Singing in the Brain:
Investigating the Role of Adult Neurogenesis in Long-Term Memory Preservation

by

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Abstract

Neurogenesis refers to the process of adding new neurons in the brain. It is typically a perinatal phenomenon. However, it can occur in very specific regions of the adult vertebrate brain, usually related to memory. The role of neurogenesis in memory is not yet clear. Some evidence suggests that neurogenesis facilitates new learning, but there is also a case for neurogenesis promoting long-term memory (Alvarez-Buylla and Kirn, 1997, Pytte et al., 2012).

The zebra finch brain contains a set of discrete regions specialized for different aspects of song learning, production, and memory. Several of these regions receive new neurons throughout life. This makes the zebra finch an excellent animal model for studying memory in vertebrates. Juveniles learn their songs from adult song tutors. The region of interest in this study is the caudomedial nidopallium (NCM), which is thought to play a dominant role in song perception, especially in memory of the tutor's song. Preliminary studies have used immediate early gene (IEG) expression and electrophysiological techniques to show that NCM preferentially responds to the tutor's song (Bolhuis et al., 2000, Phan et al., 2006). Little is known regarding the function of adult-born neurons in NCM. Could they be involved in this maintenance of the tutor song memory, a song that pre-dates them?

In order to address this question, I measured the responsiveness of new neurons to playbacks of tutor song, compared with the responsiveness of the general neuronal population. My research investigated if and how the response properties of new neurons differ from those of the general population when the bird hears its tutor's song for the first time since early life. Neuronal response properties were assessed by way

of a triple-labeling protocol that included immediate early gene (IEG) expression to identify cells that were active during song playback, as well as a neuronal marker and a birth marker to identify newly added cells. My research aimed to clarify the differences between neurons added at different points within the zebra finch's development and, more generally, provide a better understanding of the role of neurogenesis and whether it is involved in the formation of new memories, the preservation of old ones, or perhaps both. Preliminary results confirm that newly added neurons can indeed express immediate early genes. However, these new neurons appear to respond to the tutor song in a manner opposing that of the general neuronal population.

Introduction

MEMORY

The Question of Memory

The question of memory in vertebrates is among those most pressing in the field of neuroscience. Our memories inform our every move in the world, ensuring that we correctly apply knowledge learned from past experiences. Whether our actions are driven by the daily need to survive or by more intricate and long-term motives, memory is critical for their successful completion.

Memory Disorders and Mental Illness

Memory's vital role in the human experience becomes clearer in cases of disordered memory, which can take any number of forms. For example, patients with post-traumatic stress disorder (PTSD) are plagued by inappropriate and involuntary recurrences of memories of events that are "atypical in their negativity and emotional intensity" (Baddeley, Eysenck, and Anderson 2015, p. 317) . Persistent anxiety begets these intrusive memories, which in turn exacerbate the anxiety experienced by the patient. In such cases, what might be described on the most basic level as an overactive memory interferes with daily life. By stark contrast, patients who have suffered from traumatic brain injury might experience anterograde amnesia, meaning that their mechanisms to encode, store, or retrieve new memories are impaired. They must navigate daily life equipped only with memories that precede the injury. Others develop retrograde amnesia, a condition in which they struggle to retrieve their old memories (Baddeley, Eysenck, and Anderson 2015, p. 438). The exceptional case of

Clive Wearing, a man with both types of amnesia, is demonstrative of the extent to which memory shapes the human experience. Without the ability to remember the recent or the distant past, Mr. Wearing "was totally incapacitated…he could not read a book or follow a television program because he immediately forgot what had gone before. If he left his hospital room, he was immediately lost" (Baddeley, Eysenck, and Anderson 2015, p. 4).

Where to Begin?

Clearly, the effects of memory disorders are far-reaching and sometimes devastating, making the goal of better understanding memory an urgent one. Although memory touches every corner of human life, and all life for that matter, relatively little is known about its neurobiological basis compared to other cognitive processes. A weighty question addressed by this study is that of the role of neurogenesis in memory. If neurons in the memory centers of the brain are in a state of relentless turnover, how is it that we preserve and protect our precious memories over long periods of time?

ADULT NEUROGENESIS

Really?

Since the inception of the study of neuroscience, the scientific community held that neurogenesis, the creation of new neurons, was an exclusively perinatal phenomenon. The story went that each person was born with all of the neurons he or she would ever need (and it was critical to protect every single one!). This doctrine was so deeply entrenched in scientists' understanding of the brain that the original

discovery of adult neurogenesis was largely disregarded. This preliminary work, conducted by Joseph Altman in the 1960s, suggested the existence of adult-born neurons in the rodent hippocampus (Reviewed by Balthazart and Ball, 2015).

It was not, however, until Fernando Nottebohm and Steve Goldman's work in songbirds that the notion of adult-born neurons could be seriously considered. Nottebohm and his colleagues noticed that male canaries appear to undergo seasonal volume changes in HVC, RA, and X, all discrete brain regions heavily implicated in song control (Nottebohm, 1981, Grandel and Brand, 2013). Their findings seemed fitting given the knowledge that singing quality and quantity both change seasonally as well; they had identified a relationship between neural and behavioral changes (Reviewed by Balthazart and Ball, 2015). Further research using tritiated thymidine (a cell birth date marker, see *Identifying New Neurons* section below) and structural analysis established the existence of newly formed neurons in the adult canary brain. Hypothesizing that gonadal steroids may augment adult neurogenesis, Nottebohm and Goldman administered testosterone to adult female canaries and assessed resulting changes in HVC. Even control birds, who had received implantations of cholesterol, showed evidence of adult neurogenesis; testosterone only increased the number of new neurons added (Goldman and Nottebohm, 1983). Skepticism persisted in spite of Nottebohm's findings, relenting only when a subsequent study combined electrophysiology and thymidine labeling to prove that the new neurons were in fact producing action potentials in response to auditory stimuli (Paton and Nottebohm, 1984, Balthazart and Ball, 2016).

The Process of Neurogenesis

Perinatal neurogenesis takes place in the cerebellar external granule layer and the subventricular zone (SVZ) along the lateral ventricles (Reviewed by Alvarez-Buylla and Garcia-Verdugo, 2002). Contrastingly, adult neurogenesis is confined to the SVZ and the dentate gyrus (Reviewed by Braun and Jessberger, 2014). New neurons are formed in adulthood by way of adult neural stem/progenitor cells (NSPCs). NSPCs are activated to proliferate into and produce transit-amplifying cells, which in turn differentiate into the desired neuronal type, migrate, and integrate themselves into the target brain region (Reviewed by Braun and Jessberger, 2014). The signal which triggers the initial proliferation stage may take the form of neurotransmitter release, gene expression, or the presence of trophic factors. The newly formed neurons (type A cells) form chains walled by astrocytes (type B cells) that provide tunnels in order to migrate. Type C cells, those most likely to be labeled by cell birth date markers, group together alongside these chains (Reviewed by Alvarez-Buylla and Garcia-Verdugo, 2002). In rodents, this process occurs at a speed of approximately one hundred twenty microns per hour. The dynamics of axon guidance, synaptogenesis, and synaptic refinement occur after the cell has migrated to the desired location.

Identifying New Neurons

Over the past forty years, methods for the measurement of adult neurogenesis have been well established. Tritiated thymidine has long been considered the "gold standard" of cell birth date markers. The nature of autoradiography allows for the calculation of a precise threshold for number of silver grains denoting a newly formed

cell. Unfortunately, this procedure runs the risk of obfuscating fluorescent neuronal markers (Asik et al., 2014). Fortunately, when injected subcutaneously, non-radioactive thymidine analogs such as bromodeoxyuridine (BrdU) function well as cell birth date markers. BrdU is a marker of DNA synthesis that is incorporated into newly forming cells during the S phase (Reviewed by Taupin, 2007). It can be combined with fluorescent secondary antibodies and used in concert with other cell markers like Hu, which labels all neurons, and ZENK, which labels recently active cells (see *Discovering NCM* section below*)* (Bolhuis et al., 2000, Walton et al., 2012).

New Neurons in Memory

Since its relatively recent discovery, adult neurogenesis has been observed in the human hippocampus, the rodent hippocampus and olfactory bulb, and the avian song system – all regions long known to be critical for memory. The role of adult neurogenesis in memory, however, is yet unresolved. Some evidence indicates that adult neurogenesis aids in the formation of new memories. One study posited that in canaries, the birth of projection neurons in adulthood might be associated with annual renewal of the song repertoire (Kirn et al., 1991). This relationship seems intuitive – newly formed neurons could serve as raw material for the construction of new circuits, and by consequence, new memories. The "neurogenic hypothesis" paints a descriptive view of the process by which this might happen in humans – one group of authors describes how "the hippocampus is, bit by bit, remodeled, with its pattern of synaptic connections gradually modified with each generation of interloping neurons" (Baddeley, Eysenck, and Anderson 2016, p. 398). The persuasive nature of this

explanation makes it that much more difficult to interpret a related study, whose results suggested that adult neurogenesis in a specific part of the zebra finch brain aids in the preservation of long-term memory (Pytte et al., 2012). This more recent idea does not necessarily nullify prior scholarship, but it certainly indicates that the story of adult neurogenesis and memory is more complicated than one might expect.

RESEARCH IN SONGBIDS

History of Songbird Research

The songbird has an extensive history in developmental biology and neuroscience research. Song learning has often been related to the process of learning language in humans; juvenile songbirds and human babies both learn by imitating adult vocalizations, and both have sensitive periods during which vocal learning is optimized (Reviewed by Moorman et al., 2011). Birdsong is at once a rigidly stereotyped and a highly individualized behavior; it is learned from a "tutor," usually the young bird's father, but a father-son pair might produce two rather different songs depending on the species and circumstances under which the bird is raised. Studying birdsong thus has the potential to help scientists understand the "enigmatical interaction between the genetical basis of species-specific behavior and the adaptively modifying influence of individual experience" (Hinde 1969, p. xi). Chaffinches were the first birds to be considered in this capacity, one of the first observations being that their songs are highly variable until the first breeding period, after which they rarely change (Hinde 1969, p. 21). This finding was important because it was the first indication that song is learned and variable rather than inherited and stable. It also hinted at the existence

of a critical period in song learning. Later research established that when given a choice between conspecific tutors and tutors of other species, white-crowned sparrows tended to opt for the conspecific tutor. Furthermore, if they were given a choice of two heterospecific tutors, the subjects would reject both of them in favor of producing songs resembling those of white-crowned sparrows raised in isolation (Hinde 1969, p. 34). These results indicate that the species possesses an innate "template" for what song should sound like. Although neither the results from chaffinches nor those from white-crowned sparrows are universal truths in the world of songbirds, they provided preliminary insights into a model system for understanding how the brain controls behavior in the case of vocal learning. The body of research on the songbird brain has grown prolifically over the last fifty years. Much of this research involves the zebra finch (*Taeniopygia guttata*).

Why Sing?

Song is a primary means of intraspecific communication for birds. Like humans, birds have poor olfactory acuity, and thus rely on audition and vision to gather information about the environment (Reviewed by Catchpole and Slater, 2008: 6). Although vision is certainly important to birds, their line of sight is often obstructed by plants and other objects. Therefore, vocalizations are the most effective way for birds to communicate with one another over long distances. Furthermore, sounds do not appear to be energetically costly for birds to produce (Reviewed by Catchpole and Slater, 2008: 7). All birds communicate vocally, but singing is a more exclusive phenomenon. Of all bird species, only a subgroup known as the oscines are equipped

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with the appropriate vocal musculature and neural organization to produce true songs (Reviewed by Bolhuis and Eda-Fujiwara, 2003). Songs are distinct from calls in that they are longer and more complex, they must be learned, and they are produced only by males during a breeding period (Reviewed by Catchpole and Slater, 2008: 8).

Male zebra finches sing two kinds of song; directed, and undirected. Directed song is that which occurs in the presence of a female. Contrastingly, males produce undirected song in the absence of a female. Directed song has a clear evolutionary purpose, which is to attract a mate (Hinde 1969, p. 61). It is likely that complex songs are a sign of evolutionary fitness – repertoire size has been shown to have a positive correlation with reproductive success (Reviewed by Catchpole and Slater, 2008: 237). The function of undirected song is not yet known, but some evidence points to its involvement in strengthening group cohesion (Hinde 1969, p. 61). A more recent idea holds that undirected singing is a way to rehearse for occasions of directed song (Kojima and Doupe, 2011). The two forms of song are essentially identical, except that courtship songs may be performed more rapidly (Hinde 1969, p. 63). Scholars have also theorized that songbirds use song to defend their territories from competing males; this, along with attracting a mate, are generally taken to be the two most important motivations for singing (Reviewed by Catchpole and Slater, 2008: 235-236).

