From Genetics to Behavior, the Dynamics and Mechanisms of Adult Neurogenesis in a Sensorimotor Circuit

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Abstract

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The most defining attribute of the nervous system is that it shows extensive plasticity of structure and function that allows animals to adjust their behavior, physiology, and morphology to changes in their environment. One example of such plastic changes is the seasonal changes in the neural circuit responsible for singing behavior in song birds. The songbird neural circuit is comprised of the sensorimotor nucleus HVC and its target, the robust nucleus of the arcopallium (RA). Neuronal number in the HVC of adult song bird Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelli*) changes seasonally with around 25%, or nearly 68,000, neurons being born and dying each breeding and nonbreeding season, respectively. White-crowned sparrow singing behavior also changes with season such that during breeding season birds produce more songs of high stereotypy. Resultantly, the avian song control system provides a unique model that allows us to investigate the basic natural processes, regulatory mechanisms, and behavioral results of neuronal birth and addition in the adult brain, or adult

neurogenesis. Within this dissertation, I document the natural dynamics between new neuronal addition and mature neuronal death within and between breeding and nonbreeding seasons. I demonstrate that neuronal death and the resulting inflammatory response are both necessary and sufficient for increased *production* of new neurons and that neural activity is necessary and sufficient for the *addition* of the newly generated neurons into the song control neural circuit. Finally, I examine the genetic regulatory networks coordinating the multitude of processes and modulatory mechanisms of adult neurogenesis in the avian brain. Together, these studies uncover the basic cellular and genetic dynamics in addition to key regulatory mechanisms guiding successful incorporation and survival of adult-born neurons in functional neural circuits. Understanding the processes and mechanisms described herein is critical both for our basic understanding of adult neurogenesis and neural plasticity and for exploiting the clinical potential of neuronal replacement to repair damage associated with injury and neurodegenerative diseases.

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**Obviously not a real statistic as your contribution could never actually be quantified.*

Dedication

Perfect as the wing of a bird may be, it will never enable the bird to fly
if unsupported by the air.
~Ivan Pavlov

Rolf Nussbaumer Photography

I dedicate my dissertation to all of the people who have unknowingly through their everyday actions had a profound impact on shaping the scientist and person that I am. Though I strive to always express my gratitude, many of their actions went thank less.

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Chapter 1. An Introduction to Avian Adult Neurogenesis[1]

1.1 Introduction

Song behavior in oscine birds is regulated by a network of pallial and striatal nuclei. The song control system shows extensive plasticity in adults, including ongoing neurogenesis in several nuclei [1]. The addition of new neurons to the adult brain of higher vertebrates was first suggested by the pioneering studies of Altman and Kaplan [2, 3]. They reported that labeled cells were present in the dentate gyrus (DG) of rats following the injection of [^{3}H] thymidine. Their claims, however, met with skepticism and the neuronal identity of the new cells that they observed was called into question [4]. The study of neuronal addition to the adult brain was subsequently dropped for nearly twenty years in the face of the dogma that neurogenesis was completed by birth [5]. This prevailing view only started to be overturned when Nottebohm and colleagues published a series of studies showing that: new cells are added to the cortical song nucleus HVC (acronym used as proper name; [6]) (Figure 1) of adult canaries (*Serinus canarius*) [7], these new cells have neuronal morphology, some of these cells fire action potentials in response to sound [8], receive synaptic input [9], synapse on neurons in the efferent nucleus RA (robust nucleus of the arcopallium) [10], and express neuron-specific proteins [11]. Together these studies in songbirds showed that new neurons are born and incorporated into functional circuits in the brains of adults of higher vertebrates [12]. This research on adult neurogenesis in songbirds stimulated investigators to re-examine this topic in mammals. It soon became clear that new neurons are added throughout life to the dentate gyrus and olfactory bulb of mammals including humans [13-16]. Since these initial confirmatory reports, there has been explosive growth in study of the mechanisms and functions of adult neurogenesis in the mammalian dentate gyrus and olfactory bulb.

Birds continue to be a productive model for the study of neurogenesis in the adult brain, as discussed below. In this chapter we will focus on neurogenesis in the song control system as this is the most intensively studied avian model. For a review of neurogenesis in the avian hippocampus, see [17]. We will discuss the mechanisms of neurogenesis in the song system, intrinsic and extrinsic factors that influence neuronal addition, a linkage between cell death and neurogenesis, seasonal plasticity, and consider potential functions of adult neurogenesis.

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Why study adult neurogenesis in birds?

The birdsong system offers several advantages as a model:

1) Unlike mammals in which substantial neurogenesis is primarily limited to two regions of the brain (the hippocampus and olfactory bulb), new neurons are added throughout most of the avian telencephalon and so, this form of plasticity seems to be a fundamental feature of the forebrain in birds [18].

2) The song nuclei that incorporate new neurons in adult birds, HVC and area X (a basal ganglia homologue), are dedicated to the regulation of song – a sensorimotor behavior. Placing adult neurogenesis in these regions into a functional context is therefore more straightforward than is true for neuronal addition to the mammalian DG. The specific functional role(s) of the DG and the hippocampal formation (HF), in which the DG resides, continues to be a topic of debate [19]. The HF has been implicated in spatial mapping, declarative memory, episodic memory, recollection, relational memory, mood, and stress (e.g., [20, 21]). It is therefore not surprising that the function of neuronal addition to the DG is unclear [22].

3) The level of ongoing neurogenesis in the avian brain is higher by an order of magnitude than that reported in mammalian brains. In birds, 0.1-0.7% of all HVC neurons and 0.15-0.37% of hippocampal neurons are newly recruited per day on average, depending on the species [23]. By contrast, it is estimated that 0.02% of total hippocampal granule cells in mature macaque monkeys, and 0.2% in 10 week old rats, are generated per day [24, 25]. In mammals, "adult neurogenesis is not a mass phenomenon but appears to make a qualitative rather than quantitative contribution" [26]. The higher level of neuronal addition in birds makes it more tractable for experimental manipulation, and suggests that ongoing neurogenesis is a quantitative phenomenon in birds

4) Neuronal addition to mature DG is offset by cell death and total neuron number does not change throughout adult life [25]. Seasonally breeding songbirds, however, show pronounced seasonal cycles of a large increase in neuronal number in HVC of breeding adults, due to neurogenesis, followed by a decrease in nonbreeding birds, due to apoptosis (see below). This seasonal pattern facilitates relating changes in behavior to the addition or loss of neurons.

5) Most of the new neurons added to the adult HVC have long axons that project four millimeters or more to synapse on target cells in RA [10, 27]. New neurons added to the olfactory bulb in mammals, by contrast, are interneurons [28, 29], and those added to the DG

are granule cells that synapse locally on the hippocampal hilus and CA3 [30]. HVC therefore provides a unique model for investigating the incorporation of new neurons into long-range neural circuits, and facilitates manipulations of target cells to examine post-synaptic influences on the addition of new afferent neurons.

6) There are about 4,000 species of songbirds. The species studied thus far all learn to sing, possess the same conserved network of hormone-sensitive brain nuclei that regulate song, and show adult neurogenesis in HVC and area X [1, 17, 31, 32]. There is much variation among taxa in whether song learning is restricted to juveniles or continues into adulthood, the number of songs learned, plasticity of adult song, and sexual patterns of song behavior [33]. This species diversity provides rich opportunities for comparative studies to test the role of neurogenesis in regulating different aspects of learned song behavior.

1.2 Basic Processes

1.2.1 Neurogenic Niches and Proliferation

In the 1990s work by Reynolds & Weiss (1992) and Palmer et. al (1997) established neural stem cells could give rise to new neurons and glia were present within specific niches throughout the adult brain [34, 35]. Across taxa, the majority of these niches are located at or near the ventricles of the brain. Mapping studies in avians, specifically the canary, revealed that proliferating cells reside at the lateral and tectal ventricles in the adult brain (Figure 1A; [36, 37]). Nearly 0.3% of the total number of all cells proliferating in all of the canary brain arise at the tectal ventricle [37]. Cell proliferation at the ventrolateral and the dorsomedial walls of the lateral ventricles account for 93% and 6% of the total number of proliferating cells, respectively [37]. This rate of progenitor cell proliferation in the neurogenic niches of the bird brain is considerably higher than in the mammalian brain. In highly neurogenic regions of the avian brain such as the song control nucleus HVC (proper name, [6]) up to 0.7% of the total neuron population are generated in a day (reviewed in [23]), whereas only 9,000 cells - 0.2% of the total neuron population - are generated everyday in the mammalian dentate gyrus ([24, 38]).The proliferating cells in the ventricular zone (VZ) generate multiple cell types; tissue explant cultures containing the ependyma of the lateral ventricle give rise to both radial glia and new neurons that differentiate and form synapses within two weeks [39-41]. Moreover, the neural stem cell-derived radial glial cells may also serve as the neural progenitors in the adult bird brain, though this remains a topic of debate [41-43].

1.2.2 Migration of Neuroblasts

Proper migration and incorporation of new neurons is necessary for integration into functional neural circuits. In birds, the departure of neuroblasts and newly differentiated neurons from the neurogenic niche begins one to four days after birth (Figure 2; [44, 45]). These new cells must migrate over long distances to reach most of their destinations in the telencephalon. Neuroblasts translocate over the course of one to three weeks [46] by one of a few modes - radial, tangential, or undirected wander migration - depending on the ultimate fate and location of the neurons. A fraction of the neuroblasts originating at the lateral ventricles migrate along the fibers of ependymally-derived radial glia both *in vitro* [40] and *in vivo* [46]. Typical radial glial cells reside in the ventricular zone at the lateral ventricles and project their processes mediolaterally [47]. ^{3}H-thymidine positive cells with a classic bipolar migratory phenotype are found associated with radial fibers, suggesting that radial processes provide the scaffolding upon which the neuroblasts migrate through the adult avian brain [46]. Radial glia also provide trophic support for the migrating neuroblasts: insulin-like growth factor-1 is expressed heavily by radial glia cells and their fibers and promotes the migration of neuroblasts [48].

Some areas in the avian brain that incorporate large numbers of new neurons such as the olfactory bulb and HVC contain few or no radial fibers [47]. Tangential, or directed but radial-glia-independent, migration has been described extensively in mammals and occurs in avians too. As in the mammalian rostral migratory stream, chains of PSA-NCAM positive cells are present in the telencephalon dorsal to the avian olfactory bulb [49, 50]. PSA-NCAM positive chains containing a subset of differentiated neurons, astrocytes, and tanycytes [50] create extensive networks throughout the avian brain upon which PSA-NCAM positive neuroblasts migrate [49]. In contrast to the tangential model, most of the neuroblasts that give rise to new HVC neurons (about 70%) adopt a multipolar phenotype and follow a wandering, tortuous route with frequent changes in direction rather than a straight path through HVC during migration [51]. Movement is produced by translocation of the cell body along one of the processes that extends from the soma. Wandering may continue for several days, during which the cell may travel several hundred micrometers. These multipolar cells differentiate into neurons once they reach their destination [51].

It is unclear whether bipolar and multipolar neuroblasts represent different stages of migration of the same cells, or distinct neuronal types. Neuroblasts may initially migrate away from the VZ along radial glial fibers and then transition to wandering during the final stages of migration [47, 51]. Alternatively, bipolar and multipolar neuroblasts may represent different

neuronal types. Scott et al. (2012) suggest that this latter possibility is less likely, given the changes in phenotype, polarity, direction and scaffolding observed in migrating neuroblasts in mammalian brains.

1.2.3 Post-Migratory Maturation and Fate Specification

As early as eight days following birth, new neurons expressing classic neuronal markers though not fully mature and functional can be found in regions of the avian brain that regularly incorporate new neurons (Figure 2; [52]). At the end of migration, these new neurons form close contact with the soma of mature HVC neurons [9]. These new cells integrate into functional circuits and form somatal contact with mature HVC interneurons and neurons that project to area X (HVC_X) and RA (HVC_{RA}) [51, 52]. These cellular interactions may provide a "stop" signal that terminates migration [51]. In HVC the new and mature neurons form clusters that may represent a functional unit [52]. The neurons within these clusters appear to be connected by gap junctions [53]. This coupling may allow HVC_X neurons to entrain new HVC_{RA} neurons to produce the appropriate motor pattern for song production [54].

Most, in not all, of the new HVC neurons project to the afferent nucleus RA. It is unclear whether some neuroblasts differentiate into HVC interneurons [27, 55]. By two weeks of age these neurons can form synapses on RA neurons as evidenced by the uptake and retrograde transport from RA of tract-tracers [52]. Although capable of forming synapses by two weeks, nearly half of the new HVC neurons that will ultimately project to RA have not yet formed synapses even by thirty days following birth in adult canaries and zebra finches [52, 55]. By eight months, all new HVC_{RA} neurons form synapses onto RA neurons in canaries [52].

1.2.4 Survival of Newly Generated Neurons

Adult-born neurons in the avian brain persist for periods ranging from days to years (Figure 2). Between two and three weeks following birth, roughly half of all post-migratory neurons in HVC die [52]. In the hippocampus of the adult black-capped chickadee (Parus atricapillus), the number of ^{3}H-thymidine labeled neurons decreases between six and ten weeks after birth [56]. The new neurons that persist through the initial culling can survive months [57, 58] to years , depending on their time of birth, location of incorporation, and other factors discussed later in this chapter. Given that the number of newly generated HVC neurons does not decrease significantly between nine months and four years in the zebra finch (*Taeniopygia guttata*) [59], it is possible that some adult-born neurons persist in HVC and other regions of the avian brain for the remainder of the bird's life.

