A Comparison of Habituation Rates in Adult-Born and General Population Neurons in the Zebra Finch Caudomedial Nidopallium

by

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# Table of Contents

ABSTRACT .......................................................................................................................... 5

INTRODUCTION .................................................................................................................... 7

ADULT NEUROGENESIS ................................................................................................. 7

Methods for Studying Adult Neurogenesis ................................................................. 8

Adult Neurogenesis in Mammals ............................................................................... 9

Querying Functionality ............................................................................................... 11

Turning to Songbirds .................................................................................................. 11

SONGBIRDS AS A MODEL SYSTEM ............................................................................... 12

Song Learning ............................................................................................................. 13

Song Structure ............................................................................................................. 14

BIRDSONG AND THE BRAIN .......................................................................................... 16

Studying the Songbird Brain Using Immediate Early Genes .................................... 16

The Traditional Song System ...................................................................................... 17

Beyond the Song System .............................................................................................. 19

Adult Neurogenesis in the Songbird Brain ............................................................... 19

The Caudomedial Nidopallium .................................................................................... 21

Habituation in the NCM ............................................................................................... 23

EXPERIMENTAL DESIGN AND HYPOTHESES ...................................................... 24

METHODS ........................................................................................................................... 26

BRDU INJECTIONS .......................................................................................................... 26

RECORDING AND PLAYBACKS .................................................................................... 26

IMMUNOHISTOCHEMISTRY .......................................................................................... 30

IMAGING ........................................................................................................................ 30

DATA ANALYSIS ............................................................................................................ 31

RESULTS ............................................................................................................................. 33

Activation of New Neurons .......................................................................................... 33

Comparing Activation and Habituation Rates ......................................................... 33

Hemisphere Effects ..................................................................................................... 38

DISCUSSION ......................................................................................................................... 40

New Neuron Activity in the NCM ............................................................................. 40

Consequences of Plasticity for Zebra Finches ....................................................... 43

Generalizing Up: Applicability in Mammals? ......................................................... 44
List of Figures

FIGURE 1: ZEBRA FINCH SONG STRUCTURE ................................................................. 15
FIGURE 2: ZEBRA FINCH SONG SYSTEM ................................................................. 18
FIGURE 3: EXPERIMENTAL DESIGN ...................................................................... 28
FIGURE 4: CELL LABELING PROTOCOL .................................................................. 32
FIGURE 5: IMMUNOHISTOCHEMICAL IDENTIFICATION OF NEURONS .................... 34
FIGURE 6: TRIPLE-LABELED CELL ........................................................................ 35
FIGURE 7: ACTIVATION RATES OF NEW AND GENERAL POPULATION NCM
   NEURONS ............................................................................................................. 36
FIGURE 8: HABITUATION RATES OF NEW AND GENERAL POPULATION NCM
   NEURONS ............................................................................................................. 37
FIGURE 9: ACTIVATION RATES BY HEMISPHERE IN HABITUATION GROUP .......... 39
Abstract

Adult neurogenesis, or the addition of new neurons to the brain after development, is an incompletely understood process with potential implications for learning and memory. The zebra finch (*Taeniopygia guttata*) song system, which receives new neurons during adulthood and is involved in an auditory learning process that in many ways parallels that of humans, makes them a useful model for investigating these putative roles.

While some suggest that new neurons are activated during learning in much the same way as the general neuronal population, others argue that new neurons have a more plastic role in learning and memory. To probe this question, we examined auditory learning in the songbird caudomedial nidopallium (NCM, analogous to the mammalian auditory association cortex), a region that plays a role in auditory processing. Neurons in the NCM express the immediate early gene *zenk*, a correlate of neural activity, when exposed to novel conspecific song, but the response lessens as the song is repeated. This habituation is specific and long-lasting, suggesting that the NCM is specialized for learning and forming memories of new songs quickly.

To assess the degree to which new neurons participate in song learning, we compared the habituation rate in adult-born neurons and the general neuronal population. One group of birds was repeatedly exposed to a novel conspecific song to induce NCM habituation, while another group heard the song for the first time immediately prior to sacrifice. We used a triple-labeling protocol to stain for a cell birth marker (BrdU), a neuronal marker (Hu), and an activity-dependent immediate early gene protein product (ZENK). With this protocol, a triple-labeled cell was an adult-born neuron activated during song exposure.
Comparing the habituation rates of new and old neurons could reveal their relative roles in song learning. If new neurons habituate at a similar rate as the general neuronal population, this would indicate that they are incorporated into existing auditory learning circuitry and play similar roles as existing neurons. If the drop in new neuron response with song repetition is significantly more than in old neurons, this could point to a more plastic role for adult-born neurons in the NCM. We hypothesized that we would see evidence for the latter possibility. Additionally, we predicted greater activation in response to novel song in the new neuronal population than in the general neuronal population, which would also indicate relatively high plasticity. Our preliminary results support these hypotheses, and also indicate that adult-born neurons have higher baseline activity than general population neurons. Further work must be done to elaborate these findings, but they are suggestive of a role for young adult-born neurons in learning and memory.
Introduction

ADULT NEUROGENESIS

Since the 1990s, work on the topic of adult neurogenesis—the addition of new, functional neurons to the brain after an animal is fully developed—has vastly proliferated. The idea of a brain that can rejuvenate itself has excited many, prompting perhaps too-broad claims about the therapeutic potential of this process (Lowenstein and Parent, 1999). A steady increase in publications on the topic in the past decades has added nuance to these bold hypotheses, and much still remains to be elucidated about the function of these neurons.

Research on adult neurogenesis, however, predates this wide recognition. Altman and others had been presenting evidence for new neurons in adult rodent brains since the 1960s (Altman, 1963; Altman and Das, 1965a, 1965b), though their work was met with much skepticism as it went against basic neurobiological understandings of the time (Kempermann, 2011). Unlike most other cell types in the body, neurons cannot divide, so many concluded that all cells in the brain were born in the perinatal phase. Altman’s early work was unable to demonstrate an alternate mechanism for neurogenesis, and therefore was not taken seriously by many.

The discovery of neural precursor stem cells in the adult brain changed all this, as a plausible pathway for adult neurogenesis became evident (Kilpatrick and Bartlett, 1993; Palmer et al., 1995; Reynolds and Weiss, 2016). This, along with improving methodologies for the study of adult neurogenesis (see Methods for Studying Adult Neurogenesis below), has been instrumental in the expansion and mainstreaming of adult neurogenesis research. Since this time, neurogenesis has been
widely reported in rodents (Cameron et al., 1993; Corotto et al., 1993; Luskin, 1993), non-human primates (Gil-Perotin et al., 2009; Gould et al., 1997, 1999; Kornack and Rakic, 1999; Pencea et al., 2001), and songbirds (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984). Many studies have also suggested that humans exhibit adult neurogenesis (Eriksson et al., 1998; Ernst et al., 2014; Spalding et al., 2013). The debate over the extent to which neurogenesis persists in adult humans remains heated. Within a month of writing this thesis, two major studies have been published on the topic, one that shed doubt on (Sorrells et al., 2018) and one that supported (Boldrini et al., 2018) the hypothesis of persistent hippocampal neurogenesis throughout aging in humans.