Song Structure

Birdsong is delivered in "bouts," usually repeated several times in one singing session. Each bout is made up of "phrases"; each phrase contains a specific arrangement of "syllables." Each syllable may be broken down into "elements," the

smallest possible unit of birdsong (Reviewed by Catchpole and Slater, 2008: 9). For analysis, these songs are commonly represented as sonograms, images in which sound frequency is plotted as a function of time (Figure 1).

The age at which the bird is separated from his tutor is one variable that can affect the extent of tutor copying. Depending on separation date, the bird's song may closely resemble his tutor's song, or sound quite different from it. "Isolate" birds, those that are raised in total absence of a tutor, produce radically aberrant songs (Reviewed by Doupe and Kuhl, 1999). By contrast, birds that are separated from their tutors between thirty-eight and sixty-six days post-hatch, before song has crystallized, produce songs that contain mostly elements of the tutor's song, but in novel arrangements. Finally, if birds are separated from their tutors after eighty days post-hatch, they will often produce songs identical to those of the tutors (Hinde 1969, p. 65-66). However, it must be noted that in the wild, a zebra finch becomes independent from his tutor around thirty-five days post-hatch, rendering any tutoring paradigms longer than this biologically irrelevant (Reviewed by Catchpole and Slater, 2008: 67). Regardless of relevance, however, the zebra finch's ability to copy so well provides clear evidence that song is learned and is an incredibly useful tool for studying the neural bases of learning and memory.
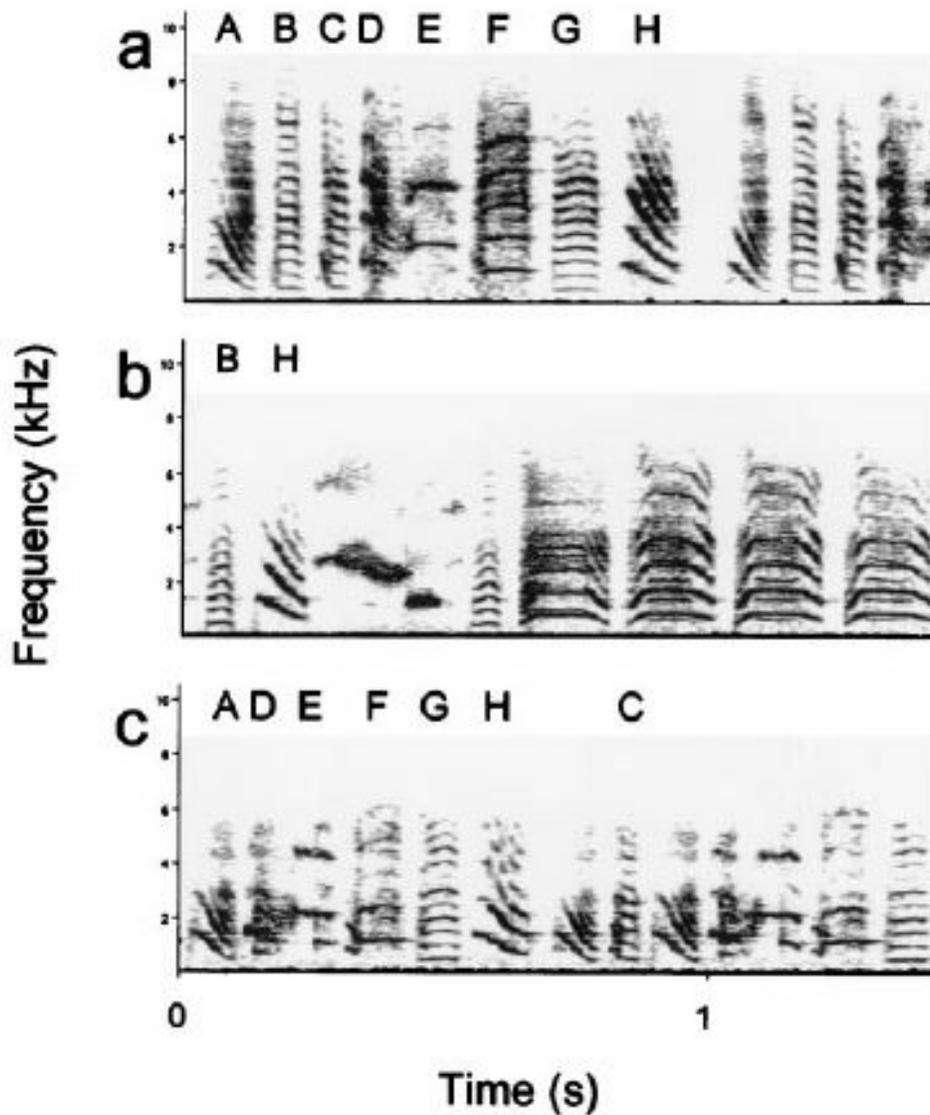
Figure 1. Sonograms from two experimental birds and a tutor. Sonogram (a) represents the tutor song. Sonogram (b) represents the song of a bird who shared only 2 syllables, B and H, with the tutor. This bird learned from a different tutor. Sonogram (c) represents the song of a bird who shared several syllables (A, C, D, E, F, G, and H) with the tutor. This bird learned from this tutor (Bolhuis et al., 2000).

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Song Learning

A mature bird's song will usually resemble, but not exactly copy, his tutor's song. The process of learning song is complex and has been the subject of much research. It has been related to the process of learning language in humans. Song learning is considered to occur in two phases; the auditory phase, and the sensorimotor phase. In the former phase, the bird hears its tutor's song and stores information regarding its arrangement and auditory properties. In the latter, the bird produces its own song and compares this to the stored information (Reviewed by Bolhuis and Eda-Fujiwara, 2003). Within the sensorimotor phase, the bird's song passes through multiple stages. The first is sub-song, which is analogous to babbling in human infants in that it will not closely resemble the fully developed song (Reviewed by Moorman et al., 2011). The sub-song then develops into plastic song, which better approximates a fully developed song but is still subject to many changes. Finally, the song crystallizes into a set and sequence of highly predictable notes (Reviewed by Kirn, 2010). Some songbirds, known as "open-ended learners," change their songs many times throughout life, usually for each breeding season. By contrast, birds like zebra finches are "age-limited learners," meaning that they no longer modify their songs once they reach around ninety days post-hatch (Bolhuis and Eda-Fujiwara, 2003, Kirn, 2010). Paradoxically, however, some studies have shown that under the right circumstances, an adult zebra finch will radically change his song. Deafening is known to elicit this effect. A seminal study demonstrated that birds deafened in adulthood still sang, but were unable to replicate more than thirty-six percent of their original crystallized songs (Nordeen and Nordeen, 1992). Another, later study found that blocking song-related

feedback with white noise prompted birds to modify their crystallized songs in order to avoid further disruption in auditory feedback (Tumer and Brainard, 2007). It seems therefore that evidence favors a corrective model of song crystallization in which birds can change their songs if deemed necessary based on auditory feedback. Furthermore, a large body of evidence suggests that zebra finches experience adult neurogenesis, traditionally associated with plasticity, in discrete brain regions known collectively as the "song control system" (Reviewed by Kirn, 2010).

The Traditional Song System

The so-called "song control system" in songbirds is understood to comprise the neural substrates of song learning and song production. It is generally subdivided into the caudal and rostral pathways, which deal most with production and learning respectively (Figure 2). The caudal pathway is sometimes called the motor pathway and consists of HVC (used as a proper name) and the robust nucleus of the arcopallium (RA). A formative study for our current understanding of the song control system found that lesions to the canary HVC caused severe song deficits (Nottebohm et al., 1976). Experimental birds exhibited "silent singing," meaning that they failed to produce actual song despite assuming a singing posture. The researchers then traced degenerating axons from lesioned HVC to two nuclei, RA and Area X. Although lesions to Area X did not affect adult stereotyped song, lesions to RA produced dramatic song deficits. Finally, degenerating axons were traced from RA, and some were found to innervate respiratory motor neurons, while others were found to innervate motor neurons that in turn synapse onto the syrinx, the muscle necessary for
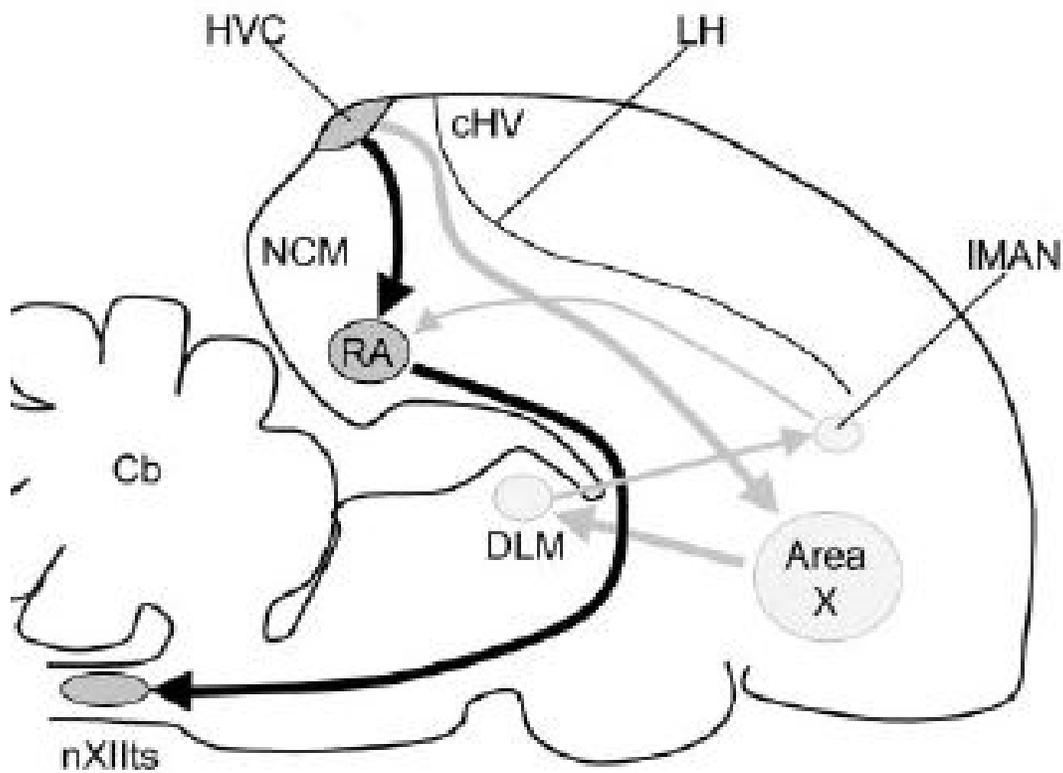
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Figure 2. A schematic representation of a parasagittal view of a songbird brain. Approximate positions of components of the song control system are labels. Black arrows correspond to the caudal or motor pathway, including HVC (proper name) and RA (robust nucleus of the arcopallium). Grey arrows correspond to the rostral or anterior forebrain pathway, including HVC, Area X, DLM (dorsal lateral nucleus of the medial thalamus), and LMAN (lateral magnocellular nucleus of the anterior nidopallium. NCM is also labeled (Bolhuis et al., 2000).

song production. Electrophysiological recordings later revealed that HVC neurons respond to playbacks of song, that auditory activity is suppressed in HVC while the bird itself is singing, and that neuronal activity in HVC precedes that in RA (Reviewed by Margoliash, 1997). One later study demonstrated that over fifty percent of neurons in the canary HVC project to RA, although the number varies depending on the time of year (Kirn et al., 1991). These results suggest a hierarchical structure in which HVC influences activity in RA, which then dictates motor neuron activity related to song production.

The rostral pathway, also called the anterior forebrain pathway, includes the remainder of HVC projection neurons which innervate striatal Area X. Area X neurons project to the thalamus, which in turn projects to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Reviewed by Kirn, 2010). To better understand the role of the rostral pathway in song control, a group of researchers lesioned LMAN in one set of zebra finches and Area X in another and compared the resulting songs to those of deafened birds to determine the roles of these two nuclei in song learning (Scharff and Nottebohm, 1991). They found that early lesions to LMAN produced repetitive songs that stabilized rapidly. By contrast, lesions in Area X yielded songs somewhat resembling those of deafened birds; they rambled, and lacked organization and stability. From these results, the researchers concluded that LMAN is necessary for plastic learning. Indeed, lesions within the rostral pathway rarely affect crystallized song in adult birds, but wreak havoc on song learning in juveniles (Bottjer et al., 1984, Kirn, 2010). Parallels have since been drawn between the rostral pathway and the mammalian basal ganglia-cortico-thalamic circuit (Reviewed by Bottjer, 2004).

