This value is affected by the average nuclear size but can still give an indication of how densely packed the HVC-RA neurons are in the image.

Modeling net addition of new neurons to HVC

Data showing how the addition of new HVC-RA neurons changes with age in the adult zebra finch was extracted from Wang et al. (2002). This paper shows that the number of ^{3}H-thymidine/fluorogold double positive HVC-RA neurons surviving for four months in HVC declines with the age at which ^{3}H-thymidine was administered (see Figure 2.8). These data were extracted from the paper and non-linear

regression was used in Matlab 6.1 (The MathWorks Inc., Natick, MA) to find rate for the decline. The following exponential decay equation provided the best fit:

$$y = 111e^{-0.002x}$$

This equation represents the number of new HVC-RA neurons that are added to HVC per day that will go on to survive for 4 months. The number added per day (*y*) declines as the bird's age increases (*x*, measured in days). For the model, we made the assumption that every new HVC-RA neuron that survived for 4 months in HVC would survive indefinitely. We integrated the rate equation from the data in Wang et al. (2000) to generate an equation that described how the total HVC-RA population would change with the age of the bird if our assumption was correct.

$$\boldsymbol{\tau_i} = \left(\int_{\mathbf{90}}^{\boldsymbol{i}} \mathbf{111}\boldsymbol{e}^{\mathbf{-0.002}\boldsymbol{t}}\, \boldsymbol{dt} \right) + \boldsymbol{c}$$

The integration was performed between day 90 and day $\boldsymbol{i}$, where day $\boldsymbol{i}$ is the age of the bird in days, because we only wanted to model the process of neuronal addition in the adult bird. We take posthatch day 90 to represent the start of adulthood in zebra finches. In this equation, $\boldsymbol{\tau_i}$ is the total number of HVC-RA neurons present in a bird $\boldsymbol{i}$ days old (as long as $\boldsymbol{i}$ is greater than 90 days). $\boldsymbol{c}$ is the number of HVC-RA neurons present at 90 days of age before any adult-born neurons have been recruited. $\boldsymbol{t}$ represents time in days. We calculated a value for $\boldsymbol{c}$ by finding the average number of HVC-RA neurons present in our 90 day old experimental birds and then used this value for to derive a set of predicted values for $\boldsymbol{\tau_i}$ at ages ranging from 90 days to 11 years. This set of predicted values show how the total HVC-RA neuron number would change with age if our assumption was correct and every new

neurons surviving in HVC for 4 months was to survive indefinitely. The predicted values for total HVC-RA number were converted to HVC-RA density by dividing them by 0.26 μm^3, the average HVC volume of all 34 experimental birds. The predicted HVC-RA density values were then plotted on the same graph as the observed HVC-RA density data (Figure 2.9). To determine how accurately the predicted values fit the observed data, we calculated the sum of squares of the residuals between the model and the observed data and used these to calculate an R-squared value.

Statistics

Linear regression was performed to calculate the R-squared and p values for each data set. For the HVC-RA density data, non-linear regression was performed using logarithmic and exponential models and the best fit equation, in this case a logarithmic model, was chosen to generate an R-squared and p value for the fit. Nuclear diameters between young (90-120 days of age) and older (> 4 years old) adults were compared using an unpaired t-test with equal variance as the groups did not fail Levene's test for equal variance.

Specific methods for Chapter 3:

Animals and surgery

For the first experiment, 40 adult males between 90 and 98 days of age were given 3 days of BrdU injections followed on the fourth day by bilateral injection of red latex microspheres to RA (Lumafluor, Naples, FL). The birds were randomly divided into 4 groups of 10 birds each and each group was sacrificed in either 1, 3, 6 or 9 months later. A fifth group of 6 birds were just given the 3 days of BrdU injections and then sacrificed 4 years later. This group received bilateral injections of green CTB into RA 6 days prior to sacrifice to enable the identification of BrdU-positive HVC-RA neurons.