Methods for Studying Adult Neurogenesis

Altman and other early adult neurogenesis researchers used autoradiographical techniques to study adult neurogenesis. They injected tritiated thymidine (3H-thymidine), which is incorporates into dividing cells, and used autoradiographic output to visualize 3H-thymidine and assay cell proliferation. Because this technique is not neuron-specific, cells exhibiting this marker then had to be morphologically identified as neurons (Altman and Das, 1965a), which contributed to skepticism around Altman’s work. Nottebohm went a step further, verifying that new cells were indeed neurons electrophysiologically in addition to morphologically (Paton and Nottebohm, 1984). Many found this methodological step more persuasive than morphology alone.

While Nottebohm’s methods were a turning point for general recognition of adult neurogenesis, the dangers and high costs associated with autoradiography
remained a limiting factor. Subcutaneously injected bromodeoxyuridine (BrdU), a thymidine analog, is incorporated in dividing cells in much the same way as 3H-thymidine. Unlike 3H-thymidine, it can be fluorescently stained when combined with one or more additional unique immunohistochemical markers, such as neuronal markers and markers of activity (Gratzner et al., 1975). This has become the method of choice for adult neurogenesis studies and is used in the present study.

Studying adult neurogenesis in humans has remained a challenge. Early research studied neuronal incorporation postmortem in cancer patients injected with BrdU for tumor monitoring purposes (Eriksson et al., 1998). Recently developed methods have made it possible to study adult neurogenesis in postmortem human brains by tracking the presence of radioactive carbon-14 isotopes in DNA and correlating intracellular levels to known global fluctuations in the isotope, expanding the potential subject base (Ernst et al., 2014; Spalding et al., 2005).

Adult Neurogenesis in Mammals

Rodent studies have consistently shown that new neurons are added in the adult hippocampus (Altman and Das, 1965a; Cameron et al., 1993) and olfactory bulb (Altman, 1969; Corotto et al., 1993; Kaplan and Hinds, 1977; Luskin, 1993). In both areas, neurons arise from limited, specific neurogenic regions that contain neural precursor cells and appropriate microenvironments to allow a series of necessary steps to occur, producing neurogenesis (reviewed by Kempermann, 2011). New olfactory neurons originate in the sub-ventricular zone (SVZ) and migrate to the olfactory bulb to differentiate (Corotto et al., 1993; Lois et al., 1996), while new hippocampal neurons originate in the subgranular zone (SGZ) and migrate to the
granule layers of the dentate gyrus (Cameron et al., 1993; Kaplan and Hinds, 1977; Kuhn et al., 1996; Stanfield and Trice, 1988), the area of the hippocampus most affected by neurogenesis.

Spalding et al. have used carbon-14 methods to demonstrate that, like in rodents, hippocampal neurogenesis does occur in human adults. The dynamics of neuronal turnover in the hippocampus differs between the species, with a higher percentage of the hippocampus replaced in humans than in mice. Additionally, in humans but not in mice, adult-born hippocampal neurons are more subject to cell death than the general population of neurons (Spalding et al., 2013). However, recent research that immunohistochemically measured proliferating cells and young neurons in post-mortem human brains has diverged on the extent or even existence of adult neurogenesis in the human hippocampus (Boldrini et al., 2018; Sorrells et al., 2018).

Unlike in rodents, little to no adult neurogenesis occurs in the olfactory bulb in humans (Bergmann et al., 2012). Instead, progeny of neural precursors in the SVZ that would migrate to the olfactory bulb in rodents instead migrate to the striatum, a region associated with reward response and motor control, in humans (Ernst et al., 2014) and some non-human primates (Bédard et al., 2002, 2006; Tonchev et al., 2005). This may be linked to an evolutionary decrease in olfactory bulb volume in primates, associated with a decreased reliance on olfaction for survival (Koscik and Tranel, 2012). There is a corresponding increase in striatal volume in primates (De Winter and Oxnard, 2001). As the striatum is involved in motor regulation, motivated behavior, and cognitive control, its increased size in primates implies increased importance of these functions. Striatal adult neurogenesis also remains controversial, with some questioning Bergman et al.’s findings (Wang et al., 2014).
**Querying Functionality**

Initial excitement about the rejuvenating potential of adult-born neurons has been tempered by increased understanding of its limitations. As described above, the process occurs only in certain brain regions in mammals, and neuronal turnover is limited. Kempermann points out that this raises its own set of questions—if the brain has the flexibility to replace neurons, why not replace more neurons, in other regions of the brain, more regularly (Kempermann, 2011)? Why these neurons only? What are the functions of the adult-born neurons?

While the answers to many of these questions remain unknown, many have speculated that new neurons may contribute to memory. Areas where adult-born neurons are incorporated include the mammalian hippocampus and the bird song system, which both have functions in learning and memory. In mammals, it has been shown that adult-born neurons exhibit increased plasticity, associated with long term potentiation (LTP) and long-term memory, for a short time after they are born (Ge et al., 2007; Nissant et al., 2009; Schmidt-Hieber et al., 2004) Evidence for the role of adult neurogenesis in these crucial cognitive tasks and, as well as potential roles in plasticity, is still debated (reviewed by Leuner et al., 2006), and much research in this area has come from an unlikely quarter—songbirds.

**Turning to Songbirds**

To probe the potential functional roles of adult-born neurons in learning and memory, songbirds have emerged as a useful model system. Even in the 1980s, when skepticism of Altman’s findings in rodents remained prevalent, many were willing to
accept that adult neurogenesis occurs in birds due to their evolutionary distance from mammals. Nottebohm published a series of articles demonstrating that fluctuations in the size of certain areas of the songbird brain, initially presumed to be related to synaptic reorganization of existing neurons (Nottebohm, 1981), could instead be attributed to adult neurogenesis (Alvarez-Buylla and Nottebohm, 1988; Burd and Nottebohm, 1985; Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984).

Songbird adult neurogenesis is described in greater detail below.

Even as adult neurogenesis in mammals and humans become more widely accepted phenomena, birds remain a useful model for studying its mechanisms and potential functions. The hippocampus, which has a major role in learning and memory, is one of the main areas of adult neurogenesis in mammals, so a model system that allows researchers to query the role of adult-born neurons in these cognitive behaviors is crucial. Though songbirds are phylogenetically further removed from humans than other mammals, they offer translational advantages that mice, rats, and even non-human primates cannot parallel. Songbirds exhibit unique behavioral homologies to human vocal learning and therefore are of great utility in invasive studies surrounding the role of neurogenesis in these behaviors.

SONGBIRDS AS A MODEL SYSTEM

Songbirds (oscines) have long been used as a model for cognitive aspects of language production, and many similarities exist between humans and songbirds in the acquisition of learned vocalizations. Both learn primarily from conspecific adult tutors, rely on vocal imitation to learn, and have a period (or periods, for some songbird species) of their lives when this learning occurs most flexibly (Marler,
Learning for both groups takes place in stages (see *Song Learning* below).