THE CAUDOMEDIAL NIDOPALLIUM

Discovering NCM

Neurons in the song system nuclei have specific response preferences regarding conspecific song, tutor song, and the bird's own song (BOS) (Reviewed by Bolhuis and Eda-Fujiwara, 2003). Electrophysiological studies have established that neurons in LMAN, Area X, RA, and HVC respond more to conspecific song than to heterospecific song. Furthermore, these regions respond more to BOS than to the tutor's song. These electrophysiological results first hinted at the existence of a neural representation of song in the avian brain. Bolstering this claim, HVC neurons in zebra finches have been shown to be selective for specific features of BOS. Some neurons required near-exact copies of syllables from BOS in order to fire above baseline, while others required two syllables, and still others required two harmonics to do the same (Margoliash and Fortune, 1992). Studies have shown, however, that neurons in the song control system are more active when the bird sings, but not when it hears song playbacks, including tutor song (Reviewed by Bolhuis and Eda-Fujiwara, 2003). These data seem to indicate that although the song system nuclei play obvious roles in song learning and song production, they do not necessarily deal with auditory processing, nor do they house memories of the tutor song. So where might such memories be stored? Researchers have sought the answer to this question by measuring immediate early gene, or IEG, expression. IEG expression is a measure of neuronal activity. Immediate early genes encode transcriptional regulators, and the degree of their expression can be determined by measuring the amount of mRNA or protein in a specific brain area (Reviewed by Catchpole and Slater, 2008). The first study to use this technique, published in 1987,

found that stimulation of sensory neurons in the rat spinal cord causes expression of c-fos protein immunoreactivity (Hunt et al., 1987, Sagar et al., 1988). A number of studies in mice established the gene zif-268/egr-1/NFGI-A/Krox-24, known better by its acronym ZENK, as a reliable marker of neuronal activity (Christy et al., 1988, Lemaire et al., 1988).

One group of researchers applied these findings in the songbird brain (Mello et al., 1992). They first established that ZENK expression in the forebrain increases rapidly upon exposure to conspecific song in both zebra finches and canaries. Expression was shown to be most robust in two previously overlooked regions, the caudomedial nidopallium (NCM) and the hyperstriatum ventrale (HV). By contrast, the song production nuclei including HVC, RA, and Area X demonstrated negligible ZENK induction in response to song playbacks. Remarkably, tape recorded song was perfectly sufficient to evoke these results. In a subsequent paper the researchers solidified their results using in-situ hybridization and posited that areas expressing ZENK in response to playback like NCM and HV could be sites of song processing and memory, as opposed to the traditional "song control nuclei" which are responsible for song production (Mello and Clayton, 1994). Intriguingly, further research revealed that differences arose in ZENK expression between birds who were exposed to song and sang in response, and those who did not sing. Birds who were permitted to sing in response to song exposure expressed significantly greater levels of ZENK in HVC, RA, Area X, NCM, and HV compared to control birds who received no stimulus at all. By contrast, birds who were exposed to song but were not permitted to sing in response experienced greater ZENK expression only in NCM and HV (Mello and Ribeiro,

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1998). It appears, therefore, that NCM and HV are involved in listening to, and presumably processing, biologically relevant sounds.

A contemporaneous study not only confirmed the above results but also elucidated the developmental regulation of ZENK expression in NCM. These researchers observed that ZENK expression in NCM could not be induced at 20 days post hatch, but could be induced at 30 days, by song exposure (Jin and Clayton, 1997). This result indicates that a developmental shift must occur in early life in order for a bird to demonstrate the appropriate responsivity to song.

Evidence suggests that a neural response to birdsong must be learned from the tutor. For example, zebra finches tutored by canary foster fathers experience the most ZENK induction in response to canary song playbacks rather than zebra finch playbacks (Jarvis, 2004, Catchpole and Slater, 2008).

Current Understanding

Research suggests that NCM is primarily involved in song processing and perhaps memory. IEG research has shown that NCM neurons habituate to playback of the same song over time, possibly indicating that the bird has memorized that song (Mello et al., 1995). The habituation effect has been shown to be contingent upon protein synthesis in NCM. Injecting inhibitors of protein synthesis immediately after stimulus presentation did not interfere with habituation, but injecting thirty to sixty minutes after exposure prevented habituation from occurring at all (Chew et al., 1995). Subsequent research probed the habituation effect by comparing IEG expression to electrophysiological results. One electrophysiological study revealed that habituation

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occurs rapidly for conspecific vocalizations, as well as heterospecific vocalizations and white noise, in accordance with IEG data (Chew et al., 1996). Later research confirmed these results but with an important caveat – unlike ZENK expression which decreases after every additional presentation of song, neuronal firing does not decrease again after the second presentation (Stripling et al., 1997). Some have drawn parallels between habituation in the avian NCM and long-term potentiation in the rodent hippocampus (Reviewed by Moorman et al., 2011).

NCM appears to be especially concerned with memorization of the tutor's song (Reviewed by Moorman et al., 2011). Studies in both juveniles learning song and adults with crystallized song have shown that IEG expression in NCM is positively correlated with number of song elements copied from the tutor, a measure of success regarding song learning (Bolhuis et al., 2000, Gobes et al., 2010). Furthermore, tutor song learning is extremely poor when the extracellular signal-regulated kinase (ERK) signaling pathway is reversibly suppressed in the caudal pallium, of which NCM is a part, during the critical period (London and Clayton, 2008). ERK is known to regulate ZENK expression, leading the researchers to infer that tutor song learning occurs not in the traditional song control system, but in NCM, and is dependent upon ZENK expression.

Immediate early gene mRNA levels in NCM peak around thirty minutes after stimulus onset (song playback, for example), while resulting protein changes might linger for a few hours. The general mechanism for IEG induction is as follows (Reviewed by Moorman et al., 2011). The presynaptic neuron releases glutamate into the synaptic cleft. The postsynaptic neuron's receptors bind glutamate, triggering the

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MAPK signaling cascade. This cascade results in phosphorylation of the ERK/MAPK complex, which then travels to the nucleus to promote IEG expression. At this point, IEG proteins either upregulate or downregulate the transcription of genes that encode late effector proteins. Some of these late effector proteins are transported to the dendrites, while others like synapsins travel to the axon via microtubules. Synapsins anchor vesicles to actin filaments in the axon and the postsynaptic density, but when they are phosphorylated by molecules such as ERK, they release their grip so that the vesicles may fuse with the membrane and exocytose. Synapsins could be the key to understanding how memories of song are formed in NCM (Reviewed by Moorman et al., 2011). Song presentation evokes greater expression of two types of synapsins in NCM. However, IEGs are known to directly inhibit synapsin expression (Velho and Mello, 2008). Clearly, more research is necessary to explicate the important role of synapsins, as well as other proteins, in songbird learning.

IEG expression in NCM is not constrained to ZENK; another gene, activity-regulated cytoskeleton-associated gene (*arc*) expression increases in the dendrites of NCM neurons after song presentation (Reviewed by Moorman et al., 2011). *Arc* codes for Arc protein, which works together with other signaling molecules to affect postsynaptic neuronal activity. For example, *Arc* is required for the phosphorylation and consequential inactivation of cofilin, a molecule that typically inhibits dendritic growth. By inactivating cofilin, *Arc* releases the inhibition and allows for spine head enlargement (Reviewed by Moorman et al., 2011). The reviewers of this research propose that when *Arc* is induced by song playback, it acts selectively on the dendritic branches that were activated by playback. *Arc,* then, may contribute to the

development of "song-selective responses," and by extension, song memories (Reviewed by Moorman et al., 2011).

ZENK has been shown to be necessary for mammalian hippocampal long-term potentiation as well as certain kinds of hippocampal learning (Velho and Mello, 2008). Perhaps a mechanism similar to that of *arc* is at play with ZENK expression, such that its expression allows for the encoding of the tutor song memory (Reviewed by Moorman et al., 2011).

NEW NEURONS IN ADULT ZEBRA FINCHES

Neurogenesis

Neurogenesis occurs in discrete regions of the songbird brain throughout life. Although the rate of new neuron formation is fastest during the mid-embryonic stage, it is by no means confined to that developmental period. Indeed, the cells of the striatum (including Area X) and the nidopallium (including NCM) as well as HVC are formed postnatally for the most part (Alvarez-Buylla et al., 1988a).

As with mammalian neurogenesis described earlier, avian neurogenesis begins in the ventricular walls (Alvarez-Buylla and Nottebohm, 1988). Radial glia are known to be involved in the process of neurogenesis as well as the migration of new neurons (Ayala et al., 2007). A key 1988 study characterized the nature and behavior of these radial glia in the canary brain. Radial glia generally exist in the ventricular zone of the lateral ventricle, with fibers traveling as far as five millimeters from the lining of the ventricle (Alvarez-Buylla et al., 1988b). Radial glia were observed to contact distinct regions in the forebrain; the caudal neostriatum is mentioned as being one area that

receives many fibers, while, perhaps paradoxically, HVC receives few. Furthermore, the researchers showed that a new cell type with an elongated nucleus frequently accompanies radial glia fibers. These elongated nuclei appeared to orient in the same direction as the fibers, and were shown to occur almost exclusively within the telencephalon.

Neuronal Migration

In a subsequent study, the same researchers inferred that these novel cells were adult-born migrating neuroblasts (Alvarez-Buylla and Nottebohm, 1988). They first established that the cells were migrating away from the ventricular zone; one day after tritiated thymidine injection, these cells were close to the lateral ventricle. At this time point, fifty percent of these elongated cells were observed to be closely associated with, if not attached to, radial glial fibers. Six days after injection, the cells had increased in number and had moved away from the ventricular zone. Their association with radial glial fibers had decreased significantly. Presumably because of this decrease in association, concomitant with a decrease in availability of radial glia, migration rate dropped significantly from day six to day twenty.

Intriguingly, a later study used two-photon microscopy to examine this alleged radial migration to HVC *in vivo* and discovered a wholly novel form of neuronal migration to be at play (Scott et al., 2012). The researchers had predicted that this might be the case, considering that the extracellular matrix of the adult brain has considerably less space than that of the juvenile brain. Indeed, instead of adhering themselves to radial glial fibers like the bipolar cells first observed in the 1988 study,

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seventy percent of young neurons appeared to be multipolar and "wandered" to their final locations by extending numerous processes in different directions into the extracellular matrix. Once they reached their stopping points, they were observed to associate themselves with established neuronal circuits. The researchers proposed that the bipolar and multipolar neurons were actually of the same type but at different developmental stages, providing a model in which cells are first guided by radial glial fibers on their journey but eventually metamorphose into multipolar cells so that they can employ "wandering" migration for the last leg of the journey.

Synaptogenesis

Once a young neuron arrives at its destination, it must make contact with other cells in the vicinity. The process of synaptogenesis is not well characterized in the adult songbird brain, but some research has been published on the subject and its relationship to gonadal steroids. An early study confirmed that treating female canaries with testosterone results in singing behavior (usually restricted to males, much like zebra finches) as well as an increase in RA volume associated with increase in dendritic field size (Devoogd et al., 1985). It then built on these results, proving that the testosterone treatment and resulting changes are correlated with a fifty-one percent increase in number of synapses within RA. Furthermore, synapses in treated birds were sixteen percent larger in diameter than those in control birds, and had forty-five percent more synaptic vesicles. Although this does not provide much information with which to proceed, it suggests that synaptogenesis is regulated at least in part by testosterone in the central nervous system.