To test for neuronal replacement in older birds, 12 adult males aged between 2 and 2.25 years were treated with 3 days of BrdU injections and then randomly split into 2 groups of 6 birds. The first group was sacrificed after 1 month and the second group was sacrificed after 3 months. To test for neuronal replacement in a complex social environment, 14 birds between 90 and 99 days of age were treated with 3 days off BrdU and then 3 days later, after sufficient time for the BrdU to have been cleared from their bodies, they were moved into a large aviary (5' x 6' x 2') containing 14 other males and 15 females. Not all 14 birds were treated at once. The first group of 7 birds received BrdU and were moved into the aviary together along with 7 untreated males and 15 untreated females. After one month, these birds were removed from the aviary and sacrificed. The second group of 7 males was then

treated with BrdU and added into the aviary with the same cohort of 7 untreated males and 15 untreated females. The second group was sacrificed after 3 months. The two groups were treated sequentially like this to ensure that each BrdU-treated male experienced the same set of other zebra finches for the duration of their survival period.

For the counts of pyknotic cells, three adult male zebra finches between aged between 150 and 200 days and 3 adult canaries aged 19-21 months were removed from stock cages and sacrificed by intracardial perfusion. The canaries were experiencing a January light-dark cycle at the time of perfusion.

Staining and immunohistochemistry

For all experimental birds, every third section through HVC was stained with the combination of BrdU and Hu antibodies to identify BrdU-positive neurons. BrdU was identified with a green secondary antibody, except in the 6 four-year survival birds in which a red secondary was used because they already had green CTB in their HVCs. In all birds, Hu was identified with a far-red secondary antibody (which is shown as blue in Figures 3.2 and 3.4).

For identification of pyknotic cells, 20 μm thick sections were cut on a cryostat and mount on plus slides. Every third section through HVC was stained with 0.9% cresyl

violet, dehydrated through ascending concentrations of ethanol, delipidized in xylene and coverslipped using Krystalon mounting media (EMD Chemicals, USA).

Image collection and neuron counts

For the 40 experimental birds that received BrdU and microsphere injection into RA, 3-color Z-stacks of HVC were collected containing the red microspheres retrogradely transported from RA, the green BrdU staining and the far-red Hu staining. For the 6 four-year survival birds, 3-color Z-stacks were collected containing green CTB retrograde label from RA, red BrdU staining and far-red Hu staining. The 12 two-year old birds and the 14 aviary-housed birds were imaged using 2-color confocal microscopy to view the red BrdU staining and the far-red Hu staining (because these birds did not receive any retrograde tracer injections).

As described in the general methods section above, retrogradely labeled HVC-RA neurons were counted using the optical fractionator method and the BrdU/Hu double positive neurons in HVC were counted using the rare-event modification of the optical fractionator method. Retrogradely labeled HVC-RA neurons were identified by the presence of punctate red microspheres clustered in a Hu-positive cytoplasm around a dimly or negatively stained nucleus. BrdU/Hu double positive neurons were counted if they had a bright BrdU-positive nucleus within an area of Hu-positive cytoplasm. Triple labeled BrdU/Hu/microsphere-positive neurons were identified by the presence of punctate red microspheres clustered around a BrdU-positive

nucleus in the center of a Hu-positive cytoplasm. BrdU-positive ventricular zone cells were identified and counted as described in the general methods section above.

Pyknotic cells were identified in tissue stained with cresyl violet on a brightfield microscope using a 100x oil objective. The entire area of HVC in every third section was scanned for pyknotic cell profiles, which were identified by the presence of either a single, very densely stained nucleus or two or more dense nuclear inclusions in an invisible or shrunken cytoplasm (see Figure 3.9). For each HVC, the total number of pyknotic cells was multiplied by 3 to estimate the number present in the entire nucleus. For each bird, an average was taken from the left and right HVC.

Statistics

Neuron counts across the multiple survival times were compared using a one-way ANOVA and when there was a significant difference between the groups, the Tukey HSD posthoc analysis was applied to identify which groups were significantly different. The number of pyknotic cells in zebra finches and canaries were compared using an unpaired t-test with equal variance as the groups did not fail Levene's test for equal variance. To look for an effect of age and social environment on neuronal survival, Hu/BrdU neuron counts from the three different conditions (young, singly housed; young, socially housed; and old, singly housed) were compared using an unweighted-means two-way ANOVA. An unweighted-means analysis was performed because there was a large difference in number of birds in each group which caused heterogeneity of variance between the three groups. BrdU-positive ventricular zone

cells in all three groups were compared using a weighted-means two-way ANOVA followed by the Tukey HSD post-hoc test.