1.3 Molecular and Physiological Mechanisms Regulating Avian Neurogenesis

1.3.1 Seasonality

New neurons are continuously added to the adult avian brain. The percent of new neurons that functionally incorporate and survive in HVC, however, changes dramatically depending on the seasonal hormonal condition (i.e, breeding vs. nonbreeding; Figure 3). Plasma T levels are high throughout the breeding season and decrease to basal levels at the end of breeding [60]. Seasonal changes in song behavior and structure [61, 62] driven by these fluctuating T levels are accompanied by changes in the morphology, electrophysiology, and gene expression of song nuclei in essentially every seasonally breeding songbird species that has been examined (reviewed by [1, 63]). The seasonal changes in the song nuclei, most of which express androgen, estrogen, or both receptors, are primarily regulated by changes in gonadal T and its metabolites [1]. More specifically, the volumes of HVC, RA, X, and nXIIts increase by up to 200% during the breeding season.

Neuron number in HVC also changes seasonally. In wild-caught Song Sparrows (*Melospiza melodia*), for example, neuron number in HVC increases from about 150,000 in the fall to 250,000 during the breeding season [62]. This change in neuron number results from seasonal patterns of cell death and ongoing neurogenesis. At the end of the breeding season, circulating T levels decrease, which prompts an increase in the death of mature HVC neurons [64]. This death of mature HVC neurons during the transition from breeding to nonbreeding seasons likely creates “vacancies” for the addition of new neurons [65, 66], discussed further below.

Field studies of wild birds show that growth of the song system occurs rapidly once plasma T levels first start to rise as day length increases in late winter, and precedes full seasonal reproductive development [62, 67]. In the laboratory, captive white-crowned sparrows (*Zonotrichia leucosphyrus gambelli*) can be implanted with a systemic T implant and exposed to a long day photoperiod to mimic breeding conditions. In simulated breeding conditions, HVC grows to its full breeding size and neuron number increased from 90,000 to 150,000 within 7 days. This addition of 60,000 neurons to an adult brain within such a short time period is unprecedented.

When circulating T drops to basal levels at the end of the breeding season, mature HVC neurons lose the trophic support provided by T, BDNF, and the electrical activity of post-synaptic neurons in RA, and so rapidly die [1, 68-71]. Within 4 days of transferring white-crowned sparrows from breeding to non-breeding conditions in the laboratory, 25% of HVC neurons die [64]. The number of new neurons present in HVC increases following the death of mature

neurons [10, 72]. In the absence of trophic support, however, most of these new HVC neurons in non-breeding birds do not persist in the song control circuit.

1.3.2 Interactions of steroid hormones and neurotrophins

Steroid sex hormones, secreted by the gonads and synthesized *de novo* in the brain of songbirds (Figure 3 and 4), promote neuronal survival primarily through their effect on the expression of neurotrophic factors. Treatment of adult female canaries with exogenous T increases the number of new neurons added to HVC [69]), but this effect requires BDNF [70]. Intracerebral infusion of recombinant BDNF (rBDNF) into HVC increases the number of new neurons by the same amount as does T treatment, whereas infusion of a BDNF blocking antibody prevents the T-induced increase in new neurons in HVC [70]. BDNF promotes neuronal addition to HVC during a discrete critical period. Infusion of rBDNF into HVC 14-20 days after the birth of new neurons increases the number of neurons that survived for at least 8 months. Alternatively, birds infused with BDNF either 4-10 or 24-30 days after birth show typical die off of 50% of new HVC neurons by four months [58, 73]. Together these observations suggest T-induced expression of BDNF increases the survival of newly generated neurons in HVC, and that there is a sensitive period soon after new neurons first reach HVC when BDNF has this effect on neuronal survival.

The interaction between T and BDNF in adult neurogenesis is mediated by local angiogenisis in HVC. T treatment expands the microvasculature in HVC through the production of vascular endothelial growth factor (VEGF) and the VEGF receptor, Quek1/VEGF-R2 [74]. One week following an increase in VEGF, newly generated HVC endothelial cells up-regulate production and secretion of brain derived neurotrophic factor (BDNF; [74]). Testosterone also stimulates perivascular production of matrix matalloproteases (MMPs), specifically MMP2 and MMP9 (Kim et al., 2008). MMPs promote neuronal recruitment by processing extracellular matrix proteins that regulate endothelial cell migration (Gottschall and Deb, 1996).

In addition to being secreted by endothelial cells, BDNF is also produced in and presumably released by the projection neurons of HVC [75]. The expression of BDNF in HVC projection neurons is up-regulated by increases in 1) testosterone [70], 2) estradiol [76], and vocal production of song [75]. In a cDNA microarray study, Thompson et. al. (2012) found that the gene for BDNF is significantly up-regulated in HVC following the transition of white-crowned sparrows from nonbreeding to breeding conditions. *In situ* hybridization confirmed that BDNF mRNA is expressed widely throughout HVC of sparrows exposed to breeding conditions, and that expression increased over the 7 days following exposure to systemic T [77]. The discrete

period in which BDNF promotes HVC neuronal recruitment (i.e. 14 to 20 days after neuronal birth [73]) corresponds to post-migratory maturation and fate specification of neuroblasts [52].

1.3.3 Behavioral Use and Neural activity

Activity within the circuit to which new neurons are added influences the addition and survival of these cells (Figure 4; [17]). Singing behavior increases neuronal survival in HVC via increased BDNF mRNA and protein expression in the population of HVC_{RA} neurons to which new neurons are added [75]. BDNF expression correlates with the number of songs produced per unit time in male zebra finches and as discussed above, and in turn this BDNF production supports neuronal survival. The number of new neurons added to the adult HVC of male canaries correlates with individual differences in the average amount of song produced [78]. Alternatively, deafening (i.e. loss of auditory neural activity) decreases neuronal addition to HVC and the auditory caudomedial nidopallium (NCM) in adult zebra finches [79, 80].

The addition of HVC_{RA} projections may be influenced by the electrical activity of target neurons in RA. The spontaneous activity of RA neurons is high in breeding sparrows when neuronal addition to HVC is high, and low in non-breeding birds when neuronal addition is low [81, 82]. The mechanism by which such activity may influence neuronal addition to HVC is not yet known. Activity-induced regulation of genes encoding molecules that promote survival of adult-born HVC neurons, axonal path finding, and/or synapse formation is likely to be important [83, 84]. Interestingly, activity-induced guidance molecules are seasonally regulated in RA neurons of white-crowned sparrows; microarray analysis of cDNA extracted from RA showed that the expression of guidance cue genes, including netrin 4 and galectin, is increased in breeding-condition birds [85]. The retrograde transport of activity-induced trophic factors produced by target neurons that influence the survival of new HVC_{RA} neurons may be modulated by activity in RA. Microarray analysis also showed that the expression of proneurogenic genes, including insulin-like growth factor 1 and neuromodulin, is increased in breeding-condition birds [85]. In HVC, the expression of mRNA for sex steroid receptors, which facilitate the retrograde transport of trophic factors bound to their receptor toward the neuronal soma, also increases during breeding conditions [86-88]. Once transported back to the soma, trophic factors likely activate signaling cascades that promote the growth and survival of new neurons (Brenowitz et al., unpublished observation; [89, 90]). Inhibition of neural activity in RA may result in a failure of new HVC neurons to form synapses on RA neurons and/or a decreased production of activity-induced trophic factors in RA. The consequence could be a lack of retrograde transport of the trophic signals and, thus, a decrease in addition of adult-born

neurons to HVC. The relationship between neural activity and neuronal addition in the adult brain, however, remain fairly unexplored.

1.3.4 Cell Death

The addition of new neurons to HVC is linked with the death of mature neurons. In adult male canaries, mature HVC neurons die when T levels decrease at the end of the breeding season. Within a few weeks there is a peak in the addition of new neurons to HVC [52]. Laser photo-ablation of both HVC_X and HVC_{RA} projection neurons in adult male zebra finches increases the addition of new HVC_{RA} neurons, but not of new HVC_X neurons [65]. In adult white-crowned sparrows, mature HVC neurons die seasonally through caspase-mediated apoptosis when T levels drop [91]. Reduction of this neuronal loss in HVC by infusion of a cocktail of caspase inhibitors reduces the number of new neurons added to HVC [66]. Consistent with these observations, in adult ring doves (*Streptopelia risoria*) lesion of the medial preoptic area of the hypothalamus (mPOA) induces neurogenesis in this region that does not normally add new neurons [92]. The mechanisms functionally linking cell apoptosis to neurogenesis are still unclear.

1.3.5 Age

Age-related decline in neurogenesis across taxa in undeniable: neurogenesis was shown to decrease with age in the first studies of mammalian neurogenesis [2] and subsequently has been shown to occur broadly across taxa ranging from humans [16] to lizards (*Podarcis hispanica*; [93]) to fish (*Apteronotus leptorhynchus*; [94]). In avians, mature neuronal replacement by new neurons begins in the juvenile canary as early as four months after hatching [95]. After four months the rate of neuronal recruitment [96] and adult-born neuronal survival [97, 98] decreases steadily with age. Albeit neurogenesis occurs at comparatively low rates in aged avians, neural progenitor cell proliferation and neuronal recruitment have not yet been observed to cease entirely in any naturally aged avian thus far. New neurons continue to be added to HVC, however, for as long as 11 years of age in zebra finches [59].

1.3.6 Sociality (and Stress)

Social setting affects neuronal recruitment not only in the mammalian brain (reviewed in [99]), but also broadly across taxa. In the male zebra finch, a highly social songbird, social enrichment (i.e. group housing with male and female conspecifics) significantly enhances neuronal recruitment to HVC and other neurogenic regions of the brain including area X and the neopallium when compared to males housed with a single female or alone [100]. Communal housing also increases new neuronal survival in the hippocampus of zebra finches [101]. The

precise mechanism through which social enrichment acts to promote neuronal recruitment and survival is entirely unexplored in birds. There are many intrinsic and extrinsic variables that could drive social alteration of neurogenesis. One factor that must be acknowledged is the stress of single or group housing on predominately social or nonsocial birds. In both solitary and social settings, stress-induced glucocorticoid elevation decreases the number of new neurons in the dentate gyrus of the mammalian brain (reviewed in [102, 103]). Thus, glucocorticoid levels likely affect avian neuronal recruitment and survival both in general and in social enrichment settings.

1.3.7 Genomics

In order for neural stem cell progeny to become mature functional neurons, newly generated cells require tight coordination of gene expression that permits reception of external signals, direct interactions with other cells, and autonomous regulation of the processes of becoming a new neuron. Very little is known about the genetic and epigenetic regulation of neurogenesis in avians. It is clear, however, that many species have evolved specialized control over neurogenesis based on selective pressures for the performance of certain behaviors. For example, food-caching black-capped chickadees have higher rates of hippocampal neurogenesis than other related non-food-caching birds [104]. Migratory white-crowned sparrows also have higher rates of neuronal migration and incorporation compared to other non-migratory white-crowned sparrow subspecies [105]. Moreover, neuronal recruitment in HVC varies widely between different species of songbirds (reviewed in [23]) and between individuals of the same species. For example, individual zebra finches incorporate new neurons into HVC at varying rates depending on their nest of origin [106]. All of these data suggest that heritability plays some role in the magnitude and localization of neuronal recruitment in the avian brain.

Gene regulatory networks that control different aspects of neurogenesis and cell death have been identified through the sequencing and analysis of the zebra finch genome and microarray analyses [85, 107]. Thompson et. al. (2012) identified 132 genes in HVC cells that changed in expression between breeding and nonbreeding physiological conditions when compared to gene expression in RA, a non-neurogenic region of the song bird brain (Figure 4; [18]). In general, genes that promote proliferation, angiogenesis, and neurite extension were up-regulated, whereas genes that support programmed cell death were down-regulated in HVC under breeding conditions. Specific genes that encode for neurotrophins known to promote neuronal migration, recruitment, and survival, including BDNF, insulin-like growth factor 1 (IGF1), and VEGF, were also up-regulated in HVC cells under breeding conditions [85]. These

studies have begun to identify the complex regulatory networks of avian neurogenesis and plasticity, and provide opportunity to discover novel genetic and epigenetic control over adult neurogenesis.

1.4 Remaining questions and objectives

Given the rich, but limited history of adult neurogenesis studies in songbirds, the clear course of study was: first, to clarify the natural dynamics between neuronal birth, survival, and death within and between breeding seasons, and second, to identify key regulators and global coordinators of these processes. Elucidating the natural dynamics between neuronal birth, survival and death will not only be essential for our basic understanding of stem cell biology and cellular interactions and dynamics, but also for uncovering additional mechanisms regulating the birth, survival, and death of neurons in the adult brain. Moreover, understanding these processes and mechanisms will identify additional levels of regulation of neural stem cell proliferation and neuronal survival that may lead to uncovering the etiologies and ultimately new intervention strategies for neurological disease and defects.

1.4.1 To this end the central research questions of my dissertation were:

1. What are the natural dynamics between neuronal birth, survival, and death *within* breeding and nonbreeding seasons?

2. What are the natural dynamics between neuronal birth, survival, and death *between* breeding and nonbreeding seasons?

3. Is the documented seasonal modulation of neural activity in RA necessary for the addition of new HVC neurons?

4. Are there genetic regulatory networks coordinating the vast array of basic cellular processes necessary for proper and successful adult neuronal addition?