Moving Forward

Clearly, our knowledge of the process of neuron addition in the adult songbird brain is ever growing. An important question that has yet to be answered is the function of adult neurogenesis. Why does it occur at all? Two studies mentioned earlier require greater scrutiny at this point. Both explore the implications of adult neurogenesis in songbirds, but they propose seemingly antithetical purposes for the phenomenon. The first study, concerning canaries, used tritiated thymidine to label adult-born HVC neurons and a retrograde anatomical tracer called fluorogold to track the projections of these newly formed cells (Kirn et al., 1991). By two hundred forty days post-hatch, eighty percent of HVC neurons born in the early fall (a time at which vocal learning is extraordinarily high for adult canaries) projected to premotor nucleus RA. The researchers observed that the majority of these neurons lived for at least eight months, suggesting that they survive in the song control system long enough to contribute to the annual renewal of song repertoire (Kirn et al., 1991). This could be taken in a more general sense to mean that adult neurogenesis promotes plasticity and continual learning. As discussed before, this story substantiates the "neurogenic hypothesis." Nevertheless, another study published twenty-one years later challenged the universality of these original findings. In this study, researchers deafened adult zebra finches in order to manipulate their highly stereotyped songs (Pytte et al., 2012). They found an inverse relationship between number of new neurons added to HVC in adulthood and number of changes made by the bird to its own song as a result of deafening. Whereas the 1991 study tried to establish a role for adult neurogenesis in learning new information, this later study suggested the opposite – that adult

neurogenesis may preserve previously learned information. Instead of choosing to reject the 1991 findings, the 2012 study proposed that the function of adult neurogenesis might encourage or discourage behavioral plasticity depending upon the target nucleus (Pytte et al., 2012).

EXPERIMENTAL DESIGN

My thesis aimed to explore the extent to which adult neurogenesis encodes new information and contributes to the preservation of previously acquired information. I examined how new neurons responded to presentation of sounds memorized long before their formation, and compared this response to that of the general neuronal population in NCM. Earlier research has confirmed that tutor song playback evokes a robust ZENK response from the general neuronal population in NCM. Furthermore, there is an established correlation between ZENK expression in the zebra finch NCM and quality of song learning (Terpstra et al., 2004). My research used a similar paradigm, but built on it by examining the behavior of new neurons formed long after song tutoring in the same context. I measured ZENK expression in all NCM neurons in response to tutor song playback like Terpstra *et al,* but I also measured ZENK expression in new, adult-born NCM neurons, and compared the two response profiles (2004). NCM is known to receive neurons throughout life (Alvarez-Buylla et al., 1988a). However, the role played there by new adult-born neurons has yet to be determined.

I processed tissue for ZENK expression as a marker of activity, BrdU as a marker of new cells, and Hu as a neuronal marker. I expected to see some triple-labeled

cells; such cells would presumably be new, active neurons. More importantly, I predicted there would be a positive correlation between number of these triple-labeled cells and quality of song-learning resembling the positive correlation between ZENK expression in the general neuronal population of NCM and quality of song-learning (Terpstra et al., 2004). This would be in accordance with the inverse correlation between number of new adult-born HVC neurons and number of song changes in deafened birds, in that both results would indicate that adult neurogenesis has a stabilizing rather than a plasticizing effect on existing memories (Pytte et al., 2012). If, instead, I observed that the response of the new neuronal population to tutor song playback differed from that of the general neuronal population, this observation would indicate that new neurons are not for maintaining old memories, but for storing new information.

Methods

Subjects and Housing

The experiment was performed in accordance with the Wesleyan University Institutional Animal Care and Use Committee and NIH guidelines. Subjects were eleven adult male zebra finches (*Taeniopygia guttata)*, all 120 days old, reared in the aviary of the Kirn laboratory at Wesleyan University. Birds were provided with food and water *ad libitum* and kept on a 14/10 hour light/dark cycle while maintaining a temperature of 70 degrees Fahrenheit. Birds were initially housed alone with their parents so that each bird's father acted as his tutor during the critical period for song learning. At 70 days post hatch (dph), experimental birds were separated from their tutors and moved to single sex housing in a separate room. This setup prevented them from hearing their respective tutor songs again until the time of song re-exposure. An age of seventy days was chosen because by eighty days the bird's song will sound very similar to his father's, but an earlier separation date could prevent the bird from learning at all (Hinde 1969, p. 65-66). During the last stage of the experiment, at 120 dph, birds were housed individually in cages within custom-made closed sound attenuation chambers for song playbacks. Time constraints resulted in a final sample size of three birds.

Song Exposure

Birds were raised until age 70 dph with their families, meaning that each bird learned song from his father. The bird's father's song will henceforth be referred to as

the "tutor song." Removal from family housing at age 70 dph marked the last time the experimental bird heard the tutor song.

Song Recording

Songs of experimental birds and their tutors were recorded in custom-built closed sound-attenuating chambers. The conditions inside the chambers replicated those of the environment in which the birds were raised (see *Subjects and Housing*, above). Each chamber was equipped with a microphone connected to a recording system (FireStudio Project; PreSonus, Baton Rouge, LA). Songs were recorded using the sound-activated computer recording program Sound Analysis Pro 2011 (Plone Foundation, Fishers, IN).

Injections

Starting at age 90 dph, each bird received injections of BrdU twice daily for four days. In every instance, the bird was removed from his cage, injected subcutaneously with 100 mg of BrdU per kilogram body weight, and replaced after sufficient recovery time. Injections occurred twelve hours apart, around 7AM and 7PM every day.

Song Re-Exposure

Each bird was re-exposed to his tutor's song around age 120 dph. This date was chosen because it allowed thirty days for BrdU labelled NCM cells to become fully integrated. In order to control for singing frequency and song amplitude, each re-exposure was achieved by way of a recording playback. Evidence suggests that for

zebra finches, just hearing a recording elicits the same degree of neural activity as a recording paired with a visual stimulus, such as that of a stuffed bird (Bolhuis et al., 2000). When an experimental bird reached 120 dph, he was housed in a cage inside a custom-built closed sound-attenuating chamber. He remained there undisturbed during a one-day acclimation period. Undirected singing, detected by a microphone installed in the chamber, indicated that the bird had become comfortable with his surroundings. After it was confirmed that the bird had acclimated, the lights in the chamber were turned off to ensure that he would remain still and silent during song playback, so as not to interfere with ZENK expression. In the darkness, all birds experienced an initial fifteen minutes of total silence, followed by song playback for thirty minutes, and then another hour of silence, in accordance with a previous study that successfully measured IEG expression in the NCM following tutor song playback (Terpstra et al., 2004).

For the song playback phase, each bird was exposed to a custom-made iTunes playlist containing thirty renditions of his tutor's song, interspersed with arbitrarily selected periods of silence ranging from 5 seconds to 10 minutes. This playlist was created in a randomized fashion and played on "Shuffle" mode in an attempt to simulate biological conditions. All birds heard around thirty song playbacks of varying durations. The playlist was broadcast through Dell A215 speakers (Dell, Round Rock, TX) at 70 decibels sound pressure level peak amplitude.

Immediately following the last hour of silence, the bird was removed from the playback chamber and sacrificed.

Perfusions and Sectioning

Birds were deeply anaesthetized with methoxyflurane (Metofane; Mallinckrodt, Mundelgn, IL) until unresponsive to a toe pinch and quickly perfused by way of the left ventricle with ten milliliters of 0.1 M phosphate buffer followed by fifty milliliters of 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The brains were subsequently removed, divided into hemispheres, and stored in the same fixative overnight at four degrees Celsius. Over the following two days, brains were sunk in 15% and 30% solutions of sucrose in 0.1 M phosphate buffer at four degrees Celsius. Brains were then stored in an anti-freeze cryoprotectant solution (500mL 0.1M PB, 300g sucrose, 10g polyvinyl pyrrolidone, 300mL ethylene glycol, bring final volume to 1000mL with dH$_2$O) at negative twenty degrees Celsius until sectioning. Sections were cut with a freezing microtome at a thickness of thirty micrometers and stored at negative twenty degrees Celsius in antifreeze until immunohistochemistry.

Immunohistochemistry

Free-floating sections were first washed with 0.1 M PBS three times for ten minutes each, then permeabilized in 0.3% Triton X-100 in 0.1 M PBS (PBST) for thirty minutes, and finally incubated in 1.5 N HCl for twenty minutes at thirty-seven degrees Celsius for twenty minutes. They were then neutralized with Tris base twice for five minutes each, washed with PBST three times for ten minutes each, and incubated in a blocking solution of 4% Bovine Serum Albumin in PBST for thirty minutes. Following this preparation, sections were incubated in a cocktail of primary antibodies including rabbit anti-Erg IgG (dilution 1:500) (SC-189; Santa Cruz Biotechnology), rat anti-

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BrdU IgG (dilution 1:100) (MCA2060; AbD Serotec), and mouse anti-Hu IgG (dilution 1:100) (45232A; Invitrogen) for two hours at room temperature or overnight at four degrees Celsius (both treatments yield the same result). Sections were then washed with PBST three times for ten minutes each and incubated in a cocktail of secondary antibodies including Alexa Fluor 555 goat anti-rabbit IgG (A21429; Invitrogen), Alexa Fluor 488 goat anti-rat IgG (A11006; Invitrogen), and Alexa Fluor 647 goat anti-mouse IgG (115-605-164; Jackson ImmunoResearch Laboratories) (all dilutions 1:500) for one hour each. After three final ten minute washes with PBST, sections were mounted on slides, cover-slipped with Aqua-Mount (Thermo Scientific, Waltham, MA), and examined under a confocal microscope.

Song Analysis

All subject and tutor songs were analyzed using Sound Analysis Pro (SAP) (Plone Foundation, Fishers, IN) and Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany). First, SASLab Pro was used to visualize recorded sounds from each recording session as spectrograms. All recordings were screened for each experimental bird and each tutor, such that songs were selected and calls and other superfluous noises were discarded. This program was used to select ten exemplary song bouts for each experimental bird as well as each tutor.

Using Sound Analysis Pro, each experimental bird's ten bouts were compared with his tutor's ten bouts. This comparison was done as a matrix, such that a single experimental bird's bout was compared to all ten tutor bouts, and vice versa. SAP compares numerous features of song recordings including pitch, frequency modulation,

amplitude modulation, entropy, and goodness of pitch. Using these attributes, SAP calculates two scores for a song: percent similarity and accuracy. Percent Similarity denotes gross similarity between whole song bouts, while Accuracy refers to the degree of song likeness on a finer temporal scale. Both scores, as well as differences in acoustic features like entropy and goodness of pitch, were recorded for each subject-tutor dyad. Figure 3 depicts the manner in which SAP compares songs. The vertical song is the tutor song, and the horizontal song is the tutee's song. Sections of the song that have passed a similarity threshold calculated by the program are denoted by red lines. The "percent similarity score" reflects the portion of the tutor song that appears in the tutee's song. The program calculates the "accuracy score" by computing degree of likeness between sections of songs that have already passed the similarity threshold. Therefore, the percent similarity and accuracy scores measure song likeness on the global and local levels, respectively.

The program calculates additional, more specific scores that can be useful for assessing quality of song learning. The Wiener entropy difference and goodness of pitch difference scores compare acoustic features in the tutor and tutee songs. "Wiener entropy" is a number between one and zero that measures acoustic randomness by evaluating the width and uniformity of elements within the song spectrogram. In order to expand the range, SAP 2011 uses a logarithmic scale for entropy, so that it ranges from zero for white noise to negative infinity for a pure tone (Mandelblat-Cerf and Fee, 2014). "Goodness of pitch" measures harmonic structure of sound; in other words, a sound is likely to be harmonic if goodness of pitch is high (Thompson et al., 2011). Differences in Wiener entropy and goodness of pitch could be due to greater entropy