Both humans and songbirds also have a series of distinct auditory and motor brain regions involved in the production and learning of their complex vocalizations. The song system of the songbird brain is well-characterized (see *The Traditional Song System* below), as are a variety of molecular and electrophysiological methods for studying it. This suggests that, on top of behavioral homologies, songbirds may also offer us an opportunity to investigate neural correlates of vocal learning.

*Song Learning*

Birds and humans alike undergo multi-phase processes for learning vocalizations. The first phase is almost exclusively auditory, in which speech or songs are memorized. In the second phase, juveniles begin to imitate these vocalizations in a preliminary way—human infants babble and young birds produce subsong, long series of sounds not organized in the same way as adult song—to develop the motor control required for song production. Over time, these emergent vocalizations organize into fully developed speech or song (Marler, 1970).

Among songbird species, zebra finches (*Taeniopygia guttata*) and canaries (*Serious canaria*), two domesticated and easily maintained birds, are the most commonly used in laboratory experiments (Williams, 2004). Zebra finches and canaries represent two of many types of vocal learning seen in songbirds. Canaries are “open-ended learners” who learn new songs every spring (Nottebohm et al., 1986), while zebra finches are “age-limited learners” who learn one song as juveniles and, generally, do not alter this song for their adult life (Immelmann, 1969).

Song learning for zebra finches occurs primarily during a critical period that
ends when the bird reaches sexual maturity at 90-120 days after hatching (Immelmann, 1969; Price, 1979). During this time, in the wild, birds live with a conspecific adult tutor, often their father. Their tutor’s song becomes the template for auditory learning (reviewed by Williams, 2004). After producing subsong, juvenile zebra finches have a period during which their song is pliable as they continue to imitate their tutor. Finally, song crystallizes and becomes stereotyped (Pytte et al., 2007). While crystallized song is not an exact copy of tutor song, it often contains overlapping elements (reviewed by Williams, 2004).

Although crystallized song remains stable throughout an individual’s lifetime, the term “age-limited learner” is perhaps misleading. While zebra finches are not, like canaries, able to learn entirely new songs after crystallization, auditory feedback remains important for the active process of song stabilization. When deafened, crystallized song degrades in zebra finches (Nordeen and Nordeen, 1992). Another “closed-ended learner,” the Bengalese finch, adjusts their pitch to remain stable when exposed to altered playbacks of their song, indicating that auditory learning may continue past puberty for song maintenance purposes (Sober and Brainard, 2009).

**Song Structure**

Zebra finch song bouts generally begin with one or more introductory notes, followed by a repeated motif. Motifs can be further divided into uninterrupted periods of sound production called notes, which in turn can be divided into constituent elements with distinct spectral characteristics. Song bouts may also include shortened versions of a motif with some, but not all, of the notes included (reviewed by Williams, 2004; see Figure 1).
Figure 1: Zebra Finch Song Structure
The structure of a zebra finch song bout (a) and individual song (b).
BIRDSONG AND THE BRAIN

The songbird brain contains a series of interconnected nuclei not present in the brains of avian species that do not produce song, termed the song system. This system is well characterized, although recent work has complicated canonical descriptions by increasingly focusing a series of auditory nuclei outside of the described pathways. Both the song system and other auditory regions provide neural substrate for studying correlates of learning and memory behaviors.

Studying the Songbird Brain Using Immediate Early Genes

Many studies of neural activity in the songbird brain rely on molecular methods measuring the expression of immediate early genes (IEGs) such as zif-268/egr-1/NFNG-A/Krox-24 (zenk). Zenk and other IEGs are associated with plasticity and they produce proteins that are upstream regulators of a number of other genes involved in plasticity, including synapsins (reviewed by Moorman et al., 2011). Many have also suggested that IEGs are crucial for the formation of long-term memories (Goelet et al., 1986; Jones et al., 2001; Wisden et al., 1990). IEG induction is used as a reliable indirect marker of activity in neurons (Jarvis and Nottebohm, 1997).

Early studies frequently used in situ hybridization to attach a radioactive marker to zenk mRNA in brain tissue sections and measure autoradiographic output (Mello et al., 1992). Much like tritiated thymidine, this methodology has become less popular as a non-radioactive alternative has emerged. Immunohistochemical techniques can be used to attach fluorescent labels to induced ZENK protein (Mello and Ribeiro, 1998). For this study, this technique has the added benefit of being compatible with fluorescent labeling of the cell birth date marker BrdU and Hu, a
neuronal marker. The tradeoff of this technique is time scale—it takes longer for the ZENK protein to accumulate after neuron activation than it does for zenk mRNA to accumulate (Kruse et al., 2000).

The Traditional Song System

The song system has traditionally been divided into two general pathways, one specialized for the motor production of song (see figure 2b) and one specialized for auditory learning (see figure 2a). These functional descriptions are over-general, and the pathways have also been referred to as the caudal pathway and the anterior forebrain pathway (AFP) respectively.

The caudal pathway, which is largely located in the posterior part of the brain, projects from the HVC (a letter-based proper name) to the robust nucleus of the arcopallium (RA), and from RA to areas of the midbrain and brainstem that control the bird’s vocal organs and respiration. Lesions to HVC and RA disrupt song production, supporting the role of the caudal pathway in motor control (Nottebohm et al., 1976).

The AFP, reminiscent of a cortico-striatal-thalamo-cortical loop in humans, projects from HVC to Area X, a nucleus in the striatum, to the lateral magnocellular nucleus of the nidopallium (LMAN) via a thalamic relay, then back to Area X (Bottjer et al., 1989; Luo et al., 2001; Vates et al., 1997). Both Area X and LMAN activate in response to conspecific song (Margoliash, 1986; Solis et al., 2000), and a series of lesion studies support AFP’s role in song learning. Lesions to LMAN cause young zebra finches to develop abnormal song but do not majorly disrupt crystallized song in adult birds (Bottjer et al., 1984), though slight natural variations in an adult’s
Figure 2: Zebra Finch Song System
A schematic representation of major auditory perception (a) and vocal production (b) regions in the songbird brain. (a) highlights in yellow regions outside of the canonical song system that activate in response to auditory stimuli. (b) includes the nuclei of the song system pathways, with areas that activate when a bird sings highlighted in orange. Abbreviations: CLM, caudal lateral mesopallium; CMM, caudal medial mesopallium; DLM, nucleus dorso-lateralis anterior, pars medialis; HVC, acronym used as a proper name; L1, L2, L3, subdivisions of Field L; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NCM, caudal medial nidopallium; nXIIIts, tracheosyringeal portion of the nucleus hypoglossus; RA, robust nucleus of the arcopallium (Bolhuis et al., 2012).
song are eliminated (Kao et al., 2005). Area X lesions also cause abnormal song development, though Area X lesioned birds exhibit different behavioral phenotypes than LMAN lesioned birds (Scharff and Nottebohm, 1991).

**Beyond the Song System**

Beyond these canonical pathways, a variety of other nuclei appear to be involved in auditory processing. These include Field L, the nidopallium terminal of ascending pathways from the ears; the caudomedial nidopallium (NCM); the caudomedial mesopallium (CMM); and regions surrounding HVC and RA (termed the sehlf of HVC and the cup of RA; reviewed by Moorman et al., 2011). NCM and CMM are higher-order regions that are likely involved in song learning and memory in addition to basic auditory processing (reviewed by Bolhuis and Gahr, 2006). Recent focus on these regions, as well as mounting evidence that both canonical pathways are implicated in both song production and learning, have moved the field away from the clearly segmented view of song production described above.