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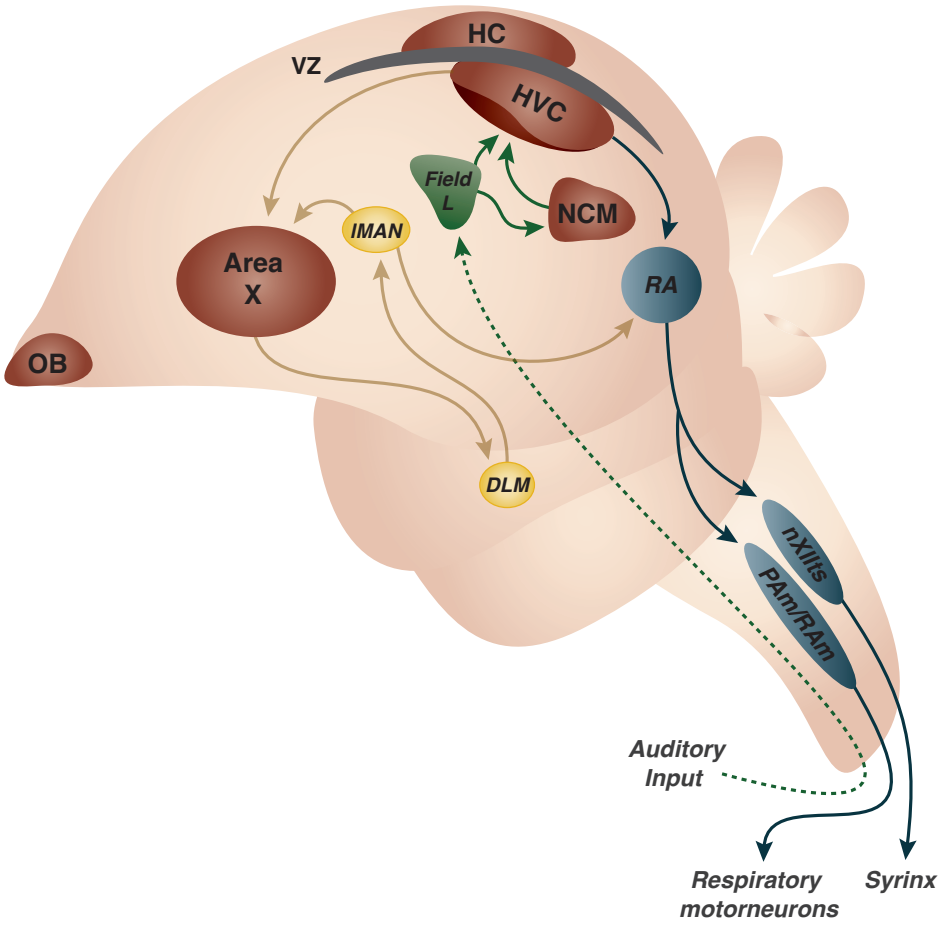

Figure 1. A schematic of the neurogenic regions in the avian brain over-laid on the avian song circuits. Neurogenic regions are shown in red. Note the proximity of HVC (and hippocampus; HC) to the VZ. A schematic version of the motor pathway for song production is shown in blue. A schematic of the ascending auditory pathway is shown in green. The dotted line indicates an indirect route through many nuclei of the ascending auditory pathway leading to Field L in the telencephalon. The anterior forebrain circuit for song learning and plasticity is shown in yellow.

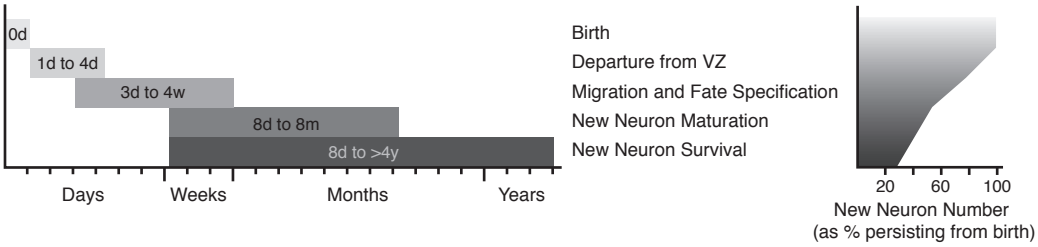

Figure 2. A timeline of the processes of avian neurogenesis and the proportion of new neurons that persist through each of these processes. The timeline is based on the work of (Alvarez-Buylla and Nottebohm 1988; Barami et al. 1994; Scott et al. 2012). The percentages of survival through the various processes of avian neurogenesis are based on the findings of (Kirn et al. 1991; Barnea and Nottebohm 1994; Nottebohm et al. 1994; Kirn et al. 1999; Scott and Lois 2007; Walton et al. 2012).

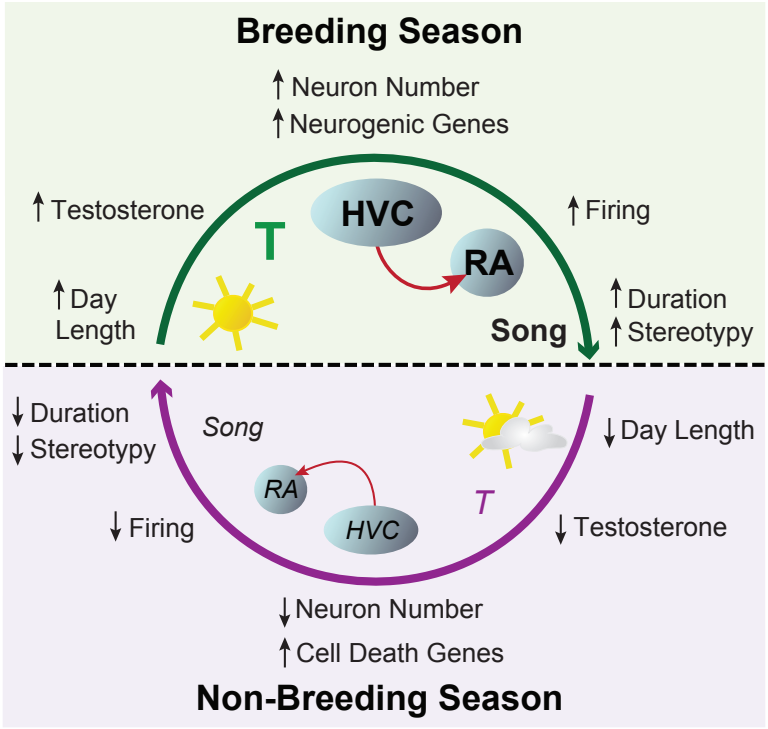

Figure 3. A model of the seasonal changes in physiology, morphology, and behavior of songbirds. As day length increases at the onset of the breeding season, plasma testosterone (T) levels increase. The increase in T drives an increase in neuronal number in HVC along with changes in gene expression, morphology and physiology of HVC and RA. All of these changes in morphology and physiology permit the production of stereotyped song. As day length decreases at the onset of the non-breeding season, T levels drop, HVC neurons die and song degrades. This cycle repeats annually.

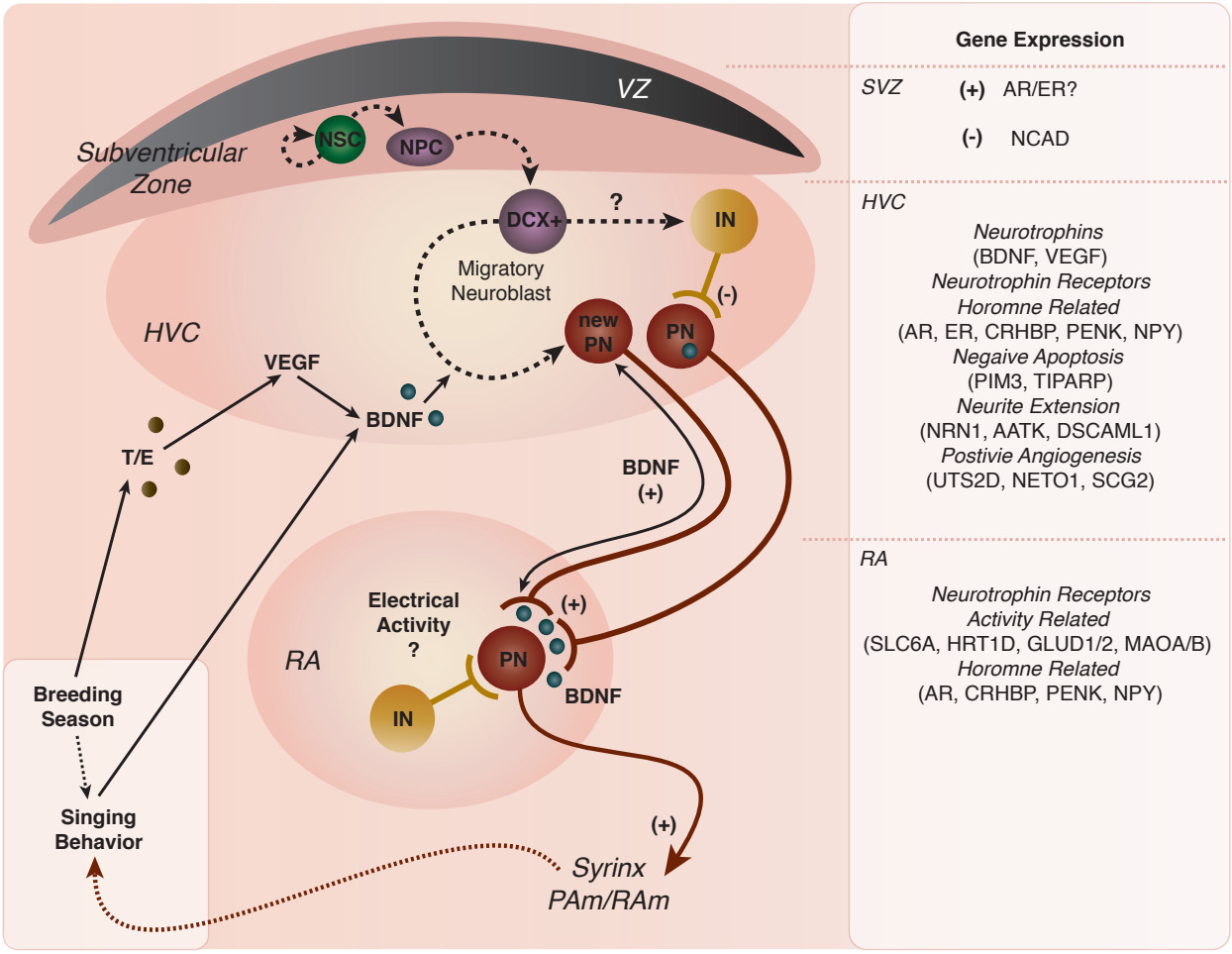

Figure 4. A schematic of the factors ranging from gene expression to behavior that influence the birth and migration of neuroblasts and the addition of new neurons to HVC. The solid black lines indicate factors that positively influence the given cell, factor, or process to which it points. The dashed lines represent changes in cell process indicated (e.g., cell divisions and migration). The dotted lines represent indirect routes of influence (i.e. through other genes, factors, etc.). The break-out panel to the right side summarizes patterns of gene expression in different brain regions that positively influence component processes of neurogenesis. In the VZ, the (+) indicates genes that promote proliferation, whereas the (-) indicates genes the promote exit from the cell cycle. Abbreviations: NSC, neural stem cell; NPC, neural progenitor cell; DCX+, doublecortin positive migratory neuroblast; IN, interneuron; PN, projection neuron.

Chapter 2. The seasonal modulation of neuronal turnover in an adult sensorimotor circuit

2.1 Abstract

The balance between neuronal addition and death is a fundamental process of adult neural plasticity and is necessary for proper maintenance and performance of behavior. Songbirds provide an excellent model for exploring the natural dynamics of neuronal turnover and its effects on behavior. The songbird neural circuit controlling song production is comprised of the sensorimotor nucleus HVC and its target, the robust nucleus of the arcopallium. Neuronal number in the HVC of adult Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelli*) changes seasonally such that around 25%, or nearly 68,000, neurons are born and die each breeding and nonbreeding season, respectively. To accommodate the large seasonal difference in HVC neuron number, neuronal turnover – or the balance between neuronal addition and death – in HVC likely differs between seasons as well. To determine if turnover of new HVC neurons changes within and between breeding and nonbreeding conditions, we labeled two cohorts of new HVC neurons separated by four, eight, and sixteen weeks with two different thymidine analogs in breeding and nonbreeding condition birds. We show that the survival of new neurons added to HVC at the onset of seasonal change is higher during breeding than nonbreeding conditions. Conversely, we found that the neuronal turnover is similar in both breeding and nonbreeding conditions once a steady-state HVC volume and neuronal number has been attained. Finally, we found that turnover of the initially labeled new HVC neurons occurs progressively with time, regardless of condition. Together, these data demonstrate that breeding conditions promote the survival of an initial population of new neurons entering HVC, but that once HVC is fully grown successive new neuronal addition and maintenance returns to levels similar to that of nonbreeding conditions.

2.2 Introduction

Since the discovery of adult neurogenesis, neuronal turnover, or the replacement of older neurons by new neurons, has emerged as a fundamental property of the adult brain. Several hypotheses regarding the function of adult neurogenesis and neuronal turnover have been put forward [1]; the replacement of older neurons by adult-born neurons has been proposed to enable the learning of new memories [2, 3], the maintenance older memories [4, 5], the replacement of over-excited neurons [5], or the replacement of neurons weakened by DNA

damage [1] or loss of trophic support [1]. Many studies have tested these hypotheses, but no studies have conclusively supported or eliminated any of these possible functions of neuronal turnover. Despite the fundamental necessity of neuronal turnover for proper establishment and maintenance of neural circuits and behavior, the basic dynamics and mechanisms regulating neuronal turnover have been rarely described in the adult context (but see [6-8]). Here, we describe the basic dynamics between neuronal birth and death and to test the role of one highly potent regulator of neurogenesis common across taxa – that is, seasonal modulation of sex steroid levels (reviewed in [9]) – in mediating neuronal turnover.