or goodness of pitch in either the tutor or the tutee song. Two highly similar songs would be expected to have small differences in Wiener entropy and goodness of pitch. Figure 4 illustrates goodness of pitch and entropy and compares them within a song spectrograph.
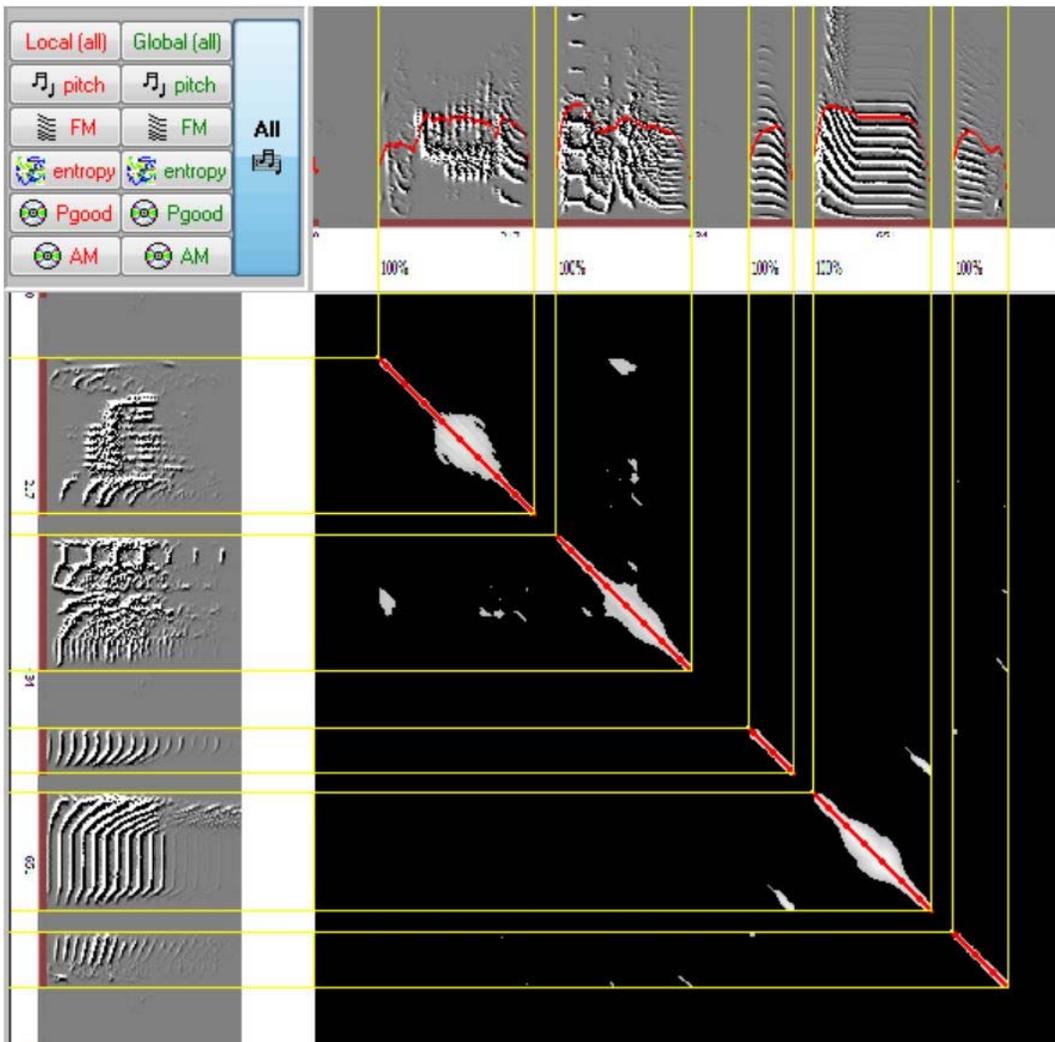
Figure 3. Pictorial representation of a song analysis from SAP 2011. Tutor song (vertical spectrogram) is compared with tutee song (horizontal spectrogram). The red lines indicate parts of songs that have passed a similarity threshold. The percent similarity score reflects the percentage of tutor song that is reflected in the tutee's song. Accuracy score is measured by calculating the extent of song likeness among the parts of songs that have passed the similarity threshold (Tchernichovski et al., 2000).

Note how strongly the 'pureness' of the harmonic stack affect the goodness of pitch

Note how the ratio between goodness of pitch and Wiener entropy changes during the whistle
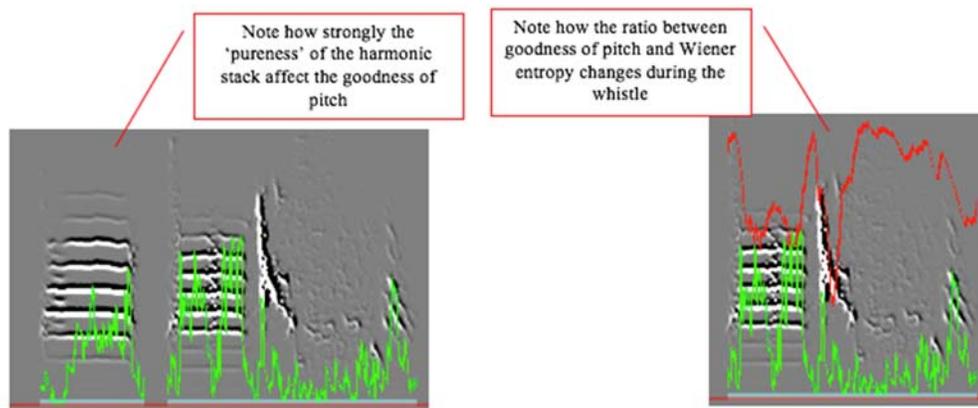
Figure 4. Sound spectrograph illustrating goodness of pitch and entropy. On the left, the green line represents changes in goodness across a song. The higher the green line, the better the goodness of pitch. On the right, goodness of pitch is contrasted with entropy (red line). Entropy is high with less structured sounds. From Sound Analysis Pro Manual (Tchernichovski et al., 2004).

Tissue Analysis

Sections containing NCM were first examined under a fluorescence microscope (Olympus BX50; Olympus America Inc., Center Valley, PA) with a 4x objective and brightfield optics. For each section, the borders of NCM were traced by hand using a program called Neurolucida (MicroBrightField, Colchester, VT). These tracings ensured that images taken on the confocal microscope would be confined to regions within NCM. Five sections containing NCM from each bird were analyzed using confocal microscopy (ZEISS, Jena, Germany) with a 25x objective. For each section, at least five images were taken of different regions within NCM. For each image, the number of Hu-positive cells was estimated using ImageJ software (NIH, Bethesda, MD). I checked each estimate and manually discounted false positives such as those created by autofluorescence. Numbers of ZENK-positive, BrdU-positive, and BrdU/ZENK/Hu positive cells were counted manually using the "Cell Counter" plugin for ImageJ. Since ZENK and BrdU label the cell nucleus, and Hu labels the cytoplasm, I only counted ZENK-positive, BrdU-positive, and ZENK-and-BrdU-positive fluorescence that appeared to be fully contained within the borders of a Hu-positive cell, in order to avoid counting false positives.

Data Analysis

Analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA). For each bird, cell counts from each section were averaged to determine mean number of ZENK-expressing, Hu-positive neurons per 1000 Hu-positive neurons and mean number of ZENK-expressing, BrdU-and-Hu-positive neurons per 1000 BrdU-and-Hu-positive neurons.

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Ten song bouts from each bird were compared in a matrix with ten tutor song bouts. Thus, a single comparison yielded one hundred scores each for percent similarity, accuracy, percent sequential match, entropy difference, and goodness of pitch difference. For every experimental bird, scores were averaged to determine mean percent similarity, accuracy, entropy difference, and goodness of pitch difference scores.

Results

NEW ACTIVE NEURONS

In accordance with my prediction, a subset of the adult-born neuron population in NCM expressed ZENK in response to tutor song playback. Figure 5 shows several high-power magnification z-stack images taken with a confocal fluorescence microscope of the same region in NCM of the same bird. Figure 5A shows Hu-positive neurons (in blue). Figure 5B is a merged image of the same Hu-positive neurons as well as BrdU-positive neurons (in green). Figure 5C is a merged image of the same Hu-positive neurons as well as ZENK-positive neurons (in red). Finally, Figure 5D is a merged image of the same Hu-positive, BrdU-positive, and ZENK-positive neurons. In each part of Figure 5, the white arrow points to the same cell, which is labeled clearly by all 3 markers. The white rectangle in Figure 5D is magnified and represented in Figure 6 to provide better resolution of this triple-labeled cell.

In the trends described below, the "general neuronal population" is defined as neurons labeled with Hu, and the "new neuronal population" is defined as neurons double-labeled with BrdU and Hu. Analyses explored how ZENK expression differed between the two populations as a function of various features of quality of song learning.
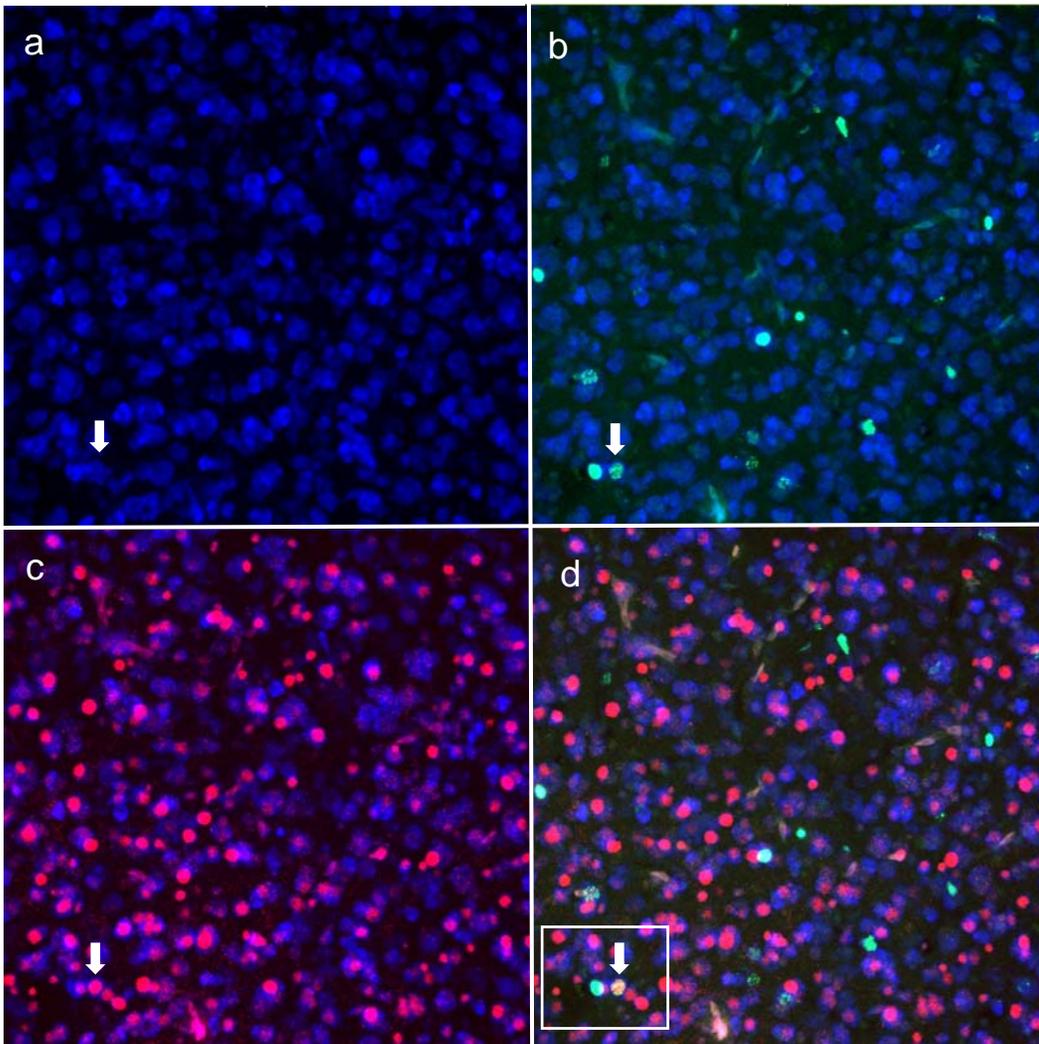
Figure 5. High-power magnification merged z-stack images of the same 30µm parasagittal section taken with a confocal fluorescence microscope. Panel A shows cells in NCM labeled with neuron-specific marker Hu (blue). Panel B shows newly added cells marked with BrdU (green) and neurons marked with Hu. Panel C shows cells expressing ZENK in response to song playback (red) and neurons marked with Hu. Panel D merges Hu (blue), BrdU (green), and ZENK (red) labeling. White arrows indicate the position of one cell marked by all three labels (boxed by a white rectangle).
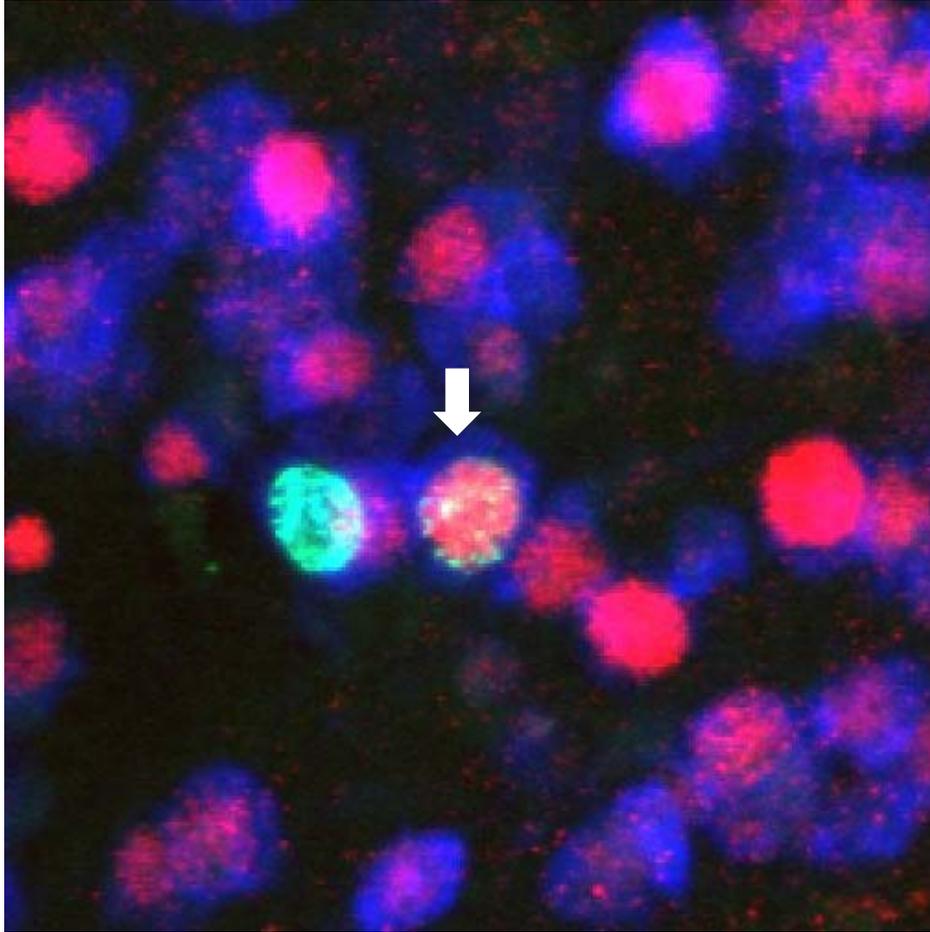
Figure 6. Close-up of the same triple-labeled neuron depicted in Figure 5, denoted by white arrow. Image is a high-power magnification merged z-stack image showing ZENK (red), BrdU (green), and Hu (blue) labeling.