**Adult Neurogenesis in the Songbird Brain**

Neurogenesis in adult songbirds is more widespread than in mammals, with new neurons added throughout the telencephalon, including in HVC (Alvarez-Buylla et al., 1990; Goldman and Nottebohm, 1983) and NCM (Adar et al., 2008; Pytte et al., 2010; see *The Caudomedial Nidopallium* below). In a similar manner as in rodents, new neurons in songbirds arise from neural precursors in localized neurogenic zones, though for birds these are “hotspots” in the ventricular wall of the subependymal zone as opposed to the SVZ (Alvarez-Buylla et al., 1990). Once born, new neurons migrate
long distances relatively quickly, aided by the processes of radial glia (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988).

A wide variety of factors have been shown to contribute to the degree of adult neurogenesis or the survival of the resultant new neurons, including factors related to learning. The number of new neurons is correlated to season (Kirn et al., 1994). In canaries, song learning is a seasonal process, so seasonal fluctuations in neurogenesis have been related to behaviorally-mediated effects of learning (Alvarez-Buylla et al., 1990; Kirn et al., 1991). Increased testosterone levels, associated with seasonal learning in males, has been shown to promote HVC neurogenesis in female birds (Goldman and Nottebohm, 1983).

Many have also proposed an element of experience-dependent regulation in less correlative ways than seasonal studies. Decreasing the activity of RA neurons, which are downstream from HVC projections, cut the number of new RA-projecting neurons in HVC by more than half, suggesting a post-synaptic activity-related regulation (Larson et al., 2013). In the auditory region NCM, deafened birds were shown to have a lower density of adult-born neurons than hearing birds (Pytte et al., 2010). These findings imply either that when new neurons are not being incorporated into functioning, active circuitry, they are more likely to die, or that lack of functional activity among existing neurons down-regulates neurogenesis in these areas. On the other hand, Scharff et al. (2000) have shown that selectively ablating HVC neurons that are often replaced during adulthood promotes neurogenesis, suggesting that when functioning neurons are lacking, they may be added.
While many of these results are suggestive of a role for new neurons in the learning and memory of song, much remains to be elucidated surrounding the precise functionality of adult neurogenesis in songbirds.

The Caudomedial Nidopallium

As outlined above, the NCM is an upstream auditory area analogous to the auditory association cortex in mammals (Bolhuis and Gahr, 2006). The majority of neurons in NCM are inhibitory (Pinaud and Mello, 2007). The NCM and nearby CMM are the highest-order areas involved in auditory processing the songbird brain, receiving projections from the primary auditory cortex Field L. NCM has roles in the processing of conspecific song (Mello et al., 1992) and has been suggested as a substrate for the memory of their tutor’s song (Bolhuis et al., 2000; Phan et al., 2006). NCM neurons’ responses to auditory cues are specific—IEG expression is higher in response to conspecific song than to heterospecific song, and no IEG induction is observed in response to pure tones (Mello et al., 1992). Additionally, preventing ZENK induction in NCM and other auditory processing areas selectively disrupts normal song memorization without affecting motor function or other auditory response patterns (London and Clayton, 2008). These results suggest that NCM is critical for auditory learning in addition to other auditory processing behaviors.

Adult neurogenesis occurs in the NCM, but little is known about the dynamics of neuronal turnover. Survival of new neurons in the NCM is impacted by the complexity of their social environment (Adar et al., 2008; Barnea et al., 2006; Lipkind et al., 2002), and deafening reduces the number of new neurons seen (Pytte et
al., 2010), suggesting that auditory stimuli and, potentially, accompanying social cues, are important for maintaining new neurons.

Peak neurogenesis in NCM occurs 40 days after the bird hatches, declining at 60 and 120 days respectively, though neurogenesis remains higher for longer if the critical period is artificially lengthened by isolating young birds (Asik, 2016). This means that, like in the HVC, NCM neurogenesis is associated with the critical period for song learning. Tsoi (2016) also found associations between NCM neurogenesis and song learning, though they were less straightforward. She demonstrated that the degree to which a bird copied their tutor’s song, a measure of the quality of song learning, is proportional to the differences between the number of new neurons added in each hemisphere of the NCM. However, she found no clear correlation between the degree to which birds learned novel conspecific songs and NCM neurogenesis rates (Tsoi, 2016). Preliminary results from our lab suggest that adult-born neurons may have a more plastic role than the general neuronal population of NCM, which could also point to a role in learning and memory (Junkins, 2016).

While these findings indicate potential correspondences between song learning and adult neurogenesis in the NCM, they establish neither causality nor the roles of these new neurons in memory tasks. They also leave open questions about the roles of new neurons in learning novel conspecific songs, as they mainly investigate learning tutor song. Much work remains to elucidate the dynamics of adult neurogenesis and the roles of young neurons in the NCM. Habituation of NCM neurons, a well-characterized behavior thought to be a correlate of learning, provides a useful avenue to pursue these questions.
Mello et al. (1992) demonstrated that for a novel conspecific song playback of ten to thirty minutes in length, neuronal activity in the NCM increased linearly. In a follow-up study, however, they found that when playbacks exceeded thirty minutes in length, activity would begin to decline until reaching levels that were not significantly different from those in control birds that received no auditory stimulus at all (Mello et al., 1995). They also presented evidence that this habituation effect could last at least a day — after five days of training on a single repeated song followed by one day of auditory isolation, activity levels were statistically indistinguishable from those in controls. Birds that had not undergone this training and were exposed to a novel song, as well as birds that had trained on one song and then were exposed to a novel song, had significantly higher neuronal activity than the controls or the habituated birds (Mello et al., 1995). This response is consistent with the hypothesis that IEGs are important in forming long-term memories (reviewed by Goelet et al., 1986; Wisden et al., 1990).

Follow-up studies have found similar habituation-like responses in neuron electrophysiology (Chew et al., 1995, 1996; Stripling et al., 1997) and bird behavior (Stripling et al., 2003). This response appears to be highly specific to both the precise auditory cue of a conspecific song and non-auditory contextualizing factors. A song to which neurons had habituated, when played again to the bird backwards, was treated as entirely novel (Chew et al., 1996), suggesting that the neurons respond to more than the spectral characteristics of a song. Neurons dishabituated when, after training on a song, playback speakers were moved, playback volume was decreased, or song bouts were paired with visual cues (Kruse et al., 2004). Habituation is also
long-lasting—habituation to novel songs has been shown to last 48 hours (Chew et al., 1995), though the ability of the NCM to retain tutor song for at least a month suggests the effect may last even longer (Phan et al., 2006). The specific and long-lasting nature of this neuronal behavior further supports its posited role in memory formation, and NCM habituation is frequently used as a correlate of song memory.

This memory system has a high capacity, with both male and female zebra finches habituating to 16 individual stimuli when trained sequentially, hypothesized not to be nearing the memory limit (Chew et al., 1996). Being able to discriminate between songs of other conspecifics is useful for zebra finches of both sexes in the wild, and this high capacity is consistent with habituation as a familiarity marker.