The seasonally induced plastic changes in morphology and function in the nuclei that control song production in songbirds, including Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), provide a robust model for examining the fundamental features of neuronal turnover (see Figure 1A for song production circuitry). The pallial song control nucleus HVC (proper name; [10]) doubles in size at the beginning of the breeding season when photoperiod (i.e. length of daylight) and plasma testosterone (T) levels increase [11, 12]. The increase of HVC volume arrises primarily from an increase in adult-born neuronal addition to HVC; HVC neuron number increases by around 25%, or 68,000 neurons [12, 13]. Most, if not all, new HVC neurons project to the HVC target nucleus, the efferent robust nucleus of the arcopallium (RA; [14], but see [15]). In the fall when photoperiod and T levels decrease, the song control circuit rapidly regresses in size [16]; around 25% of mature HVC neurons undergo natural programmed cell death, or apoptosis, within 4 days of a transition from breeding to nonbreeding conditions [17, 18]. As HVC grows and regresses, so to song becomes more and less stereotyped with breeding and nonbreeding conditions, respectively [18, 19].

In order for HVC total neuron number in HVC to vary seasonally, the balance between neuronal addition and death must also change each season. During the transition to breeding conditions, neuronal addition must exceed neuronal death for total neuronal number in HVC increase. Alternatively, during the transition into nonbreeding conditions, neuronal death must exceed neuronal addition for HVC volume and neuronal number to decrease. During stable-state periods of both breeding and nonbreeding conditions, when HVC is fully grown or regressed, the balance between neuronal addition and death likely shifts again to be equal in order to maintain a constant HVC neuronal number.

Several scenarios can be proposed regarding the particular populations of neurons (i.e. new versus mature) that survive versus die during breeding and nonbreeding conditions. Adult-born neurons that enter HVC may entirely replace older neurons [6], in which case only mature

neurons would die. In this model change in HVC total neuronal number could be due to changes in neural stem cell proliferation [20]. Alternatively, new neurons that enter HVC could be a transient population that dies either shortly after birth in nonbreeding conditions or during HVC regression upon transition from breeding to nonbreeding conditions. Previous work has shown, however, that some new neurons incorporated during breeding condition persist through HVC regression [7,18], ruling out this possibility. Another possibility is that both new and mature neurons die as new neurons enter HVC in both breeding and nonbreeding conditions, and that maintenance of a given neuron is due to factors such as blood plasma T levels, synaptic strength, neurotrophin signaling or neural activity (for reviews see [1, 21]). With this model, HVC could grow upon transition into breeding condition even while losing mature neurons so long as new neuron survival was greater in breeding than nonbreeding conditions. Here, we exploit the seasonal growth and regression of HVC in white-crowned sparrows to test these proposed dynamics between neural stem cell proliferation, new neuronal maintenance, and neuronal death within and between breeding and nonbreeding conditions.

2.3 Materials and Methods

2.3.1 Animals

Fifty male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) were collected in eastern Washington during their pre- or post-breeding season migration. Prior to the initiation of experiments, all birds were housed in indoor group aviaries and exposed to short day photoperiods (SD; 8 hr light, 16 hr dark) for at least 10 weeks to ensure that the song and reproductive systems had regressed and would be sensitive to the stimulatory effects of long day photoperiods (LD; 20 hr light, 4 hr dark) and testosterone (T). All experiments followed NIH animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee.

2.3.2 Experimental procedures

To determine the effect of breeding conditions on new neuronal turnover, we housed birds in LD photoperiods and implanted a 12-mm Silastic capsule (1.47 mm inner diameter, 1.96 mm outer diameter) filled with crystalline T (Sigma) above the scapula (Figure 1B). This LD+T manipulation induces breeding-like morphology and physiology of the song circuitry in the laboratory [19, 22]. We maintained two groups in LD+T for either eight (n=11) or twelve (n=9) weeks total. Seven days prior to transitioning birds to LD+T, each bird received five daily intramuscular injections of 3.33 µl/g of one of two thymidine analogs (TA) randomly chosen and

balanced across all groups. The two TAs used were equimolar doses of bromodeoxyuridine (BrdU; 15 mg/m in 0.09% NaCl and 0.012M NaOH; Sigma) and ethynyldeoxyuridine (EdU; 12 mg/ml in 0.09% NaCl and 0.012M NaOH; Invitrogen). Because neuroblasts take one to three weeks to migrate to HVC [23], we chose to inject birds with the first TA prior to transfer to LD+T so new neurons labeled with TA would be incorporating into HVC under breeding-like conditions. Four or eight weeks after onset of LD+T birds were injected daily for five days with 3.33 µl/g of the other of the two TAs. The two experimental groups were designed to have a four week differential period to allow for new neurons to be born and incorporate into the HVC to RA neural circuit [24, 25]. The use of two experimental groups (or three groups in SD, see below) facilitated examination of new neuronal turnover rate. Four weeks following the onset of the second TA injections birds were killed. Birds were not housed in LD+T beyond 12 wks because high T levels over extended periods of time (i.e. >12 weeks) have been found to be detrimental to the overall health of the birds (unpublished observation).

To determine the effect of nonbreeding conditions on new neuronal turnover, we synchronized the breeding cycle of another group of birds by housing birds in LD+T (n=30) for four weeks (Figure 1B). After removal of the subcutaneous T pellet birds were transferred back to SD photoperiods overnight, prompting the rapid regression and nonbreeding-like state of the song control circuitry [12, 16, 18]. Four weeks following transition to nonbreeding conditions, we injected all birds once daily for five days with 3.33 µl/g of one of the two TAs. Four (n=9), eight (n=12), or sixteen (n=9) weeks following the onset of the first series of randomly chosen TA injections, we injected 3.33 µl/g of the other TA daily for five days. Four weeks after the start of the second TA pulses, all birds were killed.

2.3.3 Blood draw and hormone analysis

Blood samples were obtained from birds in LD+T and SD every four weeks and upon termination of the experiment to measure circulating T concentrations. Blood was also collected in birds of the SD group on the final day of the LD+T synchronization period. We drew 250 µl of blood from the alar vein in the wing into heparinized collection tubes, immediately centrifuged the tubes, and stored the plasma at -80°C until assay. Using a Testosterone Enzyme Immunoassay kit (Enzo Life Sciences), we measured plasma T concentration. Minimum and maximum detectable plasma T concentrations were 0.03 ng/ml and 40.00 ng/ml, respectively. Samples with undetectable levels of T were treated as having concentrations at the lower detection limit for statistical analyses. Intra-and inter-assay coefficients of variation were 0.59% - 41.25% and 10.00%, respectively.

2.3.4 Tissue collection and processing

Five to seven hours after lights-on, brains were removed quickly after birds were deeply anesthetized with isoflurane. Brains were sectioned in the coronal plane at 40 µm on a cryostat, and each section was thaw-mounted serially. Every third section was Nissl stained and the remaining slides were stored at -80°C until immunolabeling.

2.3.5 Immunohistochemistry and analysis

To visualize BrdU-labeled cells, we processed brain sections as follows. Briefly, sections were fixed in 4% paraformaldehyde, rinsed in phosphate buffered saline with 0.5% DMSO and 0.5% Triton-X (PDTX; pH 7.4), dipped in distilled water, incubated with 2N HCl at 37°C for 30 minutes, and rinsed with PDTX. After blocking in 5% heat inactivated goat serum, slides were incubated with rat-anti-BrdU (1:200; ABD Serotec MCA 2060) and mouse-anti-HuC (1:100; Invitrogen) antibodies. Hu is a neuron-specific antigen expressed in both immature and fully differentiated neurons (Barami et al., 1995). Labeling was visualized with the fluorescent secondary antibodies goat-anti-rat Alexa Fluor 488 (Invitrogen) and goat-anti-mouse Alexa Fluor 568 (Invitrogen). All single and double labeled cells in each HVC section were counted.

To visualize EdU-labeled cells, sections fixed in 4% paraformaldehyde were rinsed in PDTX, blocked in 3% heat inactivated bovine serum, and incubated with Alexa Fluor azide 488 (1:2000; EdU Click-It Kit, Invitrogen). Slides were rinsed in PDTX and blocked again in 5% heat inactivated goat serum. Sections were incubated with mouse-anti-Hu (1:100; Invitrogen), which was visualized with goat-anti-mouse Alex Flour 568 (Invitrogen). All single and double labeled cells in each HVC section were counted. Results are reported as unilateral data obtained from the averages of counts across both hemispheres. BrdU and EdU have been shown to co-label in certain tissue, but we found no co-labeling in the white-crowned sparrow brain (unpublished results).

To identify seasonal changes in proliferative rates, TA-positive cells remaining in the ventral side of the VZ (vVZ) were counted from the hillock of the VZ medial to HVC, out to the most lateral extension of the vVZ in all sections that exhibited arching of the VZ [15, 18, 26]. Because TA-labeling become exponentially more dilute with each successive cell division [27] and neural stem cells of the vVZ overlying HVC divide for only brief periods and then become quiescent for up to at least eight months [28], the remaining TA-positive cells likely represent populations of neural stem cells that divide slowly or that became quiescent during the TA pulses, or both [29].

2.3.6 Morphometric measurements in HVC and RA

The volume of HVC in the randomly chosen hemisphere was determined by tracing the borders of the nuclei identified in Nissl stained tissue onto paper, scanning the drawings, and using Image J Software (Version 1.46; NIH; http://rsb.info.nih.gov/ij/) to determine the area of each section of the nuclei. Total volume was calculated from these areas using the formula for a truncated cone [30]. Histological measurements described below are unilateral data, reported as average volumes across both hemispheres.

2.3.7 Statistical Analysis

The thymidine analog that was injected first (i.e. EdU versus BrdU) was randomly chosen and balanced across all groups to control for any differences in the efficacy of the analogs. We therefore did not include the order of analog injection as a factor in our statistical analysis. All morphological, histological, and physiological comparisons were made with two-way ANOVAs with experiment duration and breeding condition as factors. We performed Tukey tests for post-hoc comparisons between all durations and conditions of experimental treatment. All correlations were assessed with the Pearson's *r* correlation test. For all statistical tests a p-value ≤ 0.05 was considered significant. All statistical analyses were made using JMP Version 8.0.1 (SAS, Cary NC). All data are presented as mean ± SEM.

2.4 Results

2.4.1 Validation of breeding and nonbreeding condition

We confirmed that our manipulations induced breeding-like growth and nonbreeding-like regression of HVC by comparing morphological changes within HVC and blood plasma T levels with those previously reported. Birds in the LD+T groups had final unilateral HVC volumes of 0.52 ± 0.02 mm^3 (n=18), mid-experiment T blood plamsa levels of 20.51 ± 1.73 (n=13), and final T levels of 18.29 ± 1.17 (n=14; see Tables 1 and 2 for individual LD+T group values). Birds in the SD groups had final HVC volumes of 0.30 ± 0.03 (n=14), pre-experiment synchronization T levels of 16.42 ± 2.17 ng/ml (n=12), and final T levels of 0.58 ± 0.14 ng/ml (n=21; see Tables 1 and 2 for individual SD group values). All birds will have HVC neuron number and density measured with the anticipation that these are consistent the respective condition [31]. All measures obtained thus far are consistent with those of the respective condition in previous reports [11, 12, 22].

2.4.2 Breeding conditions promote the persistence of new neurons incorporated early in HVC seasonal plasticity

To test whether breeding condition alters the addition and survival of new HVC neurons added early during HVC growth or regression, we labeled an initial cohort of new HVC neurons with one of two thymidine analogs (TA) either 28D after transition into SD conditions from LD+T (nonbreeding group) or 7D prior to transition into LD+T conditions (breeding group; Figures 1B and 2A). TA-labeled neuron numbers varied significantly with condition (i.e. breeding and nonbreeding) and the number of weeks birds were housed in a given condition (ANOVA, $F_{(4,25)}$=3.9298; p=0.0131; Table 3 and Figure 2B). All birds in LD+T had significantly more new neurons maintained in HVC than all birds in SD (LD+T, 158 ± 25, n=12; SD, 69 ± 11; post-hoc *t*-test, p=0.0008). This result suggests that breeding condition permits greater addition, survival, or both of new neurons incorporated into HVC compared to nonbreeding conditions.

2.4.3 New neuron survival returns to a common equilibrium when HVC attains steady-state breeding or nonbreeding condition

We also tested the role of breeding condition on the maintenance of new HVC neurons incorporated during stable breeding or nonbreeding condition by injecting all birds with the other of two TAs four, eight, or sixteen weeks after the initial TA pulse and four weeks prior to termination of the experiment (Figure 1B). We found that condition and length of time in condition did not vary significantly with the number of TA-labeled neurons from the final cohort of new neurons (ANOVA, $F_{(4,23)}$=1.7164; p=0.1807; Table 3 and Figure 2C). Observing no effect of condition or length in condition on the maintenance of the final cohort of new neurons demonstrates that neuronal turnover returns to similar levels in breeding and nonbreeding conditions once steady-state HVC volume and neuronal number has been attained.