Specific methods for Chapter 4:

Animals and surgery

This chapter used a total of 18 birds. Six were the 4-year survival birds already described in Chapter 2. These birds were given 3 days of BrdU injections at 90 days of age and then sacrificed 4 years later. They received bilateral injections of green CTB into RA 6 days prior to sacrifice to enable the identification of BrdU-positive HVC-RA neurons. In addition, 6 birds aged between 90 and 120 days of age and 6 birds between 2 and 2.25 years of age were taken from stock cages and sacrificed by intracardial perfusion for use in these experiments.

Staining and immunohistochemistry

For the 4-year survival birds, 2 of the 3 sets of HVC sections were analyzed. The first set was stained with for BrdU and Hu as described above and used to count both the number of BrdU/Hu double positive neurons and the number of triple labeled BrdU/Hu/CTB neurons. The second set was stained for BrdU and interneuron markers (described below) and used to look for BrdU-positive interneurons.

Primary antibodies against 3 calcium-binding proteins were tested in 40 µm thick sections of adult zebra finch tissue to ensure good antibody penetration. The antibodies used were mouse anti-parvalbumin, rabbit anti-calbindin and goat anti-calretinin (all from Swant, Bellinzona, Switzerland). Initially each primary antibody

was tested individually once each one had been optimized, they were combined into a single primary antibody mix for use on the HVC sections of the experimental birds. The final optimized protocol used to look for BrdU-positive interneurons was as follows: sections were air dried for 30 mins, rehydrated in PBS (phosphate-bufffered saline, pH 7.5) for 5 minutes, incubated in 0.2% PBST (PBS with 0.2% Triton X) for 1 hour at room temperature, blocked with 4% BSA in 0.2% PBST for 1 hour at room temperature and then incubated with the following mix of primary antibodies: mouse anti-parvalbumin, rabbit anti-calbindin and goat anti-calretinin (all from Swant, Bellinzona, Switzerland).Each primary was used at 1:5000 in blocking solution and the mix was left on the sections for 72 hours at 4ºC. Sections were then washed 3 times in PBS for 10 mins each and then incubated with the following mix of secondary antibodies: Alexa-647 goat anti-mouse, Cy5 goat anti-rabbit and Alexa-647 donkey anti-goat. Each secondary was used at 1:500 in 4% BSA in 0.2% PBST and left on the sections for 2 hours at room temperature. Sections were then washed 3 times in PBS for 10 mins each, fixed in 4% PFA for 10 mins, washed again in PBS for 10 min, incubated in 2N HCl at 37ºC for 30 minutes, incubated in sodium borate solution (0.1 M NaB_4O_7 pH8.5) for 10 mins, washed 3 times in PBS for 5 mins each, blocked with 10% normal goat serum in 0.2% PBS for 1 hour at room temperature and incubated with rat anti-BrdU (rat monoclonal primary, Axyll Corp., Westbury, NY) at 1:500 in blocking solution overnight at 4ºC. On the final day, sections were washed 3 times in PBS for 10 minutes each and incubated with Alexa-555 goat anti-rat secondary (Molecular Probes) at 1:500 in PBS for 2 hours at room temperate.

Sections were washed 3 times in PBS for 10 minutes each, rinsed in dH_20 and coverslipped with an aqueous mounting media.

In the 12 birds that had not received BrdU injections, sections were stained for the interneuron mix without BrdU. The staining protocol was the same as the one above until the fixation step in 4% PFA. After that, sections were washed once in PBS for 10 mins, rinsed in dH_20 and coverslipped with an aqueous mounting media.

For identification of interneuron nuclei using a nuclear stain, sections were stained for each interneuron marker separately using a protocol similar to the one above. After fixation in 4% PFA, sections were washed in PBS for 10 mins and then dipped into a solution of 1:1000 Hoescht 33258 (Molecular Probes) in PBS for 20 seconds, rinsed in dH_20 and coverslipped with an aqueous mounting media.