QUALITY OF SONG LEARNING

ZENK expression was assessed as a function of song learning quality in both the general and new neuronal populations. The sample size for this study was too small to justify the use of statistical analysis. However, some interesting and unexpected trends emerged when different aspects of song learning quality were considered.

Figure 7 shows song spectrograms of two zebra finch song bouts. Figure 7A is the spectrogram of the tutor's song (Bird 42), and Figure 7B is the spectrogram of the tutee's song (Bird 52). Even to the untrained eye, they appear to be quite similar. The tutee's song earned a similarity score of 40.26% and an accuracy score of 72.19%. It also received an entropy difference score of 5.36 and a goodness of pitch difference score of 2.31.

Song Similarity

The relationships between global song similarity and number of ZENK-positive neurons in NCM for both the general and new neuronal populations are shown in Figures 8A and B. There was a slightly negative trend for the general neuronal population, such that as song similarity increased, the number of ZENK-positive neurons per 1000 decreased. By contrast, the new neuronal cohort demonstrated a positive correlation between song similarity and number of ZENK-positive neurons. Thus, song similarity trended in opposite directions for new neurons and old neurons.

Song Accuracy

The trends observed for song similarity were reversed for song accuracy, shown in Figures 8C and D. The general neuronal population exhibited a positive correlation between song accuracy between tutor and tutee songs and number of ZENK-positive neurons per 1000, while the new neuronal population exhibited a negative correlation. Thus, accuracy on a more fine-grained temporal scale also trended in opposite directions for new neurons and old neurons in terms of ZENK expression.

Entropy Difference

Trends observed for entropy difference resembled those of song accuracy and are depicted in Figures 9A and B. As the difference in entropy increased, ZENK expression in the general neuronal population increased. By contrast, ZENK expression in the new neuronal population decreased. Thus, entropy difference trended in opposite directions for new neurons and old neurons in terms of ZENK expression.

Goodness of Pitch Difference

The difference in goodness of pitch revealed trends that resembled those of song similarity, depicted in Figures 9C and D. As the difference in goodness of pitch increased, ZENK expression in the general neuronal population decreased. By contrast, ZENK expression in the new neuronal population increased. Thus, goodness of pitch difference also trended in opposite directions for new neurons and old neurons.

BEHAVIOR OF NEW VERSUS OLD NEURONS

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From the above analyses, it appears that new and old neurons behave quite differently from one another in this context. To test this idea, I compared the number of ZENK-expressing cells per 1000 Hu-positive neurons to the number of ZENK-expressing cells per 1000 BrdU-and-Hu-positive neurons. I found a strong negative trend, such that as the number of ZENK-expressing neurons in the general population increased, the number of ZENK-expressing neurons in the new population decreased. This comparison is shown in Figure 9.
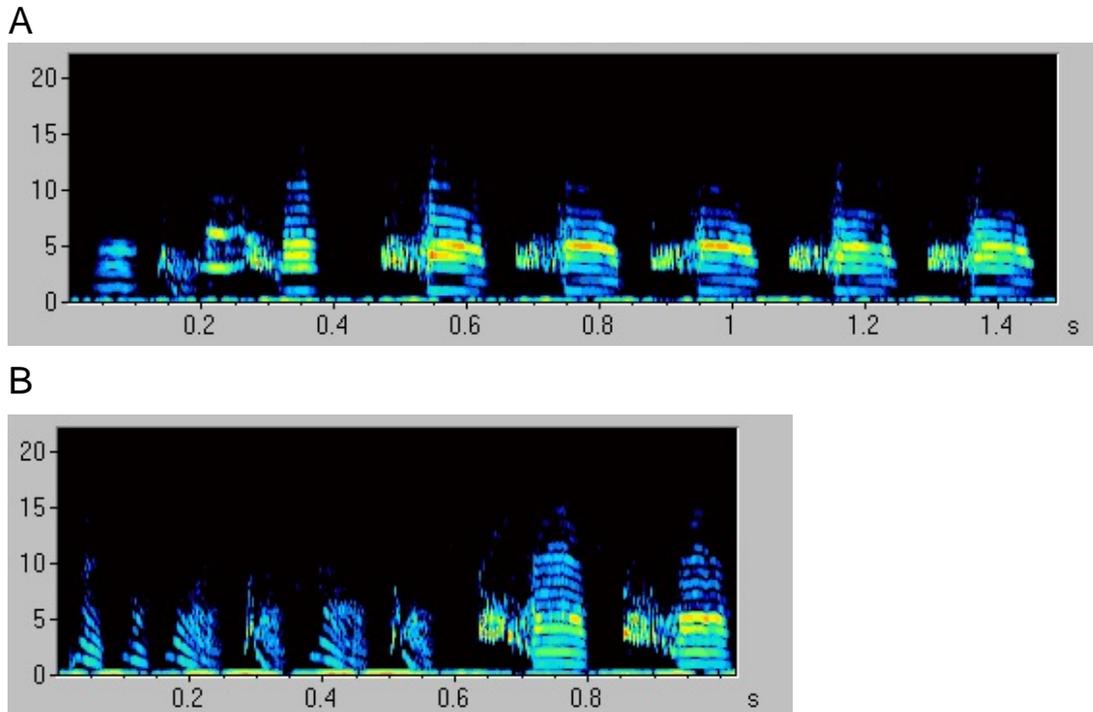
A