2.4.4 New neuronal turnover is likely progressive with time regardless of condition

To test whether neuronal turnover changed with condition, we calculated the ratio of new neurons from the initially-labeled cohort to the final cohort. This proportion did not vary significantly with condition or length in condition (ANOVA, $F_{(4,12)}$=1.2466; p=0.3432; Table 3 and Figure 3), though these analyses are still preliminary. However, with the data collected thus far, we observed a trend towards greater than 50% of new neurons persisting in HVC from the initially labeled cohort in breeding condition birds (LD+T initial cohort:final cohort, 1.45 ± 0.49, n=6; Figure 3A). Alternatively, around 50% of new HVC neurons were from the final cohort in nonbreeding condition birds (SD ratio, 1.02 ± 0.27, n=12; Figure 3A). Moreover, within a given condition (i.e. LD+T or SD) as the length between cohorts increases the proportion of the initial cohort relative to the final cohort generally decreases (Table 3 and Figure 3). Significant differences in the ratios between lengths in a given condition would suggest that neuronal

turnover of the initially labeled new HVC neurons occurs progressively with time, regardless of condition. We anticipate with the completion of data collection, all of these trends will become more pronounced and may even reach levels of statistical significance.

2.4.5 Turnover of new non-neuronal cells is not dependent on condition

We asked whether breeding condition affected non-neuronal addition in HVC both during periods of active growth and regression and once HVC reached a stable size by counting the number of TA-positive non-neuronal cells labeled concurrently with new neurons. We found that the initial cohort of TA-labeled non-neuronal cells did not vary with condition and length in condition (ANOVA, $F_{(4,25)}$=1.7070; p=0.1792; Table 3) nor did the number of new non-neuronal cells from the final cohort (ANOVA, $F_{(4,23)}$=0.4813; p=0.7492; Table 3). Furthermore, the proportions of the initial cohort to the final cohort of non-neuronal cells did not vary with condition or length of condition (ANOVA, $F_{(4,12)}$=1.2767; p=0.3369; Table 3). These results suggest that non-neuronal cells turnover in HVC at a steady rate independent of breeding condition.

2.4.6 Factors mediating maintenance of new neurons

We observed variability in numbers of new neurons from both cohorts within all experimental groups, and so we asked which of the factors measured might have contributed to the variability. Variability in the persistence of new neurons may reflect intrinsic factors such as neuronal activity and HVC growth potential or extrinsic factors like local T levels. We performed a Pearson's *r* correlation between the number of new neurons in the initial cohort and the number in the final cohort and found no significant correlation within LD+T or SD (Table 4), demonstrating that variability in one cohort of neurons is not related to variability in the other cohort. This result further suggests that intrinsic or extrinsic properties either are not contributing to variability in new neuronal survival, or are regulating the two populations of new neurons differentially. Thus, we tested the size of HVC as one intrinsic property accounting for variability in new neuronal survival by correlating HVC final size with both cohorts of neurons independently in both LD+T and SD conditions. We found that the variability in new neuron persistence from both the initial and final cohort did not correlate with HVC volume regardless of condition (Table 4). We also tested the role of T, an extrinsic factor to HVC, in accounting for variability in new neuron maintenance and we found no correlations between T levels in breeding or nonbreeding conditions and either cohort neuronal number (Table 4). Although, these data suggest that neither HVC size nor blood plasma T levels accounst for differences in levels of neuronal persistence among individuals, the data do not rule out the possibility of such

an effect nor the possibility of some other common factor mediating the persistence of new neurons.

2.4.7 Neural stem cell proliferation rate during initial cohort labeling does not differ between breeding and nonbreeding conditions

Having found that the initial cohort of new neurons survive longer in breeding condition and contribute to the growth of HVC, we asked if this change was due to increases in neural stem cell proliferative rate during LD+T. To assay proliferation rates we quantified the persistence of neural stem cells and other proliferative cells in the ventral Ventricular Zone (vVZ). Because TA availability was reduced with each successive cell division, the persistence of TA-labeled vVZ cells can be inferred to be a lack of stem cell proliferation [27, 29]. Thus, any lack of difference between persistence of vVZ TA-labeled cells can be interpreted as a lack of difference in proliferative rates totaled across the periods during and following TA pulses. The number of TA-labeled proliferative cells in the vVZ did not vary with condition or length in condition (ANOVA, $F_{(4,10)}$=1.3816, p=0.3081). Additional analyses also found no differences; the number of vVZ proliferative cells labeled with TA four or eight weeks before experiment termination did not vary significantly between SD and LD+T conditions (SD 4w, 184 ± 21, n=6, and LD+T 4w, 258 ± 44, n=6). The lack of difference in exhaustion of TA label in vVZ cells between LD+T and SD conditions suggests that proliferation rates are the same during the labeling of the initial and final cohorts of neurons, regardless of breeding condition.

2.5 Discussion

Several hypotheses exists regarding the presence and purpose of neuronal turnover none of these can be tested effectively, however, until a basic understanding of the natural dynamics between neuronal birth and death exists. Here, we describe natural neuronal turnover in one model of adult neurogenesis and test the role of sex steroids, specifically T, in modulating the balance between new and mature neuronal survival. We show that breeding conditions favor greater maintenance of new neurons added to HVC at the onset of breeding conditions than nonbreeding conditions. We found that once HVC attains a steady breeding-like or nonbreeding-like state new neuronal turnover returns to a common equilibrium. Regardless of condition and survival rate of the initial cohort of neurons, turnover of new HVC neurons occurs progressively with time. Together, these data demonstrate that breeding conditions promote the survival of an initial population of new neurons entering HVC, but that once HVC is fully grown

the survival of successive neuronal populations returns to levels similar to that of nonbreeding conditions.

2.5.1 The role of testosterone in promoting the addition and maintenance of new neurons added early in HVC growth

Breeding conditions supported a higher number of new neurons labeled with the initial pulses of TA when compared to nonbreeding conditions. This increase in survival of the early cohort of new neurons entering HVC supports the growth of HVC to its full breeding neuronal number. Our results are consistent with and provide a mechanism for previous reports of HVC seasonal growth [10 - 12]. Breeding conditions, specifically high T levels, may “lock in” new neurons, and thus facilitate HVC growth. T, however, may not act directly to support the survival of the initial cohort of new neurons. Alternatively, T may indirectly support the addition and maintenance of new neurons initially added during breeding conditions by altering the local environments to which new neurons are added (i.e. within HVC), or form synaptic connections (i.e. within RA), or both. For example, the survival of new neurons early after exposure to breeding conditions could result from an increase in available "vacancies" for new neurons to reside and form synaptic contacts due to the hypertrophied volume of HVC and the increase dendritic arbors of RA [32, 33]. T and its estrogenic metabolites increase expression of the genes for vascular endothelia growth factor (VEGF) and brain-derived neurotrophic factor (BDNF), which provide increased trophic support for new neurons within the local HVC environment [34, 35]. Increased activity of target neurons in RA [36] could also account for higher recruitment and survival of the initially born new neurons in nonbreeding conditions. Regardless of the specific mechanism, our results demonstrate that breeding conditions, specifically LD photoperiods and the presence of high T plasma levels, promote a high survival rate of the new neurons initially added to HVC during seasonal growth. Alternatively, in nonbreeding conditions, T and its downstream trophic effectors, remain low, and the new neurons that enter HVC continue to turn over.

Increased survival of new neurons added to HVC during the early stages of breeding condition growth may seem to contradict a previous report that new neurons persist longer in nonbreeding conditions than breeding conditions [7]. Nottebohm et. al. (1994) reported that male canaries pulsed with ^{3}H-thymidine in fall (nonbreeding season) had a significantly higher number of ^{3}H-thymidine positive cells around 40 and 120 days following new neuron labeling than canaries pulsed in spring (breeding season). A direct comparison of our results to the canary work can not be made however for the following reasons: 1) the timing of ^{3}H-thymidine

pulses was not precisely controlled so that each canary received pulses at the same physiological time into breeding or nonbreeding season, 2) the breeding season group of canaries was allowed to progress into nonbreeding conditions as evidenced through a reduction in HVC volume in the spring 140 day experimental group [7], and 3) canaries exhibit reduced seasonality compared to white-crowned sparrows [37].

2.5.2 New neuron survival returns to a common equilibrium when HVC attains steady-state condition

We found that the survival of new neurons labeled after HVC reaches its fully grown or regressed state does not differ, suggesting neuronal turnover returns to a common level in both breeding and nonbreeding conditions to maintain a breeding or nonbreeding-like HVC. Moreover, a lack in difference in the number of proliferative cells in the vVZ suggests that proliferation rate also does not change once HVC enters this steady-state condition. These data are consistent with previous reports that HVC volume and neuron number reach their breeding maxima by seven days following onset of LD+T [22] and are fully regressed four to fourteen days following transition from LD+T to SD [16, 18]. Measurements beyond these time points do not indicate a significant change in HVC volume or neuronal number. Our work is the first to report the effect, or lack thereof, of breeding condition on the survival of neurons added *after* HVC stabilization.

2.5.3 Turnover of new non-neuronal cells is not dependent on condition

We found no significant effect of condition (i.e. LD+T or SD) on the maintenance of individual cohorts of new non-neuronal cells or the proportion of new non-neuronal cells that survive, suggesting non-neuronal turnover rate may be constant over all seasons. A constant turnover rate may initially appear contradictory with previous reports of increased angiogenesis [20, 34] and the glial cell population [20] with T administration. However, a constant turnover rate of non-neuronal cells would not necessarily preclude the expansion of endothelial and glial cell populations. Alternatively, the apparent differences in results between our and previous work may be due to differences in timing of TA labeling and experiment length. Both previous studies labeled new glial and endothelial cells born shortly after the administration of T (2-19 d) and assayed the number of these cells shortly after their birth (10-35 d). Thus, the increase in non-neuronal cell numbers in these prior studies suggest that T influences the proliferation, migration, and maybe, in the longest experiments, the maturation of these non-neuronal cells. These studies, however, do not assay the long-term survival of such cells. In our study, T may have transiently increased the rate of non-neuronal cell proliferation immediately after the onset

of breeding condition to contribute to the expansion of the vasculature and glial population, but our results suggest that the period that T has a positive effect on proliferation and addition of non-neuronal cells is in fact very transient. In both the LD+T and SD groups with TA pulses separated by four weeks we did not observe a significant effect of breeding condition on the number of new non-neuronal cells. Thus, our results demonstrate that T did not alter the addition and survival of new non-neuronal cells labeled 1 week prior or 4 weeks after the onset of T. Because the majority young cells leave the VZ and begin wandering within HVC three to six days after birth [24, 26], our finding of no difference in the survival of non-neuronal cells labeled one week prior to T onset further suggests that previous reports of increases in TA-labeled non-neuronal cells is may be due to increases in neural stem cell proliferation rather than survival rate.

2.5.4 Model of Neuronal Turnover

By comparing the initial cohort of new neurons to the final, we found that new neurons progressively decrease with time regardless of condition. The number of new neurons from the initial cohort decreased steadily across both LD+T and SD groups. Moreover, we find that the rate of vVZ proliferation is not different in stable breeding and nonbreeding conditions. Prior to our study two different models of neuronal turnover existed; in one model new neurons enter HVC and replace only mature neurons whereas in the other model new neurons enter HVC and replace both new and more mature neurons. In order for the first scenario to accomodate increases in total HVC neuronal number, neural stem cell proliferation must be higher in breeding conditions. Given that we find the initial cohort of neurons are replaced by new neurons in as little as four weeks and that we find no change in vVZ proliferation, our data supports the second model as a model of HVC neuronal turnover. Thus, the maintenance of a given neuron, be it new or more mature, must be due to factors besides neuronal age alone. Factors that promote neuronal survival independent of age likely include blood plasma T levels [38-40], synaptic strength [33], neurotrophin signaling [35, 41] or neural activity [36], among others.

2.5.5 Factors mediating maintenance and turnover of new neurons

The null hypothesis regarding which HVC neurons survive, new versus mature neurons, is that survival is stochastic and not dependent on any intrinsic or extrinsic properties of HVC or seasonal physiology in general. We found that of the properties related to HVC seasonal growth and regression that we measured, none correlated with the variability in neuronal survival rates within cohorts and within experimental group. Our results suggest that neither HVC volume nor

T levels contribute to the level of neuronal survival within the initial or final cohorts of new neurons. These results are limited, however, by the fact that we could not measure HVC volume, total neuronal number, neuronal death, or plasma or local T levels at the precise time of incorporation of new neurons. Moreover, given the experimental design we cannot tease levels of neuronal addition apart from levels of new neuronal survival. Thus, we cannot entirely rule out HVC space availability or plasma or local T levels as a factor regulating the rate of new neuronal incorporation, survival, or both.

2.5.6 Conclusion

We have shown that a pallial nucleus in adult songbirds undergoes neuronal turnover such that during breeding conditions new neurons enter HVC and become “locked in” to support the seasonal growth of HVC. Once HVC growth stabilizes at breeding condition neuronal numbers, new neurons enter HVC and replace more mature neurons at a rate similar to nonbreeding conditions. These observations demonstrate that the turnover of neurons is quite dynamic and sensitive to both testosterone levels and exposure length. The pronounced and dynamic nature of neuronal turnover in HVC provides an excellent system for future studies examining the mechanisms regulating neuronal replacement and promoting new and mature neuronal survival.

2.6 References

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Table 1. Blood plasma T levels

	LD+T		SD			Two-Way ANOVA
	4w	8w	4w	8w	16w	
Testosterone (ng/mL)						
LD+T (pre-synchronizing or mid-experiment)	17.07 ± 2.93	21.54 ± 2.02	N.D.	16.58 ± 2.54	15.6 ± 4.4	**$F_{(4,21)}$=1.0258; p=0.4013**
Final Day	17.75 ± 2.52[a]	18.58 ± 1.42[a]	0.39 ± 0.10[b]	0.57 ± 0.22[b]	1.18 ± 0.59[b]	**$F_{(4,30)}$=78.4508; p<0.0001**

All values are mean ± S.E.M. Superscript numbers denote significant differences across groups with post-hoc Tukey test.