Image collection and neuron counts

In the 4-year survival birds, 3-color Z-stacks of HVC were collected containing the green CTB retrogradely transported from RA, the red BrdU staining and the far-red Hu staining. These stacks were used to count the number of BrdU/Hu double-labeled neurons and the number of BrdU/Hu/CTB tripled neurons using the rare-event modification of the optical fractionator method, as described in the general methods section. BrdU/Hu neurons were counted if there was a brightly strained red BrdU-positive nucleus in the centre of a Hu-positive cytoplasm. BrdU/Hu/CTB neurons were identified by the presence of green CTB rimming a red BrdU-positive

nucleus in the center of a Hu-positive cytoplasm. These counts were then used to determine the percentage of BrdU-positive neurons that had been retrogradely labeled by CTB from RA.

To look for BrdU-positive cells that were also positive for the mix of interneuron markers, 3-color Z-stacks of HVC were collected containing the green CTB retrogradely transported from RA, the red BrdU staining and the far-red interneuron mix staining. The green CTB signal was not technically needed for the counts in these stacks but was included to allow demarcation of the boundaries of HVC. Every stack was examined for the presence of BrdU-positive nuclei in the center of a cytoplasm that was positive for the mix of interneuron antibodies. Both left and right HVC was examined for each bird.

Total interneuron counts were performed using the optical fractionator method in confocal Z-stacks using the following disector probe dimensions: grid square= 100 µm, counting frame= 50 µm and disector height= 10 µm. Interneurons were identified by bright staining with the mix antibodies against the three calcium-binding proteins (parvalbumin, calretinin and calbindin). No nuclear stain was used for these neuron counts.

Statistics

Interneuron counts in the 3 age groups were compared using a one-way ANOVA followed by the Tukey HSD post-hoc test.

Specific methods for Chapter 5:

Animals and experimental timeline

28 adult males were used in this experiment. There were three groups: Deaf (10 birds), L+D (10 birds) and Controls (8 birds). All birds had their songs recorded in a sound isolation chamber at the start of the experiment, before any surgical manipulation had occurred. Birds in the Deaf group received bilateral cochleae removal at 130 days of age (range 127-136) and were then continuously recorded for the next month to document resulting changes in their song. Birds in the L+D groups received bilateral electrolytic lesions of lMAN at 118 days of age (range 115-122), then had their songs recorded again before receiving bilateral cochleae removal 14 days later. They were also recorded continuously for the month after deafening. Controls received no surgery but were recorded in a sound proof recording chamber for a full month starting at 130 days of age to mimic the housing conditions of the other groups. All 28 birds were removed from the recording chambers at the end of the month and housed in single cages on racks in the same room for the remainder of the experiment. At two later time points, posthatch day 220 and posthatch day 340, they were returned to recording chambers for a few days to get song recordings. After the final recording session, all 28 birds received bilateral injections of green CTB to RA to label HVC-RA neurons and were then sacrificed 6 days later. One month prior to sacrifice, at posthatch day 310, the birds were given 3 days of BrdU injections to birthdate label new neurons that were then

counted in HVC one month later. The experimental timeline is illustrated in Figure 5.1.

Bilateral cochleae removal

Birds were food and water deprived for 1 hour prior to being injected with the anesthetic Nembutol (pentobarbital sodium, 60 mg/kg; Ovation Pharmaceuticals, Deerfield, IL) into the breast muscle at a dose of 5.7 microlitres per gram of body weight. Once under the anesthetic, birds were placed in a specially-built stereotaxic device for surgery and feathers were plucked from the back of the head. The scalp was dissected along the midline and the muscle at the back of the skull moved aside with skin hooks. A small window was made in the skull overlying the semicircular canal and then a small hole was made in the cochlea dome. A fine hook made from a tungsten wire was lowered through the hole and used to hook and pull out the cochlea. The same procedure was then performed on the other hemisphere. Removal of both cochleae was confirmed by examination of the intact organs under the dissecting microscope. To close, the muscles were pulled back into place, the scalp was closed with cyanoacrylate and an antibiotic (Neosporin ointment) was applied to the skin. Birds were kept warm under a heat lamp (33°C) until they had recovered enough to perch. They were examined for normal balance and posture before being returned to the recording chamber.