B

Figure 7. Spectrograms of tutor and tutee songs. Panel A shows the tutor song (Bird 42) and Panel B shows the tutee's song (Bird 52). The tutee's song earned a similarity score of 40.26% and an accuracy score of 72.19%. It also received an entropy difference score of 5.36 and a goodness of pitch difference score of 2.31.
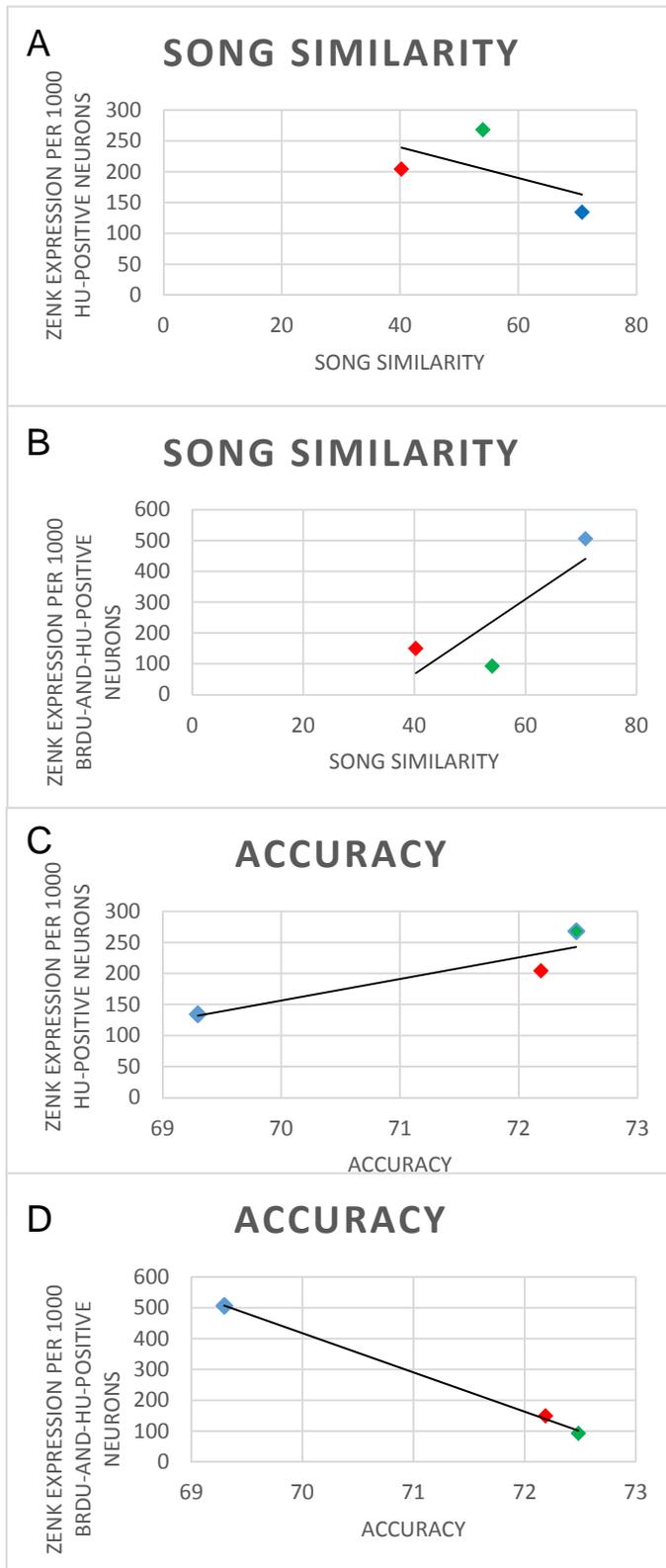
Figure 8. Trends for song similarity and accuracy scores in the general and new neuronal populations. Each color represents a different bird. Panel A shows a negative trend between song similarity score and the amount of ZENK expression in the general neuronal population (Hu-positive neurons). Panel B shows a positive trend between song similarity score and the amount of ZENK expression in the new neuronal population (BrdU-and-Hu-positive neurons). Panel C shows a positive trend between accuracy score and ZENK expression in the general neuronal population. Panel D shows a negative trend between accuracy score and ZENK expression in the new neuronal population.
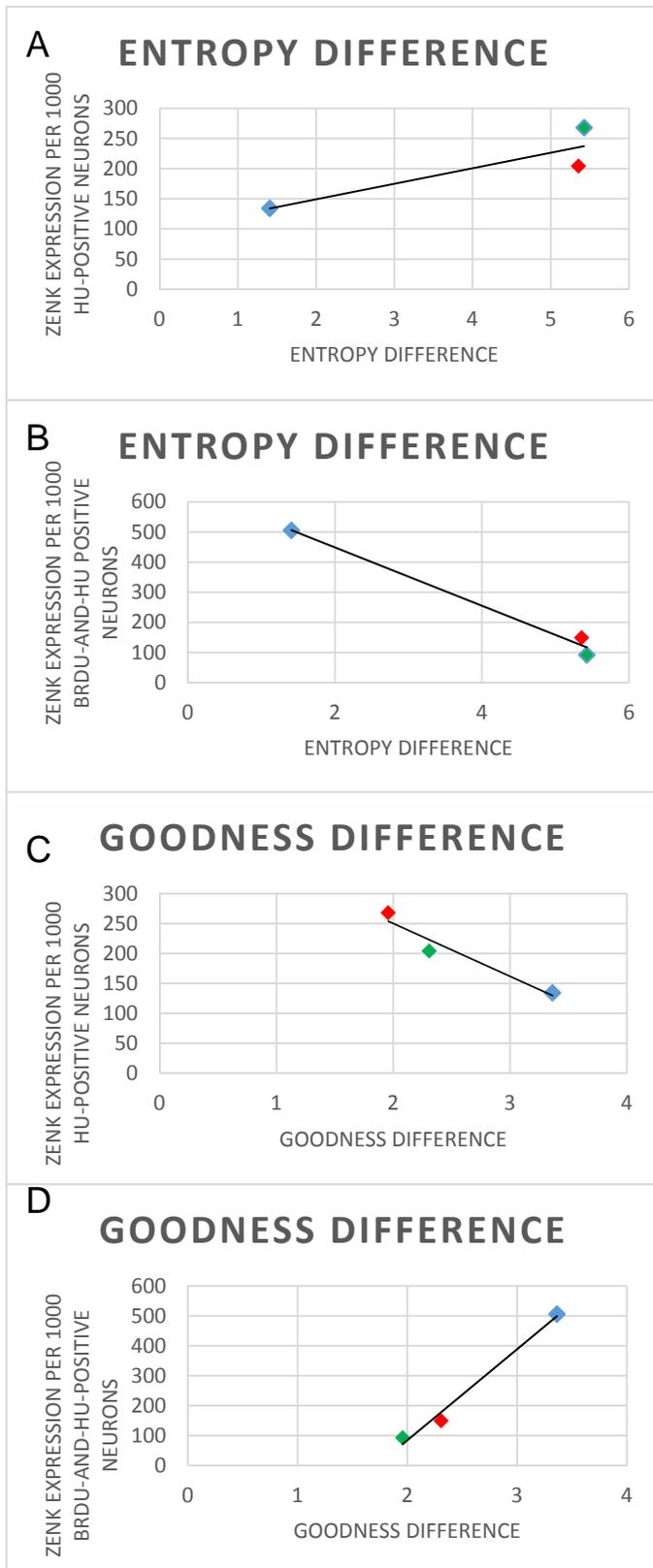
Figure 9. Trends for entropy difference and goodness of pitch difference in the general and new neuronal populations. Each color represents a different bird. Panel A shows a positive trend between entropy difference and the amount of ZENK expression in the general neuronal population (Hu-positive neurons). Panel B shows a negative trend between entropy difference and the amount of ZENK expression in the new neuronal population (BrdU-and-Hu-positive neurons). Panel C shows a negative trend between goodness of pitch difference and ZENK expression in the general neuronal population. Panel D shows a positive trend between goodness of pitch difference and ZENK expression in the new neuronal population.
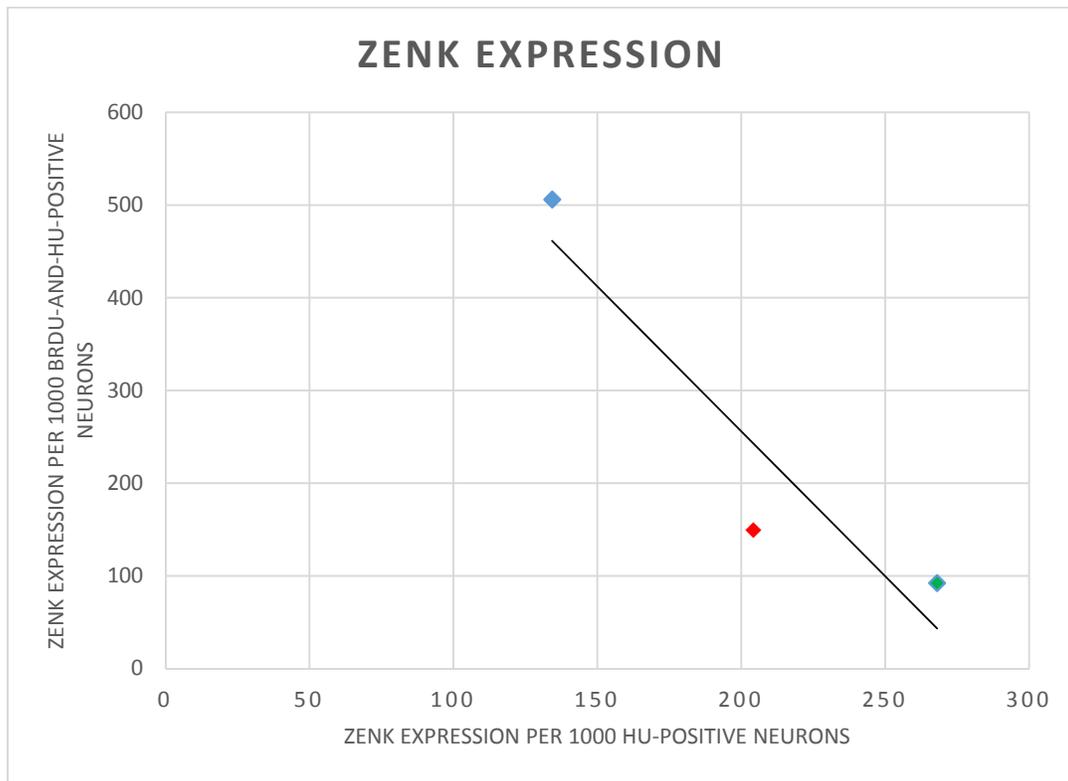
Figure 10. ZENK expression in the general neuronal population (Hu-positive neurons) compared to ZENK expression in the new neuronal population (BrdU-and-Hu-positive neurons). There is a negative trend such that as ZENK expression increases in the general population, ZENK expression decreases in the new population.

Discussion

YOUNG ADULT-BORN NEURONS IN NCM EXPRESS ZENK

Here I have shown systematic changes in ZENK expression in new adult-born neurons in response to tutor song playbacks as a function of various acoustic features. It is well known by now that the general neuronal population in NCM responds to song playbacks. NCM neurons have been shown to prefer conspecific song to pure tones and tutor song to novel conspecific song (Mello et al., 1992, Terpstra et al., 2004). Some information is available about the integration of new adult-born neurons into preexisting circuits in other parts of the songbird brain. For example, research in canaries has shown that some newly arrived neurons have associated themselves with mature HVC projection neurons as early as eight days after labeling (Kirn et al., 1999). Indeed, by thirty days new neurons are found all over HVC and have been shown to fire action potentials and to innervate other neurons (Goldman and Nottebohm, 1983, Paton and Nottebohm, 1984, Kirn et al., 1991). Although the fates of new neurons are well-characterized in HVC, they are less so for other regions of the songbird brain like NCM.

NCM is known to receive new neurons, but the fates of these new neurons are not yet clear. Survival time for adult-born neurons in the zebra finch caudal nidopallium (NC, which contains NCM) has been shown to be influenced by social experience, social change, age, and position (Adar et al., 2008). A study comparing hearing-intact and deafened adult male zebra finches has revealed a significantly lower rate of neuron addition to medial NCM in deafened birds, indicating that survival of adult-born NCM neurons probably depends upon use of this brain region (Pytte et al.,

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2010). None of the studies described here, however, have attempted to investigate the behavior of newly added neurons. Here I have provided evidence that adult-born neurons in NCM express ZENK as early as thirty days. This evidence may suggest that young adult-born neurons can participate in a neural response to tutor song.

NEURAL RESPONSE TO TUTOR SONG IN NCM

The General Neuronal Population

In the general neuronal population of NCM, I found that amount of ZENK expression evoked by tutor song playback correlated negatively with percent song similarity. This result was unexpected because it contradicts previous findings that amount of ZENK expression evoked by tutor song playback correlates positively with song similarity (Terpstra et al., 2004). There are a few potential explanations for this apparent discrepancy. Most importantly, the positive correlation from the 2004 study was observed only in lateral NCM. My thesis examined NCM as a whole and did not account for medial-lateral differences, a factor that likely contributed to my inability to replicate Terpstra and colleagues' results. Additionally, my sample size was simply too small to establish any true correlations. It could be that with a larger sample size my apparently negative correlation would disappear altogether.

Intriguingly, although ZENK expression correlated negatively with the song similarity score in the general neuronal population, I found that it correlated positively with the accuracy score, another measure of song learning quality. The reasoning for this discrepancy may be rooted in SAP 2011's calculations for similarity and accuracy. As described earlier, the similarity score provides a percentage of the tutor's song that

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is contained in the tutee's song, based on sections of song that have passed a threshold for sameness set by the program (I used the default setting, "zebra finch"). By contrast, the accuracy score measures the similarity between sections of song that have already passed the threshold. These two measures do not necessarily have to correlate positively; two birds may share many syllables, but the shared syllables will not always be perfect matches in terms of acoustic features like entropy and goodness of pitch.

Also intriguing was the positive correlation between entropy difference and amount of ZENK expression in response to tutor song. Terpstra *et al* showed that ZENK expression is higher in lateral NCM for those birds who exhibited better learning of tutor song (2004). Better learning of tutor song would imply smaller entropy and goodness of pitch differences, because the bird's song and tutor song would presumably have highly similar acoustic properties. Logically, then, it would follow that amount of ZENK expression would correlate negatively with entropy and goodness of pitch differences. This was the case for goodness of pitch difference, but it was not for entropy difference. This discrepancy is puzzling and an explanation is not readily available, as researchers who use SAP 2011 tend to focus on the percent song similarity and accuracy scores and have rarely published data regarding entropy and goodness of pitch difference.

The New Neuronal Population

With this research I sought to find out if new neurons encode previously acquired memories. I predicted that the new neuronal population would respond to tutor song playback in a manner that resembled that of the general neuronal population.

Specifically, I expected to see ZENK expression in both the general and new neuronal populations correlate positively with SAP 2011's song similarity score. Contrary to my prediction, however, the general and new neuronal populations did not respond similarly to tutor song playback. The general neuronal population exhibited a negative correlation, which was unexpected to begin with, but the new neuronal population exhibited a positive correlation. Furthermore, this antagonistic behavior was observed not only with regards to the song similarity score, but also every additional measure of quality of song learning discussed in this research.

Although my sample size was too small to justify statistical analysis, it is impossible to ignore the fact that the new neuronal population behaved in a manner directly opposing that of the general neuronal population. In birds who experienced a great deal of ZENK expression in established neurons, new neurons did not respond much at all. The converse was also true; in birds whose older neurons exhibited a modest response to tutor song playback, new neurons exhibited a more robust response as shown in Figure 10. At present, my findings suggest that new adult-born neurons participate the most when the general neuronal population participates the least, and vice versa. Although the sample size is too small to draw conclusions, the fact that ZENK responses in the general population differed from those of new neurons is inconsistent with my hypothesis that new neurons help maintain old memories. This fact is more consistent with the idea that new neurons are for new memories. In order to draw conclusions from these results, it is essential that this study be repeated on a larger scale with more decisive measures regarding quality of song learning.

Adult Neurogenesis is related to Plasticity

The role of adult-born neurons in songbirds, rodents, and humans is yet unclear. Seminal studies on this topic have argued convincingly for a plasticizing model of adult neurogenesis. In this model, new neurons are incorporated into new circuits, promoting the modification of preexisting memories and creation of new ones. For example, Kirn *et al* (1991) established the existence of a positive correlation between neurogenesis and song repertoire renewal in canaries. Similarly, research in zebra finches has shown that HVC neuron recruitment is higher before song crystallization than after in juveniles, and that it decreases throughout adult life (Reviewed by Wilbrecht and Kirn, 2004). In other words, adult neurogenesis appears to be positively correlated with plasticity in HVC of two different songbird species. Relatedly, some have proposed that adult neurogenesis in the hippocampus is critical for pattern separation (Reviewed by Barnea and Pravosudov, 2011). The idea is that new, adult-born neurons allow the animal to learn new information without disrupting circuits encoding previously learned information (Wiskott et al., 2006). Most evidence for the role of neurogenesis in pattern separation comes from spatial memory research. For example, one study showed that blocking hippocampal neurogenesis in mice interferes with performance on a delayed non-matching to place radial arm maze task when two locations were close to one another, but not when they were far apart (Clelland et al., 2009). No analogous research has been done in the avian brain, but it would be interesting to explore whether adult neurogenesis in NCM affects a songbird's ability to discriminate between two highly similar songs, as a sort of auditory pattern separation. There is

currently no way to ablate neurogenesis in the songbird brain, but perhaps in the future X-irradiation or chemical treatment may be used to suppress neurogenesis in NCM of adult male zebra finches. Subjects could subsequently be subjected to behavioral testing in which they are required to distinguish between two highly similar conspecific songs. Such manipulations would provide insights into the mechanism by which NCM recognizes and responds preferentially to tutor song.