Table 2. Morphometrics of HVC

	LD+T		SD			Two-Way ANOVA
	4w	8w	4w	8w	16w	
HVC						
Unilateral Volume (mm^3)	0.50 ± 0.03[a]	0.55 ± 0.03[a]	0.24 ± 0.04[b]	0.42 ± 0.06[a,b]	0.31 ± 0.04[b]	**$F_{(4,27)}$=12.9651; p<0.0001**
Neuron Density ($x10^3/mm^3$)	N.D.	N.D.	N.D.	N.D.	N.D.	
Unilateral Neuron Number ($x10^3$)	N.D.	N.D.	N.D.	N.D.	N.D.	

All values are mean ± S.E.M. Superscript numbers denote significant differences across groups with post-hoc Tukey test.

Table 3. Numbers of new neurons and non-neuronal cells from both cohorts

	LD+T		SD			Two-Way ANOVA
	4w	8w	4w	8w	16w	
Initial Cohort						
TA Positive Neuron Number	172 ± 50[a]	144 ± 12[a,b]	89 ± 22[a,b]	71 ± 12[a,b]	36 ± 16[b]	**$F_{(4,25)}$=3.9298; p=0.0131**
TA Positive Non-Neuron Number	169 ± 51	129 ± 44	95 ± 28	105 ± 13	47 ± 13	$F_{(4,25)}$=1.7070; p=0.1792
Final Cohort						
TA Positive Neuron Number	160 ± 37	117 ± 14	135 ± 27	86 ± 22	78 ± 17	$F_{(4,23)}$=1.7164; p=0.1807
TA Positive Non-Neuron Number	92 ± 17	127 ± 30	115 ± 33	117 ± 22	86 ± 14	$F_{(4,23)}$=0.4813; p=0.7492
Proportions						
Initial Cohort/Final Cohort Neurons	1.70 ± 0.73	0.97 ± 0.20	0.87 ± 0.16	0.98 ± 0.28	0.13 ± 0.02	$F_{(4,12)}$=1.2466; p=0.3432
Initial Cohort/Final Cohort Non-Neuron	1.20 ± 0.49	0.30 ± 0.16	0.89 ± 0.15	1.31 ± 0.41	0.35 ± 0.15	$F_{(4,12)}$=1.2767; p=0.3369

All values are mean ± S.E.M. Superscript numbers denote significant differences across groups with post-hoc Tukey test.

Table 4. Comparisons between several factors and variability in new cell survival

	LD+T Groups	SD Groups
Pearson's Correlation		
TA Neurons from Cohort 1 to Cohort 2	R_2=0.01201; p=0.8363	R_2=0.0214; p=0.6500
Correlations for Cohort 1		
TA Neurons to HVC V	R_2=0.0271; p=0.2781	R_2=0.0004; p=0.9619
TA Neurons to mid-/pre-experiment T levels	R_2=0.0029; p=0.8987	R_2=0.1555; p=0.3336
TA Neurons to final T levels	R_2=0.2816; p=0.2204	R_2=0.0264; p=0.6541
Correlations for Cohort 2		
TA Neurons to HVC V	R_2=0.0805; p=0.7002	R_2=0.0057; p=0.8594
TA Neurons to mid-/pre-experiment T levels	R_2=0.0788; p=0.4278	R_2=0.1903; p=0.3872
TA Neurons to final T levels	R_2=0.0383; p=0.2388	R_2=0.0007; p=0.8140

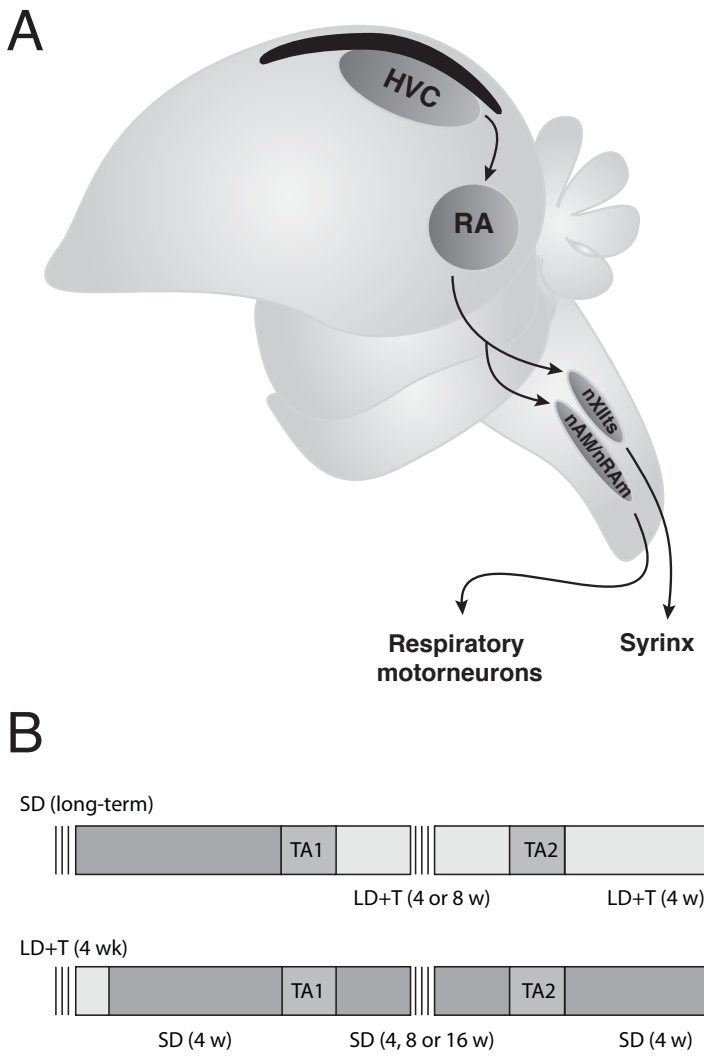

Figure 1. Experimental design. *A)* A schematic of the song control system. *B)* The experimental design of both breeding and nonbreeding condition groups. The breeding condition group is on the top and the nonbreeding condition group on the bottom. TA1 = initial thymidine analog pulses of either EdU or BrdU, TA2 = final thymidine analog pulses with the TA not used for TA1.

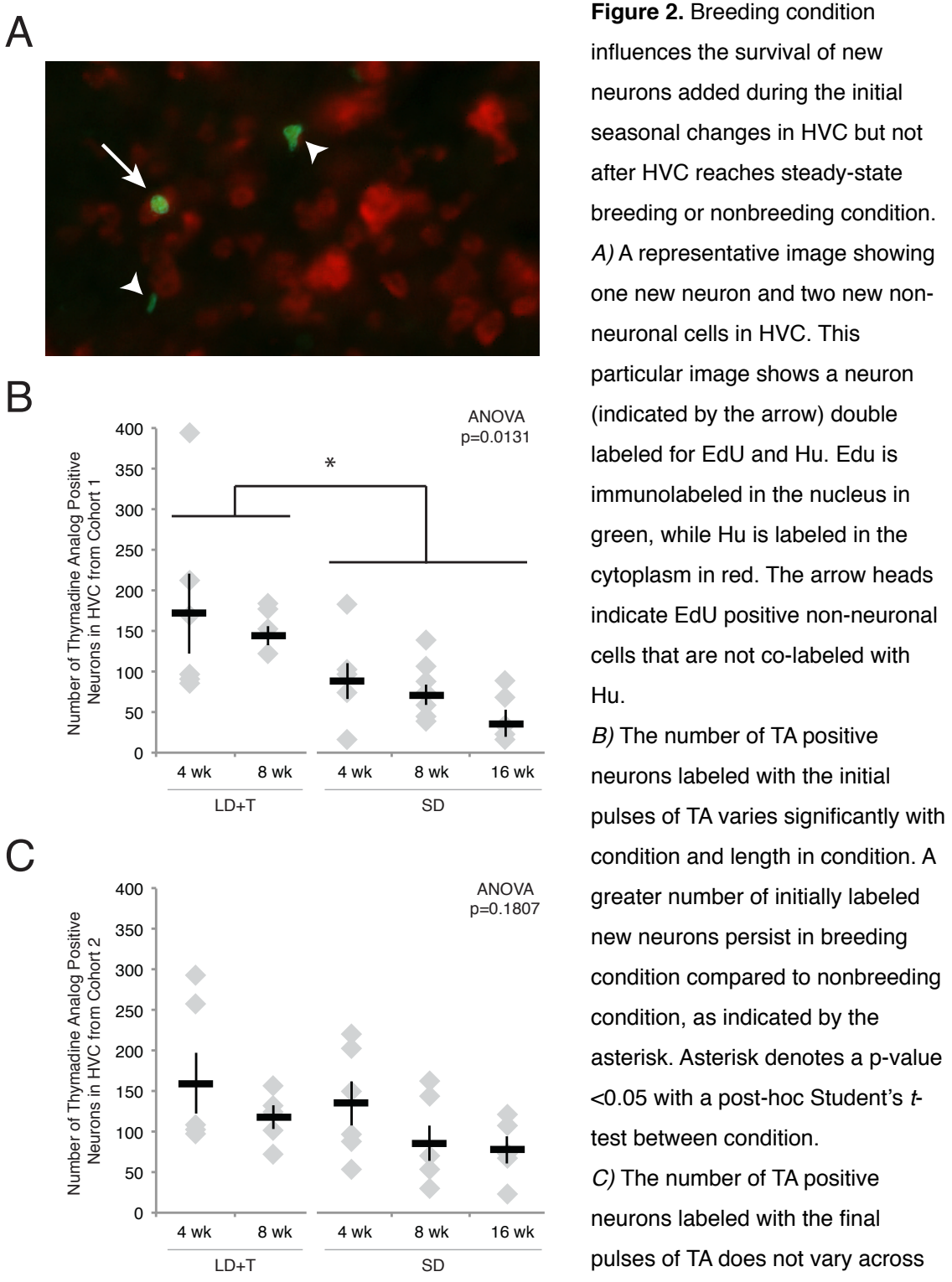

Figure 2. Breeding condition influences the survival of new neurons added during the initial seasonal changes in HVC but not after HVC reaches steady-state breeding or nonbreeding condition. *A)* A representative image showing one new neuron and two new non-neuronal cells in HVC. This particular image shows a neuron (indicated by the arrow) double labeled for EdU and Hu. Edu is immunolabeled in the nucleus in green, while Hu is labeled in the cytoplasm in red. The arrow heads indicate EdU positive non-neuronal cells that are not co-labeled with Hu. *B)* The number of TA positive neurons labeled with the initial pulses of TA varies significantly with condition and length in condition. A greater number of initially labeled new neurons persist in breeding condition compared to nonbreeding condition, as indicated by the asterisk. Asterisk denotes a p-value <0.05 with a post-hoc Student's *t*-test between condition. *C)* The number of TA positive neurons labeled with the final pulses of TA does not vary across experimental groups. Each grey dot represents the number of TA positive neurons from one bird while the black bars indicate the group mean S.E.M.

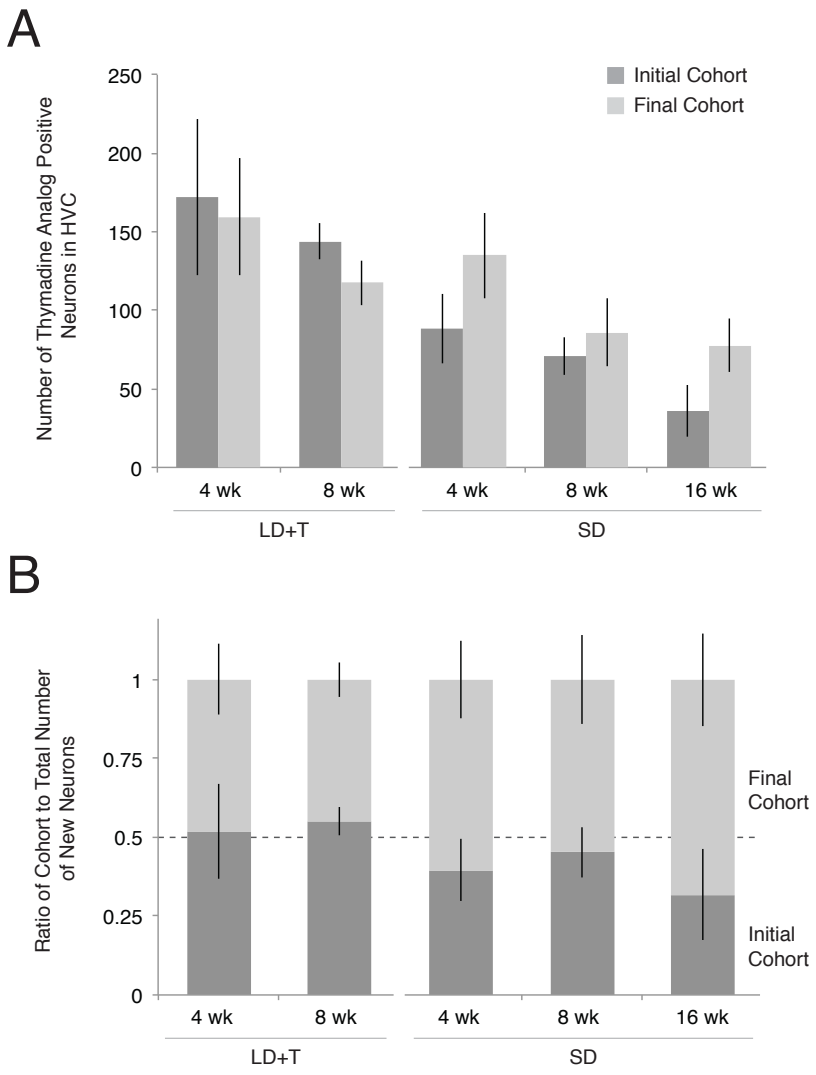

Figure 3. Neuronal turnover differs, although not significantly, between breeding and nonbreeding conditions. *A)* The number of TA positive neurons in HVC reploted as averages to show the trends in turnover of the initial cohort of new neuons. In breeding conditions the number of neurons present from the initial cohort is higher than the new neurons from the final cohort. Alternatively, in nonbreeding conditions the final cohort number is higher than the initial cohort. *B)* The ratio of each cohort of new neurons to the total number of new neurons across experimental groups. The ratio of initially- versus finally-labeled new neurons is around or above 0.5 (indicated by the dashed line, or a one to one ratio of neurons from each cohort) in breeding condition but below 0.5 in nonbreeding conditions.