Habituation of neurons in the NCM represents a clear, specific, well-characterized neuronal behavior that has been correlated to learning and memory. As such, it provides a useful locus for investigating the contribution of adult-born neurons to these complex cognitive processes. This study characterizes the habituation behavior of adult-born neurons in the NCM, as compared to the NCM’s general neuronal population (born during perinatal development), to investigate if any differences exist in these patterns.

EXPERIMENTAL DESIGN AND HYPOTHESES

When comparing the habituation rate of adult-born and general population neurons, we anticipate three potential outcomes. The first is that new and old neurons habituate at the same rate, indicating that new neurons are incorporated into functional circuitry and act in much the same way as the general population. The second is that new neurons habituate at a higher rate than the general population,
which would suggest that these neurons have a specialized, plastic role in learning and memory. The third, and most perplexing, possibility is the general population habituates at a greater rate than the new neurons. Were this the case, it would imply that new neurons are not as active in learning and memory behaviors as the other neurons in this region, leaving open the question of what they are doing instead.

We hypothesized that we would find evidence for the second scenario—new neurons habituating at a greater rate than the general neuronal population. We additionally predicted that new neurons would respond at a higher rate to novel conspecific song in a group of birds that was not habituated by song repetition. Many have suggested that young neurons play a specialized, plastic role in the adult rodent brain, especially related to memory (Ge et al., 2007; Nissant et al., 2009; Schmidt-Hieber et al., 2004). Our hypotheses are based on the premise that this applies to young neurons’ role in the songbird brain, especially as it pertains to song learning. The preliminary results described in this study support both of these hypotheses.

We used a protocol based on the experiment by Mello et al. (1995; discussed at length above) that first described the NCM habituation effect. We slightly altered their method by using a longer playback prior to perfusion (one hour instead of thirty minutes) to accommodate our use of IEG immunohistochemistry instead of the in situ hybridization performed in the original paper. This method allowed us to easily combine measures of neural activity with cell birth marker BrdU, and Hu, a neuronal marker, so we could explore functions of new neurons. The staining method necessitated a longer playback period prior to perfusion to ensure the induction of the ZENK protein during activity (see above), but this was the only major change to the protocol described in Mello et al.
Methods

SUBJECTS

This study examined 16 zebra finches (Taeniopygia guttata) raised in closed colonies in the Kirn laboratory at Wesleyan University. Due to time limitations, results for only 6 birds were quantified. Birds reflected in these results were raised in single family (n = 3) or multi-family (n = 3) housing. All subjects were moved to single-gender group housing for injections and isolated in custom sound-attenuating chambers for playbacks. Access to food and water was ad libitum, and birds were maintained on a 14/10 light/dark cycle. All animal use was in accordance with the Wesleyan University Institutional Animal Care and Use Committee.

BrdU INJECTIONS

BrdU injections began at approximately 90 days post-hatch (mean = 129, range = 85-229, standard deviation = 45.26). Subjects were injected twice a day for four days with 0.05 mL of bromodeoxyuridine (BrdU; Roche Diagnostics GmbH, Manneheim, Germany) in solution. For each injection, birds were removed from their cages and replaced after sufficient recovery time.

RECORDING AND PLAYBACKS

At approximately 30 days after their first injection (mean = 35, range = 30-47, standard deviation = 5.33), birds were moved to custom-built sound-attenuating chambers for playback and recordings. These chambers contained microphones that were connected to a recording system (FireStudio Project; PreSonus, Baton Rouge,
LA), as well as identical speakers (Harman Kardon, Stamford, CT). At this point, subjects were divided into a habituation group, a non-habituation group, and a control group (see Figure 3).

After a one-day acclimation period, birds in the habituation group began a five-day playback period. During this time, birds were exposed to three identical one-hour playback sessions each day. All playbacks were constructed using a single three-second conspecific song, recorded from an individual in a colony at Rockefeller University that none of the subjects had any contact with to ensure song novelty. Using the sound editing program Audacity®, the sound clip was amplified and background noise was removed. This recording was repeated five times for a fifteen second song “bout,” followed by forty five seconds of silence, for each minute of each hour-long playback, which played at a peak amplitude of approximately 70 dB. One day after the final training, birds received one more identical hour-long playback immediately after the lights were turned on in the morning. This timing was chosen because birds are generally less active in the morning, and excessive motion or vocalizations could have been a confounding variable for brain activity. After the final playback, birds were sacrificed (see Perfusions and Sectioning below).

The non-habituation and control groups were in the sound-attenuating chambers for the same length of time as the habituation group (7 days) but remained in silence, with no playbacks. On the final morning, birds in the non-habituation group received a one-hour playback, in the manner described above, prior to sacrifice. Birds in the control group were kept in silence for the hour after the lights turned on and then were sacrificed.
Figure 3: Experimental Design
A summary of the injection and playback paradigm used for the three experimental groups.
During the final hour prior to perfusion, all birds were recorded to determine whether or not they sang.

PERFUSIONS AND SECTIONING

Birds were removed from sound-attenuating chambers and deeply anesthetized using methoxyflourine (Metofane; Schering-Plough Animal Health, Union, NJ). When unresponsive to a toe pinch, they were quickly perfused. 10 mL of 0.01 M phosphate-buffered saline (PBS) were delivered via the left ventricle, followed immediately by 50 mL 4% paraformaldehyde in 0.01 M PBS (PFA).

The brains were removed from the skull and stored overnight in PFA at 4º C. Over the subsequent two to three days, brains were sunk in 10%, 20%, and 30% solutions of sucrose in 0.01 M PBS, also at 4º C. They remained in the 30% sucrose solution until sectioning.

Brains were then cut into hemispheres and sectioned with a freezing microtome at a thirty-micron thickness. Sections were stored in a cryoprotectant anti-freeze solution (500 mL 0.1 M PBS, 300 g sucrose, 10 g polyvinyl pyrrolidone, 300 mL ethylene glycol, dH₂O to bring to a final volume of 1000 mL) at -20º C until immunohistochemistry could be performed. Brains were frozen to the freezing microtome with the medial side down, so the most medial portion of the brain could not be sectioned. This remaining medial piece was thawed and stored in cryoprotectant anti-freeze solution as well. It was then measured and used to estimate the coordinates of all sections (see Imaging below).
IMMUNOHISTOCHEMISTRY

All immunohistochemistry was performed on free-floating sections. Sections were rinsed three times in 0.01 M PBS for ten minutes each. They were then permeabilized in PBST for thirty minutes and incubated in 1.5N HCl for 20 minutes at 27ºC. This was followed by two five-minute neutralizations in Trisma base, and then three ten-minute washes in PBST. Sections were blocked using a 3% Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO) solution for two ten-minute periods. At this point, sections were incubated in a primary antibody cocktail containing a 1:100 dilution of rat anti-BrdU (Bio-Rad, Hercules, CA), a 1:500 dilution of rabbit polyclonal anti-Egr-1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and a 1:100 dilution mouse monoclonal anti-Hu (Invitrogen, Eugene, OR). This incubation was either done overnight at 4º C or for two hours at room temperature, with both processing methods yielding the same results.