Many attempts have been made to disentangle causality regarding adult neurogenesis and plasticity in the songbird brain (Reviewed by Wilbrecht and Kirn, 2004). One group of researchers prevented young zebra finches from imitating their tutors during the sensorimotor phase of song learning and observed the resulting effects on adult neurogenesis (Wilbrecht et al., 2002). Unilateral denervation of the syrinx resulted in a doubling of number of new neurons recruited to ipsilateral HVC. These birds were still able to learn from their tutors, unlike those who had undergone bilateral denervation of the syrinx. Furthermore, deafening abolished the unilateral surge in neurogenesis. Taken together, these results imply that learning new song promotes adult neurogenesis.

Despite convincing tales of the relationship between adult neurogenesis and plasticity, however, further research has obfuscated rather than clarified matters. The temporal correlation between adult neurogenesis rates and singing renewal in canaries is enticing, but some research has suggested that these processes are not actually causally related. Could it be that adult neurogenesis is simply a vestigial process, not actually necessary for learning and memory?

Adult Neurogenesis is Unnecessary

Wilbrecht and Kirn have suggested that, given that adult neurogenesis occurs on a much smaller scale than developmental neurogenesis, it may not serve any function at all (2004). This idea is supported by research in some animals in which adult neurogenesis does not seem to be at all necessary for learning and memory (Reviewed by Barnea and Pravosudov, 2011). For example, some species of bat do not experience adult neurogenesis, yet they possess an exceptional capacity for spatial learning. Studies in rodents have attempted to show that experimentally reducing hippocampal neurogenesis negatively affects learning and memory, but Leuner *et al* caution scientists against jumping to conclusions when interpreting such results (2006). Taking them as proof of a relationship between neurogenesis and learning is risky because it does not account for the fact that many methods for suppressing neurogenesis (like irradiation and antimitotic agent methylazomethanol) can affect other unrelated brain and bodily processes.

Furthermore, though many studies suggest the necessity of adult neurogenesis in learning and memory, a comparable number of studies suggest the opposite (Reviewed by Leuner et al., 2006). Experimental studies in mice, who are known to receive new neurons to the hippocampus and the olfactory bulb throughout life, have suggested that adult neurogenesis might be uncoupled from learning and memory. For example, cyclin D2 knockout mice, whose adult neurogenic ability is greatly suppressed, can still learn new tasks (Reviewed by Barnea and Pravosudov, 2011). Research in rats has shown that neither methylazomethanol treatment nor irradiation within the hippocampus result in any difficulty on the Morris water maze task.

However, the assessment of adult neurogenesis as being unnecessary for learning and memory fails to address a number of uncertainties. It could be that currently employed measures of learning are not sensitive enough to catch the deficits brought on by impaired neuron addition (Reviewed by Leuner et al., 2006). Furthermore, if adult neurogenesis is unnecessary, how do we account for the fact that neuron addition and survival can be regulated seasonally and experientially, at least in the adult songbird brain (Reviewed by Wilbrecht and Kirn, 2004)?

Adult Neurogenesis is Necessary for Song Maintenance

Happily, more recent research has advanced a possible role for adult neurogenesis in the songbird brain. Zebra finches are closed-ended learners, meaning that once their songs have crystallized, they do not change throughout adulthood. Based on the idea that adult neurogenesis plasticizes the brain and makes it more receptive to new information, zebra finches would be expected not to require new neurons after song learning is complete. Curiously, however, one group of researchers showed that zebra finches still receive new neurons in the song control nuclei long after crystallization has occurred (Alvarez-Buylla et al., 1990). Similarly, another group has shown that western song sparrows, who are closed-end learners like zebra finches, continue to undergo seasonal fluctuations in neurogenesis rates in HVC long after their songs have crystallized (Tramontin and Brenowitz, 1999). This information prompted some scholars to consider a new potential function for adult neurogenesis – maintenance of learned information. Pytte, George *et al* have recently shown that among birds who have been deafened after their songs have crystallized, the number of

adult-born neurons recruited to HVC correlates negatively with the number of changes made to the bird's own song as a result of deafening (2012). This is the first study to reveal a negative correlation between new neuron number and behavioral plasticity in songbirds. The correlation suggests that new neurons play a role in stabilizing preexisting behaviors. It also substantiates preexisting evidence from the same researchers which has shown that recovery of preexisting song structure following Botox injections to the syrinx is best in zebra finches with the highest rates of HVC neuron addition (Pytte et al., 2011). Unfortunately, until now few attempts have been made to investigate this newly proposed function of adult neurogenesis, likely due to the diverse, confusing, and contradictory information on the subject derived from research on several rather different species.

Finding Common Themes

The function of adult neurogenesis appears to differ dramatically across species (Reviewed by Leuner et al., 2006). Mice with suppressed neurogenic ability perform worse on spatial tasks like the Morris water maze and radial arm maze tasks than controls, but, as discussed above, these results are not replicable in rats. Additionally, some species of bat do not depend upon adult neurogenesis at all for their superb spatial memories. Whereas a positive correlation between neurogenesis and learning has been demonstrated in canaries, tree shrews show a negative correlation. Innumerable other studies have put forth possible roles for adult neurogenesis in different animals that at times directly contradict one another.

Nonetheless, research on adult neurogenesis occasionally converges on common themes. For example, as described above, mice whose hippocampal neurogenesis has been ablated by X-irradiation struggle with pattern separation as it relates to spatial learning and memory (Clelland et al., 2009). Their performance appears normal when spatial cues are far apart, consistent with data from cyclin D2 knockout mice. However, when spatial cues are close together, the mice experience more difficulty completing the task. Similarly, NCAM (-/-) mice receive very few SVZ neurons to the olfactory bulb after birth, and demonstrate impaired olfactory discrimination abilities, despite having intact olfactory memories (Reviewed by Alvarez-Buylla and Garcia-Verdugo, 2002). It appears that as techniques to suppress neuron addition and tasks to measure learning and memory impairments become more sensitive, a clear role for adult neurogenesis in murine pattern separation may soon emerge.

The same kind of convergence may someday be attainable for research in songbirds, if the appropriate measures become available. However, since it is currently not possible to ablate adult neurogenesis in the avian brain, researchers in this field must resort to creative correlational studies. Examining the behavior of newly added neurons and relating it to the behavior of the general neuronal population is a useful and relatively simple place to start. However, more information about the process and conditions of adult neurogenesis in songbird brain regions other than HVC is needed before progress can be made. Knowing as much about NCM as we do about HVC will advance the field towards a "big-picture" understanding of adult neurogenesis.

FUTURE DIRECTIONS

HVC Neuronal Composition

HVC contains three types of neurons; RA-projecting, Area X-projecting, and local interneurons (Reviewed by Balthazart and Ball, 2016). It was originally thought that most adult-born neurons recruited to HVC were interneurons, because injection of retrograde tracer horseradish peroxidase in RA and Area X did not successfully label any new HVC neurons. However, more sensitive retrograde tracers have shown that most if not all adult-born HVC neurons are actually of the RA-projecting type.

The Process of Adult Neurogenesis in HVC

The songbird HVC is known to undergo a distinct type of adult neurogenesis known as neuronal replacement. In adults, total HVC neuron number remains stable over time, even though adult neurogenesis is a documented phenomenon in this nucleus (Reviewed by Wilbrecht and Kirn, 2004). This stability is taken to be a result of the dynamics between neuronal death and new neuron addition in HVC. Dying HVC neurons have been observed at every age in zebra finches and canaries, albeit less so in zebra finches. Cell death appears to drive neurogenesis in HVC; selectively killing HVC-RA projection neurons results in a surge in neurogenesis (Reviewed by Wilbrecht and Kirn, 2004). The relationship is possibly bidirectional, as experimental suppression of cell death results in diminished recruitment of new neurons (Reviewed by Barnea and Pravosudov, 2011).

Conditions of Adult Neurogenesis in HVC

The neuronal composition of HVC fluctuates seasonally (Reviewed by Balthazart and Ball, 2016). Fluctuations in new HVC neuron number are associated with fluctuations in cell death, such that peaks in new neuron numbers are concomitant with troughs in dying cell numbers. Peaks occur in October and March and are preceded by periods of abundant neuronal death. These seasonal changes are known to be influenced by changing levels of circulating sex steroids. Experimental studies have shown that increasing plasma testosterone concentration results in greater neuronal recruitment to HVC. Furthermore, testosterone treatment in female canaries results in a tripling of new RA-projecting neurons (Rasika et al., 1994). Testosterone is thought to have this effect by extending the survival time of adult-born neurons, rather than stimulating neuronal proliferation, but this explanation is not yet proven.

Sex steroids are not the only way to manipulate adult neurogenesis in HVC. Singing activity, for example, is known to affect HVC volume (Reviewed by Balthazart and Ball, 2016). Singing upregulates the release of brain-derived neurotrophic factor (BDNF), which is known to promote HVC expansion. Furthermore, preventing male canaries from singing results in lower BDNF mRNA and protein levels in HVC compared to birds who were allowed to sing at will. BDNF appears to promote the survival of new neurons, because BrdU-labeled RA-projecting neurons survived longer in those birds who were allowed to sing.

What about NCM?

Adult neurogenesis is clearly well-characterized in the songbird HVC. However, while HVC is useful for exploring the process of song learning, NCM is better suited to research regarding role of adult neurogenesis in long-term memory preservation because it receives new neurons throughout life and is considered to be the location in which tutor song memory is stored (Reviewed by Bolhuis and Moorman, 2015). Unfortunately, there is limited information available on the process of adult neurogenesis in NCM. The conditions of neuron addition in the adult songbird brain vary from region to region; new neuron numbers do not fluctuate seasonally in Area X, and adult neurogenesis does not occur in RA at all (Reviewed by Balthazart and Ball, 2016). Therefore, we cannot assume that the process of adult neurogenesis is controlled in the same way in NCM as it is in HVC.

Most of what is known about adult neurogenesis in NCM concerns the conditions for survival of new neurons. As mentioned earlier, survival of new neurons in the zebra finch caudal nidopallium (NC, which contains NCM) has been shown to be influenced by a number of internal and external factors. Researchers first established that birds housed in complex social environments had more new neurons than birds housed singly or in pairs (Lipkind et al., 2002). Later studies found that change in social environment influences new neuron survival, but this effect depends upon age of the new neurons in question. Specifically, change from a simple to a complex social environment promotes survival of younger (1 month-old) new neurons, but the converse is true for older (3 month-old) new neurons (Adar et al., 2008). Additionally, these effects on survival depended upon rostrocaudal position within NC.

The researchers have touted their results as evidence of an "anatomical representation of time" in the brain. In this model for memory storage, parts of the brain that are more sensitive to new information (i.e., newer neurons) are used to house recent memories, while parts of the brain that are more "set in their ways" (i.e., older neurons) house older memories.

This research is relatively unique and highly valuable for its focus on the relationship between adult neurogenesis and long-term memory. By elucidating the conditions for survival of new neurons in NC, it provides insights into the process of memory storage in this region. This is a good start, but to fully understand the role of adult neurogenesis in long-term memory preservation, we must understand adult neurogenesis in NCM to the degree that we understand it in HVC. By knowing the types of neurons contained by NCM and the factors – perhaps quality of song learning is a good starting point – that influence their addition in adulthood, we can approach an understanding of why new neurons are needed there and how they operate. My research suggests that new neurons and old neurons respond differently to song playback, and the nature of this difference depends on various features of quality of song learning. My results may indicate that new neurons in NCM encode new memories rather than the tutor song.

Conclusion

Here I have shown for the first time that adult-born neurons in NCM can express ZENK as early as thirty days after their formation. In order to determine if these new neurons are actually contributing to an old memory of the tutor song, this study must be repeated with a larger sample size and a control group who does not receive song playback prior to sacrifice. Nonetheless, it is impossible to overlook the fact that the new neuronal population behaved in a manner opposing that of the general neuronal population for all measures of song similarity tested. Indeed, comparing ZENK expression in the general population to ZENK expression in the new population for each bird revealed a strong negative trend, suggesting that new neurons become more involved in the neural response to song playback when old neurons are less involved. The trends reported here provide useful information about the behavior of new adult-born neurons in NCM and suggest that they are more associated with neural plasticity and learning new information than with the maintenance of long-term memory.

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