Chapter 3. Reactive neurogenesis in response to naturally occurring apoptosis in an adult brain[2]

3.1 Abstract

Neuronal birth and death are tightly coordinated to establish and maintain properly functioning neural circuits. Disruption of the equilibrium between neuronal birth and death following brain injury or pharmacological insult often induces reactive, and in some cases regenerative, neurogenesis. Many neurodegenerative disorders are not injury-induced, however, so it is critical to determine if and how reactive neurogenesis occurs under non-injury induced neurodegenerative conditions. Here, we used a model of naturally occurring neural degradation in a neural circuit that controls song behavior in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) and examined the temporal dynamics between neuronal birth and death. We found that during seasonal-like regression of the song control nucleus HVC (proper name), caspase-mediated apoptosis increased within two days following transition from breeding to nonbreeding conditions and neural stem cell proliferation in the nearby ventricular zone (VZ) increased shortly thereafter. We show that inhibiting caspase-mediated apoptosis in HVC decreased neural stem cell proliferation in the VZ. In baseline conditions the extent of neural stem cell proliferation correlated positively with the number of dying cells in HVC. We demonstrate that as apoptosis increased and the number of both recently born and pre-existing neurons in HVC decreased, the structure of song, a learned sensorimotor behavior, degraded. Our data illustrate that reactive neurogenesis is not limited to injury-induced neuronal death, but also can result from normally occurring degradation of a telencephalic neural circuit.

3.2 Introduction

The adult brain is capable of incorporating new neurons into existing neural circuits, a process broadly termed neurogenesis. Given that the absolute neuronal number in neurogenic regions typically does not increase in adult birds and mammals (reviewed in [1], but see [2]), the addition of new neurons must be offset by neuronal death. Proper equilibrium between neuronal birth and death is critical for maintaining neural circuits that control behavior, as is apparent from the pathological consequences that occur when neuronal death exceeds birth in chronic

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[2] Larson TA, Thatra NM†, Lee B†, Brenowitz EA. (2014) Reactive neurogenesis in response to naturally occurring apoptosis in an adult brain. The Journal of Neuroscience. 34(39): 13066-76 doi: 10.1523/JNEUROSCI.3316-13.2014 PMID: 25253853

depression, Parkinson's Disease, Alzheimer's Disease, and age-related cognitive decline (reviewed in [3]).

Neuronal birth and death in healthy brains are tightly coordinated and causally linked. During early brain development, excess neurons are produced and subsequently die as neural circuits are refined (reviewed in [4]). In adult vertebrates, neuronal death caused by ischemia or injury can increase neurogenesis within 7 days (reviewed in [5] and [6]). Neuronal addition following neuronal death, referred to as reactive neurogenesis, may repair damaged areas and thereby restore function and behavior [7]. Although the effects of injury-induced neuronal death are well-documented, the temporal dynamics between non-injury induced neuronal birth and death—and their effects on behavior—remain unclear.

Seasonal regression of the avian motor pathway for song production (Figure 1A) provides a tractable model for investigating the balance between non-injury induced neuronal death and replacement, and their contribution to performance of a learned sensorimotor behavior, song. In Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*) the number of neurons within the pallial song control nucleus HVC (proper name, [8]) changes seasonally [9]. Each breeding season HVC incorporates nearly 68,000 new neurons [10], most or all of which project to the robust nucleus of the arcopallium (RA) ([11], but see [12]). As sparrows transition from breeding to nonbreeding conditions, an equal number of HVC neurons die via apoptosis [13]. Song behavior also changes seasonally; white-crowned sparrows typically sing one song type that in breeding conditions is more stereotyped and longer than in nonbreeding conditions [14].

Gambel's white-crowned sparrows are useful for studying neurodegeneration and reactive neurogenesis because in HVC these processes occur rapidly, robustly, and naturally (as opposed to being injury- or pharmacologically-induced). Here, we induce natural neural degeneration by transferring birds from breeding to nonbreeding conditions and characterize the relationship between neural stem cell proliferation in the ventricular zone (VZ) and cell death in HVC. Moreover, we manipulate HVC cell death to show that it is necessary for the up-regulation of neural stem cell proliferation. We also identify changes in song that accompany the observed HVC cellular changes. Our study provides important insights into temporal dynamics between non-injury induced neuronal birth and death and how modulation of this equilibrium relates to behavior.

3.3 Materials and Methods

3.3.1 Animals

Seventy-four adult male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*; white-crowned sparrows) were collected in eastern Washington during their pre- or post-breeding season migration. The minimum ages of birds were estimated by plumage coloration upon capture: at least 14 months for adults (white and black crown) or at least 2 months for juveniles (white and brown crown). Prior to the initiation of experiments, all birds were housed in indoor group aviaries exposed to short day photoperiods (SD; 8 hr light, 16 hr dark) for 12 weeks to ensure that the song and reproductive systems had regressed and would be sensitive to the stimulatory effects of transfer to long day photoperiods (LD) and testosterone (T) implant. All experiments followed NIH animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee.

3.3.2 Experimental Procedures

To examine the addition and survival of newborn neurons in HVC, birds housed in SD for 12 weeks were injected intramuscularly with BrdU (15 mg/ml in 0.09% NaCl and 0.012M NaOH; Sigma) at a dose of 50 mg/kg once daily between 6 and 8 hours after lights-on for 5 days. Two days following the final BrdU injection, each bird was implanted subcutaneously above the scapula with a 12-mm Silastic capsule (1.47 mm inner diameter, 1.96 mm outer diameter) filled with crystalline T (Sigma). We kept all birds in LD for 28 days after T implantation to allow full breeding-like growth of the song control nuclei [10]. All birds except LD+T control birds (n=8) had T pellets removed after 28D of LD+T and were transferred overnight to SD, which mimics nonbreeding season conditions [9]. To assess neuronal and song degradation, birds were housed in SD until sacrifice at different time points used in previous studies: 1 day (n=8), 2 days (n=9), 4 days (n=9), 7 days (n=10), 14 days (n=10), or 28 days (n=9) following transition into SD [14-16]. To label proliferating neural stem cells and their progeny, all birds were injected with one dose of BrdU (50mg/kg, 15 mg/ml) intraperitoneally two hours prior to tissue collection.

To determine whether stem cell proliferation in the VZ is functionally linked to cell death in HVC, we used caspase inhibitors to inhibit cell death in HVC during natural HVC regression. Previous work showed that neuronal loss from HVC results from caspase-dependent apoptosis and that infusing HVC with a cocktail of caspase inhibitors decreased the number of apoptotic cells and newborn neurons in HVC of adult white-crowned sparrows transferred from breeding to nonbreeding conditions [13, 17]. Thus, to inhibit apoptosis within HVC upon transition from breeding to nonbreeding conditions, we stereotaxically implanted cannulae bilaterally adjacent

to HVC and infused the same cocktail of caspase inhibitors. Two days prior to transition from breeding to nonbreeding conditions, eleven 22- to 27-month old birds were anesthetized with isoflurane (2%). We lowered cannula near HVC 0.8 mm ventral, 3.0 mm lateral to the intersection of the midsagittal and transverse sinuses. Histological analysis demonstrated that the ends of the cannulae track were 224 ± 33 μm from HVC. We fixed the cannula to the skull with dental cement and attached a micro-osmotic pump (Alzet model 1007D) to the cannula. Six birds received osmotic pumps containing a cocktail of caspase inhibitors (0.015 mg in 100 μl of 1% DMSO in equal concentrations: pan-caspase inhibitor [Z-VAD-FMK; R&D Systems], caspase-3 inhibitor [Z-DEVD- FMK; Calbiochem], and caspase-9 inhibitor [Z-LEHD-FMK; Calbiochem]), while five control birds received only vehicle. We placed osmotic pumps into sealed microcentrifuge tubes filled with saline and mounted the tubes into custom-made "backpacks" that allowed the birds to fly freely. Two days after surgery, birds were transitioned from 28D of LD+T to SD for 3D. Two hours before sacrifice on the last day, all birds were injected IP with one dose of BrdU (50 mg/kg, 15 mg/ml) to label proliferating stem cells and their progeny in the VZ.

3.3.3 Blood draw and hormone analysis

Blood samples were obtained from birds in LD+T at 28 D and upon euthanasia to measure circulating T concentrations. We drew 250 μl of blood from the alar vein in the wing into heparinized collection tubes, immediately centrifuged the tubes, and stored the plasma at -20°C until assay. Using a Testosterone Enzyme Immunoassay kit (Enzo Life Sciences), we measured plasma T concentration. Minimum and maximum detectable plasma T concentrations were 0.03 ng/ml and 40.00 ng/ml, respectively. Samples with undetectable levels of T were treated as having concentrations at the lower detection limit for statistical analyses. Intra-and inter-assay coefficients of variation were 2.27 - 5.43% and 27.20%, respectively.

3.3.4 Tissue collection and processing

Five to seven hours after lights-on, brains were removed quickly after birds were deeply anesthetized with isoflurane. Within 2 min, brains were bisected at the midline and one randomly chosen hemisphere was frozen on dry ice for histology as described below. Halved brains were sectioned in the coronal plane at 40 μm on a cryostat, and each section was thaw-mounted serially. Every third section was Nissl stained and the remaining slides were stored at -80°C until immunolabeling. Because only one hemisphere of each brain was sectioned, all histological measurements described below are reported as unilateral counts or densities. The other half of each brain was stored at -80°C for future studies.

3.3.5 Immunohistochemistry and analysis

To visualize BrdU-labeled cells, we processed brain sections as follows. Briefly, sections were fixed in 4% paraformaldehyde, rinsed in phosphate buffered saline with 0.5% DMSO and 0.5% Triton-X (PDTX; pH 7.4), incubated with 0.1 mg/mL proteinase K for 5 minutes, and post-fixed. Slides were dipped in distilled water, incubated with 2N HCl at 37°C for 30 minutes, and rinsed with PDTX. After blocking in 5% heat inactivated goat serum, slides were incubated with rat anti-BrdU (1:200; Accurate) and mouse anti-HuC (1:100; Invitrogen) antibodies. HuC is a neuron-specific antigen expressed in both immature and fully differentiated neurons [18]. Labeling was visualized with diaminobenzidine (DAB) staining following incubation with biotinylated goat anti-rat and anti-mouse IgG secondary antibodies (1:200; Vector) and amplification with avidin-biotin peroxidase (ABC) complex (Vector). To obtain a purple precipitate for the BrdU antigen, we used DAB-nickel stain (0.05% DAB, 0.05% Nickel Ammonium Sulfate and 0.015% H2O2 in PBS). All single and double labeled cells in each HVC section were counted. BrdU-positive cells in the ventral side of the VZ (vVZ) were counted from the hillock of the VZ medial to HVC, out to the most lateral extension of the vVZ in all sections that exhibited arching of the VZ (see Figure 4A & B) [12]. New HVC neurons are born in this region of the VZ [19]. Because BrdU pulses were administered at two experimental time points, the counts of BrdU positive cells in the vVZ represent both neural stem cells that divided and became quiescent prior to LD+T onset, and vVZ proliferation two hours prior to tissue harvesting. Baseline labeling of neural stem cells with the first pulses of BrdU likely remains constant across experimental groups because the neural stem cells of the vVZ overlying HVC divide for only brief periods and then become quiescent for up to at least eight months [20].

Activated caspase-3 (AC-3) immunolabeling was used to identify cells undergoing apoptosis [21]. Sections were blocked with 5% heat inactivated goat serum, incubated with rabbit anti-AC-3 antibody (1:400; Santa Cruz), and then incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector) [13]. DAB staining was performed following amplification of the secondary antibody with ABC. Sections were lightly counterstained with thionin, a Nissl stain, as previously described [22]. AC-3 positive cells were counted from four HVC sections using a random, systematic sampling protocol as described below [23]. All measurements were made blind to treatment group.

All IHC was performed in batches of up to 28 slides with tissue from randomly assigned birds. Each batch included positive and negative controls.

3.3.6 Morphometric measurements in HVC and RA

Volumes of HVC and RA in the randomly chosen hemisphere were determined by tracing the borders of the nuclei identified in Nissl stained tissue onto paper, these drawings were scanned, and the area was determined using Image J Software (Version 1.46; NIH; http://rsb.info.nih.gov/ij/). Total volume was calculated from areas of the tracings using the formula for a truncated cone [23]. We used a random, systematic sampling protocol to measure neuron density in HVC; this protocol yields estimates that do not differ from stereological methods [23]. Samples were collected from sections through the full extent of each brain region. All Hu positive neurons that fell within an ocular grid (ca. 1.9 X 10^3 mm^2 at 1000X) randomly positioned in each section of HVC were counted to calculate neuronal density. Neuronal number was obtained by multiplying neuronal density for each bird by that bird's unilateral HVC volume.