Electrolytic lesions of nucleus lMAN

Birds were food and water deprived for 1 hour prior to being injected with the anesthetic Nembutol (pentobarbital sodium, 60 mg/kg; Ovation Pharmaceuticals, Deerfield, IL) into the breast muscle at a dose of 5.7 microlitres per gram of body weight. Once under the anesthetic, birds were placed in a specially-built stereotaxic device for surgery and feathers were plucked from the back of the head. The scalp was dissected along the midline and nucleus lMAN was located in both hemispheres using stereotactic co-ordinates. A small window was made in the skull above each and a small slit was made in the dura with a 30 guage hypodermic needle to facilitate entry of the electrode (an insect pin coated in insulex except at the tip). The electrode was lowered into the brain to the required depth and connected to a circuit with a 45V battery. The circuit was completed by attaching a clip to the bird's foot claw and ensuring a good connection with saline. A continuous current of 50 microAmps was passed into the brain for 90 seconds. The electrode was then retracted and re-inserted at different co-ordinates to make a second lesion; two lesion sites were used to ensure the entire lMAN nucleus was removed. The electrode was then retraced and the same procedure was repeated in the other hemisphere. After the lesions had been made, the windows of skull were replaced and the scalp was closed with cyanoacrylate and an antibiotic (Neosporin) was applied to the skin.

Staining, image collection and neuron counts

Every third section through HVC was stained for BrdU and Hu for identification of BrdU-positive neurons. Three-color Z-stacks of HVC were collected on a confocal microscope that contained the green CTB signal, the red BrdU staining and the far-red Hu staining. These stacks were used to count (1) the number of HVC-RA neurons retrogradely-lableled with CTB from RA; (2) the number of BrdU/Hu double-labeled neurons; (3) the number of triple-labeled BrdU/Hu/CTB new HVC-RA neurons; and (4) the number of BrdU-positive ventricular zone cells lining the ventricle above HVC. These slides were also imaged at 10x to measure HVC volume. The HVC-RA neurons were counted using the optical fractionator method and number of both BrdU/Hu neurons and BrdU/Hu/CTB neurons were counted using the rare-event modification of the optical fractionator method (see general methods above). For all counts and volume measurements, both left and right hemispheres were analyzed and then combined to give an average value for each bird.

Quantifying lMAN lesions

Every other section through lMAN was stained with 0.9% cresyl violet, dehydrated through ascending concentrations of ethanol, delipidized in xylene and coverslipped using Krystalon mounting media (EMD Chemicals, USA). Stained sections containing lMAN were imaged using brightfield microscopy at 20x magnification. For the Control and Deaf birds, lMAN was identified by its characteristic large neurons and the boundaries were traced using NeuroLucida software (MBF Biosciences,

Williston, VT). The area of lMAN in every section was measured and lMAN volume was estimated by summing the areas and multiplying by 40 μm (the sampling interval multiplied by the section thickness). For the L+D birds, every section spanning the normal location of lMAN was examined for the characteristic large neurons. For most birds, part of the original lesion site was still visible as a glial scar. If any part of lMAN was still remaining, the volume of the remaining section was measured in the same was as the intact lMAN was measured in the Deaf and Control groups. The volume of lMAN remaining, if any, was averaged between the left and right hemispheres and expressed as a percentage of the average lMAN volume measured in the 8 Control birds. Two of the L+D birds had greater than 60% (on average across left and right sides) of lMAN remaining and so were excluded from further analysis, leaving a total number of 8 birds in the L+D group.

Song analysis

Songs were recorded using the live recording module of Sound Analysis Pro software (Tchernichovski et al. 2000). Analyses were performed in 3 parts: firstly, songs produced by birds in the Deaf group, before deafening, and at several time points after deafening, were analysed using Sound Analysis Pro to determine the progressive changes that occurred as a result of cochleae removal; secondly, songs produced by all 3 groups were visually scored by observers blind to the experimental design to look for group effects of the different surgical procedures on song structure; thirdly, the motif stereotypy of the Control group was determined at

posthatch day 130 and day 340 to get an indication of whether the song had got more stereotyped over the course of the experiment.