After the primary incubation, sections were washed three times for ten minutes each with PBST, then incubated for an hour at room temperature in a secondary antibody cocktail containing 1:500 dilutions of Alexa Fluor 555 goat anti-rat (Invitrogen, Eugene, OR), Alexa Fluor 488 goat anti-rabbit (Invitrogen, Eugene, OR), and Alexa Fluor 647 donkey anti-mouse (Invitrogen, Eugene, OR). Finally, three more ten-minute PBST washes were done. Sections were mounted and coverslipped with Aqua-Mount for examination on a confocal microscope.

IMAGING

For each subject, the most medial portion of the brain, which remained unsectioned, was examined on the confocal microscope. In all subjects reflected in
these results, this medial piece contained intact NCM or hippocampus just dorsal to the NCM, and the thickness of this region was measured. Using the thickness and the relative location of each slice, distances from the midline in micrometers was estimated. The most intact medial slices, all under an estimated 1050 micrometers from the midline to ensure that they contained the NCM, were chosen for analysis.

Each of the chosen sections was then imaged on a confocal microscope (ZEISS LSM 510; Jena, Germany). Three Z-stacks were taken in each section at a magnification of 25x in three color channels (though not all images were quantified). Images were imported into ImageJ (NIH, Bethesda, MD), where cells were counted manually using the Cell Counter plugin. All cell counts were performed blind to the animal’s identity. Multiple individuals performed cell counts, but a single individual manually corroborated these counts to ensure inter-observer reliability. Hu+ cells (neurons), Hu+/ZENK+ cells (active neurons), Hu+/BrdU+ cells (adult-born neurons), and Hu+/ZENK+/BrdU+ cells (active, adult-born neurons) were counted (see Figure 4).

DATA ANALYSIS

For each bird, the counts of Hu+ and Hu+/ZENK+ cells from six Z-stacks (two images from each of three sections) were totaled, as were the counts of Hu+/BrdU+ and Hu+/ZENK+/BrdU+ from nine Z-stacks (three images from each of three sections). The proportion of general population and adult-born neurons that were active were calculated. Due to the small number of subjects, no inter-group statistics were performed. All calculations were done in Excel (Microsoft; Redmond, WA). Plots were created in MATLAB (MathWorks; Natick, MA).
Figure 4: Cell Labeling Protocol
A schematic representation of the three immunohistochemical labels used and which overlapping labels were counted, along with their biological significance.
Results

ACTIVATION OF NEW NEURONS

Data were generated by counting the number of new neurons added to the NCM, as well as the proportion of new neurons and general population neurons that were active during the playback the hour prior to perfusion. An example of a new, active neuron (triple labeled) is depicted in Figures 5 and 6, as contrasted with an inactive new neuron and active and inactive neurons from the general population.

COMPARING ACTIVATION AND HABITUATION RATES

We compared the rates of activation in new and general population NCM neurons in the three experimental groups—control birds, non-habituated birds, and habituated birds—and used these to calculate the rate of habituation in the two neural populations. We found that, across all three experimental conditions, a greater proportion of adult-born neurons were active than general population neurons, though this trend was not as pronounced in the habituation group (see Figure 7). For general population neurons, the habituation effect did not reduce activity to baseline levels seen in control subjects, with habituated activity at 165.21% of baseline. This was not seen to the same degree in new neurons, which activated at 113.26% of baseline in the habituation group. The rate of habituation, calculated as the percent decrease from the non-habituation to habitation group was over twice as high for new neurons (30.31%) than for general population neurons (14.07%; see Figure 8).
Figure 5: Immunohistochemical Identification of Neurons
Merged z-stack of immunohistochemically stained tissue taken on confocal fluorescence microscope at 50x magnification. Neurons are labeled with Hu (blue; a), active cells are labeled with ZENK (red; b), and new cells are labeled with BrdU (green; c). A merged image (d) highlights a new, active neuron (Hu+/BrdU+/ZENK+; red box); a new, inactive neuron (Hu+/BrdU+/ZENK-; yellow box); an active neuron from the general population (Hu+/BrdU-/ZENK+; green box); and an inactive neuron from the general population (Hu+/BrdU-/ZENK-; white box).
Figure 6: Triple-Labeled Cell
A close-up, taken at 75x magnification, of a merged z-stack highlighting the triple-labeled cell (red box). This cell is a new, active neuron.
Across the three experimental groups, more active neurons were seen in the new population than in the general population. Habituation did not reduce activation levels to baseline in either population, though it came closer to doing so in the new neuronal population. Number of subjects in each group was 1, 3, and 2 respectively.

**Figure 7**

Across the three experimental groups, more active neurons were seen in the new population than in the general population. Habituation did not reduce activation levels to baseline in either population, though it came closer to doing so in the new neuronal population. Number of subjects in each group was 1, 3, and 2 respectively.
New neurons exhibited a greater loss of activity (30.31%) after training on a song—that is, a greater degree of neuronal habituation—than general population neurons (15.66%).

Figure 8
New neurons exhibited a greater loss of activity (30.31%) after training on a song—that is, a greater degree of neuronal habituation—than general population neurons (15.66%).
HEMISPHERE EFFECTS

The habituation group contained two subjects. For one of those subjects, the left hemisphere was examined, while for the other, the right hemisphere was examined. This was the only group to contain examples from both hemispheres. While inter-subject differences between only two subjects have severely limited interpretive utility and may not generalize, they did provide the opportunity to look at differences in neuronal activity between hemispheres. Another major difference between these subjects, besides hemisphere, was the new neuron incorporation time, or the days between the first injection and perfusion. The right hemisphere subject had 38 days for neuronal incorporation, while the left hemisphere subject had 47.

Activity patterns differed between the two subjects (see Figure 9). Both new and general population neurons had higher activity levels in the left hemisphere subject than the right, though this trend was much more pronounced in the new neuronal population. In the right hemisphere subject, new neurons exhibited lower ZENK expression than general population neurons, counter to the trends seen in group averages and in the left hemisphere subject. Relative difference in activity between new and general population neurons was greater in the left hemisphere subject.
Figure 9
Differences in activation rate in the new and general neuronal populations in the two habituated subjects. Higher activation levels were seen in the left hemisphere than in the right hemisphere in the new neuronal population (102.63% increase in ZENK expression in the left compared to the right hemisphere). In the general neuronal population, the left hemisphere was also more active, but only slightly (11.30% increase in ZENK expression). Activation rates were higher in the general population than the new neuronal population in the right hemisphere, but this trend was reversed in the left hemisphere.
Discussion

Our findings support both of our major hypotheses—we saw trends towards a higher degree of habituation in adult-born NCM neurons than general population neurons, as well as an increased response to novel song in the new neurons. While these results are limited by a variety of factors (see Potential Confounds below) including, crucially, that they represent too few subjects to determine statistical significance, they support a putative plastic role for young adult-born neurons in the zebra finch song system and raise additional questions. Little is known about patterns of neurogenesis in NCM, and less still about activity of those new neurons after incorporation into existing NCM circuitry, so much further elucidation will be required to contextualize these results.