Analysis of the Deaf birds:

To determine how the songs of the Deaf birds had changed over the 7 month period, Sound Analysis Pro software was used to compare songs produced at several points after deafening to the pre-surgery motif. First, a canonical pre-surgery motif was identified for each bird by examining several pre-surgery song files and finding a motif that represented the most consistently produced syllable order and syllable quality. To get a baseline score for how much variability was present in the pre-surgery song, 25 motifs produced on posthatch day 130 were compared to the selected canonical motif using the 'asymmetric' and 'timecourses' settings in the Sound Analysis Pro similarity scoring module. The software breaks down the motifs into their component syllables and compares each syllable using a range of acoustic features including pitch, frequency, duration, pitch modulation and entropy. Each syllable in the canonical motif is matched to the most similar syllable in the motif under comparison and the syllable pair is scored on how much of their length can be matched and how accurate the match is. Each syllable score is averaged to give a final composite 'similarity score' which also takes into account the order in which the matched syllables appear in two motifs. At each time point after deafening, 25 motifs were compared to the canonical pre-surgery motif and an average of the 25 similarity scores was taken. When the song was too degraded to identify a motif, a string of vocalizations that was preceded by introductory notes and was similar in

length to the original motif was selected for comparison. Finally, the average similarity score for each time point was divided by the baseline similarity score generated from the 25 pre-surgery motifs to produced a final 'normalized similarity score'. This last step allowed the scored from the 10 deaf birds to be directly (See Figure 5.4).

Visual scoring:

Although the songs produced by the L+D birds in the final recording session on day 340 showed by eye that the pre-surgery motifs were mainly intact, they all showed dramatic increases in tempo resulting in a shortening of the motif length. As a result, Sound Analysis Pro did a bad job of matching pre- and post-surgery syllables. To remedy this, we used 2 observers to visually score syllables from all 3 groups of birds. A similar approach has been used by the other groups looking at the effects of lMAN lesions and deafening (Brainard & Doupe 2000; Brainard and Doupe 2001; Scott et al. 2007), probably because they had similar problems with automated sound analysis in cases where the song tempo had increased. Two observers, who were blind to the experimental design but familiar with looking at zebra finch sonograms, were presented with panels of syllables that had been cropped out of a single song file. For each bird, a set of all the pre-surgery syllables from day 130 were presented along with a set of all the syllables types sung on day 340. For the Deaf birds, there was so much variability in their vocalizations what it was not possible to represent every syllable type produced on day 340. A sample of syllables was selected that covered all the main types of sound produced on that day. The

observers were asked to match each pre-surgery syllable to a syllable produced on day 340 and score it on a scale of 0-3 based on how well matched the two syllables were. A score of 0 meant there was no matching syllable present and a score of 3 meant there was a well matched syllable amongst the syllables sung on day 340. Once a syllable from day 340 had been matched, it could not be matched to another pre-surgery syllable so that deletions of the original syllables would be reflected in the final score. The mean score for all syllables in the pre-surgery motif was then calculated for each bird. In addition, the observers were given 2 other sets of comparisons. The first set comprised of pairs of syllable sets produced by the same bird on day 130 but taken from different song files. This comparison was designed to find the maximum score possible from the 2 observers using this rating method. The second set comprised of pairs of syllable sets produced by completely unrelated birds on day 130 and was designed to find the baseline score produced when unrelated songs were compared. This second set was important because there is only a limited set of sound types present in zebra finch song so even unrelated songs will not produce a score of 0. The scores offered by the 2 observers were well correlated (Pearson correlation = 0.91) so they were averaged for each bird.

Control song stereotypy:

To get an estimate of how stereotyped the songs of the Control birds were at the beginning and end of the experiment, we assessed how consistently the motif was produced in a given song bout, for songs produced on posthatch day 130 and posthatch day 340. For each day, 5 song files were chosen at random. For each

song file, the motifs were isolated and each motif was compared to every other motif in the bout using Sound Analysis Pro similarity scoring (as described above). This generated a total of 60-100 motif comparisons per time point per bird, depending on the number of motifs contained in the original song files. The similarity scores for each comparison were averaged to produce a 'mean motif similarity score'. To determine how much the stereotypy of the song motif had improved over the 7 months, the mean motif similarity score from day 130 was subtracted from the score from day 340 to produce a 'shift in similarity score'. This shift was then correlated to the number of HVC-RA neurons present at posthatch day 340 and the number of BrdU-positive neurons present in HVC 1 month after BrdU injection.

Statistics

All measures from Deaf, L+D and Control groups were compared using a one-way ANOVA followed by the Tukey HSD posthoc analysis. The mean motif similarity scores from Control songs on day 130 and day 340 were compared using a paired-samples t-test. To correlate HVC-RA neuron density with song features, standard linear regression was used to generate R-squared and p values.

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