NEW NEURON ACTIVITY IN THE NCM

This study examined habituation in the NCM. Because habituation is specific (Chew et al., 1996; Kruse et al., 2004), long-lasting (Chew et al., 1995; Mello et al., 1995), and has the capacity to occur for many songs in a short period of time (Chew et al., 1996), it has been hypothesized that it is a neural correlate of forming memories of and learning conspecific songs. Habituation in the ZENK response is particularly interesting in this regard, as ZENK and other IEGs are required for LTP, crucial to the formation of new memories (reviewed by Davis et al., 2003; Jones et al., 2001).

Our results show that habituation to repeated conspecific song happens at a higher rate in adult-born neurons in NCM than in NCM’s general neuronal population. These trends suggest that adult-born neurons are involved in long-term
memory formation to a greater degree than other neurons in the NCM. This would be consistent with the putative role of new neurons in plasticity and learning.

While the habituation rate in new neurons is higher than in the general neuronal population, this trend may be at least partially attributable to the fact that far less habituation was seen in the general neuronal population than anticipated. Mello et al. (1995) measured approximately a four-fold increase in zenk mRNA induction in non-habituated birds as compared to control animals. Levels were virtually indistinguishable between the control and habituated groups. In contrast, we see a less dramatic increase from the control to the non-habituation group in both new and general population neurons and habituation ZENK levels farther from baseline control levels than expected, especially in the general neuronal population.

Our methodology approximated as closely as possible that of Mello et al. (1995), but we were limited by the fact that, due to our need to measure both BrdU and ZENK, we had to measure ZENK protein rather than zenk mRNA. No study, to our knowledge, has attempted to measure habituation using IEG protein expression. As protein takes longer to be induced than mRNA, our final playback was twice the length of that described in the original study—an hour instead of half an hour. The 1995 study reported depressed activation levels in response to song after only sixty minutes, suggesting that our playback time would be sufficient to cause habituation even in naïve non-habituated subjects.

While theoretically, by perfusing an hour after beginning the playback, we were measuring ZENK expression that was induced around the start of the playback session, the mechanisms of ZENK expression are incompletely understood. It is possible that our change to the protocol and our choice to measure protein instead of
mRNA is responsible for the lower-than-anticipated increase in expression in the non-habituated group. Additionally, in the vocal control center RA, increases in zenk mRNA induction do not always correspond with an increases in ZENK protein levels, a decoupling that seems to be mediated by behavioral development (Whitney et al., 2000). While this has only been seen in areas associated with song production, it is possible that, due to a similar behaviorally mediated dissociation, the habituation effect is much more pronounced in mRNA than in protein levels. However, a variety of other confounds may also have contributed to this unexpected result (see Potential Sources of Error below). More data must be examined to determine if our methodology is appropriate for measuring NCM neuron habituation.

In every experimental condition, we also observed higher ZENK expression in adult-born neurons than we did in the general neural population, suggesting greater baseline activity. While we hypothesized that we would see increased activation in response to novel conspecific song in the new neurons, as this would be consistent with a plastic role in learning new song, the fact that this trend is present in the habituation and control groups is puzzling. As discussed above, the habituation rate of new neurons still exceeded that of the general neuronal population despite higher levels of ZENK expression in new neurons than general population neurons in the habituation group. Habituated birds also exhibited activation levels similar to those of control birds in the new, but not the general, neuronal population. Thus, relative to the new neurons in the non-habituated and control groups, these results are expected.

Interpreting the heightened baseline firing rate in a condition with no stimulus, however, is more complex. As the single bird included in the control group in the present data was relatively inactive, singing only two bouts during the hour prior to
perfusion, these neurons were likely not responding to the bird’s own song. This suggests that alongside increased plasticity, increased excitability in the absence of auditory stimulus may be a feature of young adult-born neurons in NCM. Interestingly, in zebra finches younger than 20 days old, zenk induction in NCM is high even without auditory stimulation (Stripling et al., 2001). Our results suggest that constitutively high IEG expression may be a feature of young neurons in adult birds as well.

CONSEQUENCES OF PLASTICITY FOR ZEBRA FINCHES

A plastic, high capacity system for recognizing conspecific songs could be highly beneficial for zebra finches and other songbirds in the wild. The necessity of recognizing a variety of conspecific songs and well as the behavioral correlates of doing so have been widely reported in many songbird species (Krebs, 1976; Kroodsma and Byers, 1991; Searcy et al., 1981). Based on the habituation response, which encodes novelty and is thought to correlate to long-term memory formation, it seems that the NCM may be specialized for this task (Chew et al., 1995). As the expediency of learning new songs likely remains high throughout life, with birds consistently encountering new conspecifics, it makes sense to have a flexible and plastic system to continue forming new memories as the bird ages.

Our results suggest that such a system may be found in the addition of new neurons to this region during adulthood. Much remains to be elucidated about the dynamics of neurogenesis in the NCM, including survival rates of new neurons and the degree to which new neurons are replacing dying neurons, and it is challenging to interpret these results without this framework. However, our preliminary data support
the hypothesis that new neurons are specialized for song learning. As habituation of NCM neurons is thought to be associated with memory formation, a greater degree of habituation in new neurons compared to general population neurons suggests they may have an enhanced role in this process. Additionally, high firing rates in response to novel song may indicate that new neurons encode novelty to a greater degree than the rest of the neuronal population. Both memory formation and novelty encoding could contribute to this putative regenerating song learning system. Such a system, in which young neurons are constantly added to the NCM to increase circuit plasticity, could help explain mechanistically songbirds’ ecologically necessitated ability to learn many new songs quickly.

GENERALIZING UP: APPLICABILITY IN MAMMALS?

The songbird system presents a unique opportunity to study the mechanisms of vocal learning with a behavioral model analogous to humans. Additionally, widespread neurogenesis in the adult songbird brain provides a range of ways to study the process of adult neurogenesis and its functional implications. However, birds and humans are evolutionarily far removed from each other. Despite homologies, mammalian and avian species differ in many ways on every level from cellular to behavioral, so we must use caution when attempting to make general statements about adult neurogenesis.

Even when only considering anatomical locations in which adult neurogenesis occurs, inter-species differences abound, including between mammal species, and controversy among the scientific community remains prevalent. While rodents exhibit neurogenesis in the hippocampus (Altman and Das, 1965a; Cameron et al., 1993) and
olfactory bulb (Altman, 1969; Corotto et al., 1993; Kaplan and Hinds, 1977; Luskin, 1993), localization is less clear in humans.

It is well-accepted that olfactory bulb adult neurogenesis does not occur at high rates in humans (Bergmann et al., 2012). Some say neurons born in the SVZ that would migrate to the olfactory bulb in rodents instead are incorporated into the striatum (Ernst et al., 2014), but others cast doubt upon this (Wang et al., 2014). Some have argued that hippocampal neurogenesis is relatively consistent between adult humans and rodents (Eriksson et al., 1998; Spalding et al., 2013; Boldrini et al., 2018), while new evidence questions whether hippocampal adult neurogenesis exists at all in humans (Sorrells et al., 2018). Debate as to whether adult neurogenesis exists in humans, seemed to have settled in the 1990s, has been reopened with the recent persuasive results of Sorrells et al. (2018), though their study examined only the hippocampus, not the striatum, and has yet to be replicated. In fact, equally recent results suggest exactly the opposite (Boldrini et al., 2018), and this debate will likely continue to develop. Regardless, in birds, incorporation of new neurons is widespread, not limited to certain brain areas like for mammals (Alvarez-Buylla et al., 1994). These basic mechanistic differences and open questions pose challenges to applicability of songbird neurogenesis studies.

When it comes to functionality, there is similar disagreement in the field. The relationship between adult neurogenesis and learning is not consistent between species, and much conflicting evidence exists (reviewed by Leuner et al., 2006). However, many have demonstrated that young neurons have enhanced plasticity in mice (Ge et al., 2007; Nissant et al., 2009) and rats (Schmidt-Hieber et al., 2004). As this plasticity is associated with high rates of LTP and correlated to long-term
memory behavior, this evidence points to memory-related functionality for young adult-born neurons in mammals. Our results suggest that high plasticity, and thus enhanced role in memory, for young neurons may converge between songbirds and mammals. Further inter-species comparative work will be crucial for allowing us to understand any fundamental functions of adult neurogenesis, as well as evolutionary divergences in adult-born neurons’ roles.

POTENTIAL SOURCES OF ERROR

Many variables in our study could have affected the rate of neurogenesis or the degree of NCM neuronal activation. While our data are too limited to assess the contributions of most of these factors to the results presented here, a brief overview of potential confounds is provided to help understand the caveats around interpretation.

The subjects presented here are strongly biased towards the left hemisphere, with only one out of six tissue samples obtained from the right hemisphere. We compared neuronal activation between this one subject and a matched left hemisphere pair in the same experimental group. Lateralization of habituation and response to conspecific song has been shown in electrophysiology (Phan and Vicario, 2009) and IEG studies (Tsoi et al., 2014). While we were unable to estimate habituation rates for each hemisphere, our activation results suggest that responses of new neurons differ more between hemispheres than responses of general population neurons. Despite the severe limitations of this data, this raises questions about lateralization and neurogenesis. More work must be done to characterize similarities or differences in lateralized response patterns between new and general populations.
Another factor that may have affected our data was whether or not birds sang during their final playback. Some neurons in NCM respond to the bird’s own song (Yanagihara and Yazaki-Sugiyama, 2016), so it is possible that singing could increase activity even in auditory areas. While playbacks were performed immediately after the lights turned on in the mornings to minimize birds’ levels of activity, all but two subjects reflected here sang extensively in response to the recorded playback. The other two birds sang less than three song bouts each, so their singing behavior is counted as negligible. Our data were too limited to directly examine this effect.

Time between labeling new neurons and final playbacks and perfusions—that is, the time allowed for new neurons to be incorporated into the brain—may also have affected the trends seen here. New neuron plasticity in mice is restricted to a critical period of approximately one to 1.5 months after neuron birth (Ge et al., 2007). The incorporation period varied between our subjects, ranging from 37 to 52 days to allow at least a full 30 days between injections and the start of training. If a similar limit to young neuron plasticity exists in the songbird brain, this variability could affect the activity patterns we see. As we have too few subjects to look more closely at correlations between incorporation time and activity levels, this remains speculative (see Future Directions below).

Other factors, including bird age, incorporation time, hemisphere, and degree of auditory stimulation during isolation may have affected degree of neurogenesis and new neuron survival, though the precise effects of these variables in NCM is unclear. Regardless, our activation data for the new neuronal population are presented as ratios, which should be unaffected by rates of new neuron addition or survival.
Hemisphere, singing during playback, and time until incorporation may have influenced response patterns, as may have the differences between our protocol and other studies of NCM IEG habituation. Despite these limitations, our results and their implications for plasticity suggest many exciting directions for future work.

FUTURE DIRECTIONS

Much remains to be done in order to more fully interpret these results and their potential applicability to mammals. First, this study could be extended in multiple ways. The initial number of subjects in the present study was much larger, and tissue has been processed for 10 additional birds that are not represented in the results here. Including these birds would expand the control group to 3 birds, the non-habituation group to 7 birds, and the habituation group to 6 birds. This would be enough subjects to measure statistical significance.

The study could also be expanded methodologically. Electrophysiologically examining adult-born NCM neurons would allow us to examine habituation dynamics on a finer time scale and within a single bird. Paton and Nottebohm (1984) pioneered a technique for recording from new neurons in the canary HVC, but did not report differences in auditory response patterns between new and general neuronal populations. Applying a technique like this could give us the capacity to examine habituation on a finer scale within single subjects. This study is also correlative, and causal data linking new neurons to song learning, such as data obtained by selectively ablating adult-born neurons, could help us understand better why new neurons are added to the NCM at all. As no ablative methodology exists at this time for birds, further correlative work could at least reveal nuances in the role of this plasticity.
As discussed above, this work suggests that previous findings of increased plasticity in young neurons in rodents could be applicable in the bird song system. In the mouse hippocampus, enhanced LTP amplitude and lowered threshold for LTP induction persist for a critical period spanning from one to 1.5 months after neurons are born (Ge et al., 2007). NCM habituation could be used as a measure of functional plasticity in new neurons in birds. Systematically varying the amount of time to incorporation and measuring this response could allow us to establish whether a similar critical period timeline exists for adult-born neurons in birds as in mammals.

The extent of this putative plasticity must also be assayed. Certain neurons in NCM are tuned to respond primarily to tutor song, to the bird’s own song, or to both (Yanagihara and Yazaki-Sugiyama, 2016), and many hypothesize that NCM is a substrate for tutor song (Bolhuis et al., 2000; Phan et al., 2006). This then raises questions about whether new neurons’ increased plasticity in song memory is also involved in the memory of the bird’s own song and the tutor song, even if these memories were formed prior to their genesis. Comparing response patterns in adult-born and general populations of neurons to these other stimuli could elucidate the functional significance of this plasticity.

Prior to answering questions of comparative activation in these populations, there is also much to be learned about the dynamics of adult neurogenesis in NCM. We have a good understanding of the timeline of adult neurogenesis in the HVC, projections of new neurons, and factors that affect the rate of neurogenesis (reviewed by Barnea and Pravosudov, 2011), but lack this basic knowledge in NCM. Gaining such fundamental information will allow us a more thorough framework within which to trace potential confounds and interpret the preliminary results presented here.
Conclusion

In the NCM, where the behavior of adult-born neurons is very incompletely characterized, information about activity patterns of young neurons, especially as distinct from the general neuronal population, may be crucial for elucidating the roles of these neurons. The preliminary results reflected in this study point towards young adult-born neurons being highly plastic and excitable in the zebra finch NCM, to a greater degree than neurons born in the perinatal period. We demonstrated trends towards plasticity in new neurons’ relatively high rate of habituation, a correlate of learning and memory, and trends towards excitability in their heightened levels of activity in all stimulus conditions. While further validation of this experiment must be performed to improve our interpretations of these trends, plasticity and excitability indicate a role for young neurons in learning and long-term memory in this auditory region of the songbird brain.
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Derived from the Medial Ganglionic Eminence But Not from the Adult Subventricular Zone. J. Neurosci. 34, 10906–10923.