3.3.7 Song recording and analysis

To analyze song behavior we housed all birds individually in sound isolation chambers (Industrial Acoustics AC-1) beginning 14 D after LD+T onset. By 14 D song achieves the stereotyped structure typical of breeding song [14]. Using Syrinx software (J. Burt, www.syrinxpc.com), we continually recorded vocalizations throughout the remainder of the experiment. To encourage birds to sing, we provided each bird with a mirror and 5 minutes of playback song from group-housed Gambel's white-crowned sparrows in breeding condition every 20 minutes during lighted conditions. Eight different song attributes (minimum and maximum frequency; frequency range; duration; aggregate entropy; average entropy; average power; and maximum power) of 30 randomly chosen digitized whole songs per bird and each syllable within all chosen songs were measured with Raven Pro version 1.4 (Cornell Lab of Ornithology, http://www.birds.cornell.edu/brp/raven/ravenoverview.html). Variability in measures of these song attributes did not change when we sampled more than 30 songs. We calculated the mean of each song or syllable attribute for a given day for each bird (as in [14]). We calculated the coefficient of variation of minimum and of maximum frequency for whole song and for each syllable by dividing the standard deviation by the mean of a syllable or whole song from each bird for a given day [14]. We measured the cross correlation (similarity of two waveforms) between whole songs produced during day 21 of LD+T and day 23 of LD+T or day 1, 2, 4, 7, 14, or 28 of SD using the same 30 digitized songs with the batch-processing, cross-correlation function in Raven. We calculated song percent completeness from the number of syllables produced in each of the same 30 songs by dividing the total number of syllables by the maximum number of syllables produced by the given bird - typically five syllables in Gambel's

White-Crowned Sparrows. To control for individual bird variability in song completeness, we obtained a relative percent completeness value for individual birds' songs throughout the time-course by dividing the given day's value by the value from LD+T of the same bird. We measured song rate as the maximum number of songs produced during any two hour period of the day. Two hour binning was chosen to obtain enough songs to perform statistical analysis from birds that sang infrequently (i.e. 14 D and 28 D of SD). All measures of song were performed on song collected during 21 and 23 D LD+T and during 1, 2, 4, 7, 14, and 28 D of SD. If a bird did not sing at least 30 songs on one of these days, we did not include that bird's songs for that day in the analysis. Birds in the caspase inhibitor study never sang more than 30 songs during SD 1, 2, and 3D, and so they were not included in the analyses.

3.3.8 Statistical analysis

Comparisons were made with one-way ANOVAs with experiment duration as the factor, and Tukey tests for post-hoc comparisons between durations of experimental treatment. For the caspase inhibitor experiment, comparisons between experimental groups were made with Student *t*-tests. All correlations were assessed with the Pearsons' *r* correlation test. For all statistical tests a p-value ≤ 0.05 was considered significant. All statistical analyses were made using JMP Version 8.0.1 (SAS, Cary NC). Data are presented as mean±SEM.

3.4 Results

3.4.1 Validation of full growth and regression of the song control system

We confirmed that our manipulations induced breeding-like growth and nonbreeding-like regression of the song production pathway (see Figure 1B for experimental design) by showing that plasma T levels and morphological changes within HVC and RA were consistent with LD+T and SD condition values from other studies. Systemic T plasma levels were elevated at 28D of LD+T (Table 1) and morphological features of HVC and RA were typical of birds in breeding condition (Table 2, Figure 2A) [9, 10]. Plasma T levels decreased by 1D of SD and remained basal through the full 28D of SD (Table 1). HVC and RA morphology of birds transitioned into nonbreeding conditions were consistent with those of nonbreeding white-crowned sparrows (Table 2, Figure 2A) [9, 15].

3.4.2 Apoptosis within HVC peaked rapidly after transition into nonbreeding conditions

We determined the time course of cell death in HVC as birds transitioned into nonbreeding conditions by quantifying the number of apoptotic cells within HVC during rapid regression using the apoptosis marker AC-3 (Figure 2C). AC-3 positive cell number increased by 200%

compared to breeding condition as soon as 2D following transition from LD+T to SD (post-hoc Tukey, p=0.0342; Table 3 and Figures 2B). The number of AC-3 positive cells in HVC was not elevated significantly during any other time points of SD when compared to the number of AC-3 positive cells in HVC of breeding condition birds (Table 3 and Figure 2B). Cells expressing AC-3 demonstrated features typical of apoptosis, including membrane blebbing, cytoplasm or chromatin condensation, and nuclear fragmentation. Apoptotic neurons no longer consistently expressed the neuronal marker Hu and we therefore could not reliably confirm the neuronal identity of AC-3 positive cells (Figure 2C).

3.4.3 Both new and pre-existing neurons died during rapid regression of HVC

We determined whether or not new adult-born neurons are selectively targeted for death during regression, by counting the number of new BrdU positive neurons that persisted throughout regression (Figure 1B and 3A). Adult-born neurons that enter HVC each breeding season might: (1) replace older neurons that died during the transition into nonbreeding conditions, (2) be a transient population that emerges and dies each breeding and nonbreeding season without persisting, or (3) incorporate and die along with pre-existing neurons. The number of new BrdU positive neurons present in HVC decreased by 53% over the 28D following the transition from breeding to nonbreeding condition (Table 3). This decrease in the population of new neurons closely matches the near 50% reduction in total neuron number (new and pre-existing neurons) in HVC during regression (Tables 2 and 3). Moreover, the timing of death in both populations of neurons is similar: by 1D of SD both new and total neuron numbers decrease by around 35%, and by 14D of SD both neuronal populations decrease by around 60% (Tables 2 and 3). These findings suggest that factors besides a neuron's age alone govern whether it lives or dies during regression of HVC**.**

3.4.4 Cellular proliferation in the vVZ is temporally linked to apoptosis in HVC

We identified the temporal dynamics of neural precursor cell proliferation during transition to nonbreeding from breeding conditions by quantifying cellular proliferation in the ventral VZ (vVZ; i.e. cells labeled with a two-hour pre-pulse of BrdU) during the time-course of regression. Basal rates of proliferation in the vVZ did not differ significantly between long-term breeding (28D LD+T) and long-term nonbreeding (28D SD) birds (*t*-test, p=0.1120; Table 3 and Figure 3C). vVZ proliferation levels, however, varied significantly with the number of days of active regression (ANOVA, $F_{(6,35)}$=6.6854, p<0.0001). Proliferation became significantly elevated by 4D of SD compared to LD+T (Tukey, p=0.0447;Table 3 and Figure 3C). Proliferation decreased to near basal LD+T levels by 7D SD and remained basal through 14D and 28D SD

(Table 3 and Figure 3C). The peak in vVZ proliferation was delayed temporally compared to the peak in HVC cell death (Figures 3C and 4A), suggesting that the two phenomena were functionally related.

We determined that changes in vVZ proliferation were related to changes in HVC cell death number by comparing vVZ proliferation with HVC cell death from each day within the time-course of regression (Table 3). The levels of proliferation in the vVZ correlated positively with the number of AC-3 positive cells in HVC during stable breeding and nonbreeding conditions (i.e. 28D of LD+T or SD, respectively; R^2=0.8801, p=0.0002, n=9; Figure 4B). There were no significant correlations on days in the time-course that corresponded to a peak in HVC cell death (i.e. 2D SD), a peak in vVZ proliferation (i.e. 2D and 4D SD), or any other times during active regression of the song control circuit (i.e. 1D, 7D, and 14D of SD; R^2=0.0002, p=0.9433, n=21; Figure 4B). These results demonstrate that proliferation in the vVZ follows HVC cell death during active regression of the song production circuit, and that under baseline conditions the amount of proliferation in the vVZ positively correlates with HVC cell apoptosis.

3.4.5 The proliferation of stem cells in the vVZ is functionally linked to cell death in HVC

We tested the necessity of HVC cell death for the observed increase in vVZ proliferation by experimentally inhibiting HVC cell death with an infusion of a cocktail of caspase inhibitors for the first three days of rapid HVC regression. The caspase inhibitor cocktail prevented the normal decrease in total neuronal number (see Table 4 for values and statistics) and the increase in the number of AC-3 positive apoptotic HVC cells (Table 4 and Figure 5A). To control for possible effects of age or testosterone on neuronal turnover, all birds receiving infusions were age matched and testosterone levels were confirmed to not be significantly different between experimental groups (Table 4). Upon quantification of vVZ proliferation, we found that birds infused with the caspase inhibitor cocktail had significantly fewer BrdU positive cells in the vVZ than those infused with the vehicle control (Table 4 and Figure 5B). These data indicate that the increased proliferation of neural stem cells in the vVZ is dependent on HVC cell death and that the increase in vVZ proliferation following naturally occurring HVC cell death is indeed a process of reactive neurogenesis.

3.4.6 Neuronal turnover is related to age but not testosterone levels

Neuronal survival and neural stem cell proliferation may be influenced by both T and age (reviewed in [24, 25]). We assessed the relationship of T and age to cell death and proliferation by comparing systemic plasma T levels and the minimum age of the birds (minimum age upon capture plus time spent in captivity) with cellular attributes of HVC and the vVZ. When all values

for breeding and nonbreeding conditions were pooled, T levels of birds sampled in breeding and nonbreeding conditions did not correlate with the number of AC-3 positive cells in HVC (R^2=0.0115, p=0.4539, n=39), the number of new neurons (R^2=0.0109, p=0.4410, n=40), the number of BrdU positive cells in the vVZ (R^2=0.0042, p=0.7002, n=36), or minimum age (R^2=0.0100, p=0.3931, n=39; data not shown). Furthermore, comparisons made with pooled baseline condition values (i.e. LD+T and 28D of SD) or pooled active regression values (i.e. 1, 2, 4, 7, and 14D of SD) revealed no significant correlations for the above measures.

Age correlated negatively with the number of AC-3 positive cells in HVC during baseline breeding and nonbreeding conditions (R^2=0.5667, p=0.0075, n=11; Figure 6A) and the number of BrdU positive cells in the vVZ (R^2=0.6546, p=0.0026, n=11; Figure 6B), such that older birds had lower numbers of AC-3 cells and fewer BrdU positive cells in the vVZ than younger birds. These data indicate that as a bird ages, steady state neuronal turnover, or the birth and death of neurons in stable breeding and nonbreeding conditions, decreases.

By contrast, AC-3 cell number did not correlate with age (R^2=0.0115, p=0.2674, n=23) during active regression of HVC (i.e. SD 1-14D). Moreover, HVC volume and total neuron number did not correlate with age at maximal breeding condition (i.e. LD+T 28D; HVC volume, R^2=0.1095, p=0.2458; HVC Hu density, R^2=0.5562, p=0.2542; n=4; Figure 6C) or when HVC was completely regressed (i.e. 28D of SD; HVC volume, R^2=0.0008, p=0.9507; HVC Hu density, R^2=0.0092, p=0.9393; n=7; Figure 6C). Taken together these data suggest that HVC in all birds, regardless of age, regresses the same amount during the transition from breeding to nonbreeding conditions. Alternatively, during active regression of HVC, the vVZ proliferation did correlate with age (R^2=0.2259, p=0.0342, n=18). These findings further suggest that because all birds exhibit similar amounts of cell death but reduced vVZ proliferation with age during active HVC regression, older birds would likely take more time than younger birds to repopulate the neurons lost during the transition from breeding to nonbreeding conditions.

3.4.7 Song structure degrades as the song control circuit regressed

A relationship between neuronal addition to HVC and the production of new or more stereotyped song in breeding condition birds is well documented [14, 26, 27]. We quantified the time-course and manner in which song degrades during transition into nonbreeding conditions, as this has not been documented previously. Upon regression of the song production pathway, maximal song rate (i.e. the greatest number of songs that birds sang during two consecutive hours) declined significantly during regression with a decrease in rate observed as early as 1D of SD (Table 5). By 14D of SD, five of nine birds had stopped singing entirely and by 28D six of

eight remaining birds were no longer singing. Song completeness and duration decreased over time of regression (Figure 7B and Table 5). Aggregate entropy (i.e. the unpredictability of song structure), variability of maximal frequency, and song similarity (Figure 7C) changed as a function of day in the time-course of regression (Table 5). Significant changes in spectral properties of individual syllables were not detected (data not shown). We found no significant correlations between age or T levels and song quality or quantity in breeding and nonbreeding conditions. Six of the eight birds recorded for song stopped singing between 14D and 28D of SD, thus we could not determine whether cellular attributes of HVC correlated with either song quantity or quality. The overall timing of song degradation, however, reflects the timing of neural regression in HVC (summarized in Figure 8). These data demonstrate that song structure reflects the overall condition of the song control circuits.

3.5 Discussion

The question of how neuronal birth and death are balanced in the adult brain is of considerable interest because the proper balance is necessary for normal neural maintenance and function [28]. The relationship between natural cell death and reactive proliferation, and resulting changes in behavior are not well understood. Here, we demonstrate that during rapid seasonal-like regression of the song production pathway in adult sparrows, apoptosis increases two days following transition from breeding to nonbreeding conditions and that vVZ neural progenitor cell proliferation increases shortly thereafter. Moreover, we find that this change in vVZ proliferation is dependent on HVC cell death; inhibiting caspase-dependent apoptosis in HVC decreases vVZ proliferation. We demonstrate that baseline vVZ proliferation correlates positively with HVC cell apoptosis and that neuronal turnover decreases with age. We show that song, a learned sensorimotor skill, degrades as HVC regresses (Figure 8). Our study shows that reactive neurogenesis is not limited to injury-induced neuronal death, but also appears to occur with non-injury induced degradation of a neural circuit.

3.5.1 Relationship between cell death and neural stem cell proliferation

We found that HVC apoptosis peaked two days following initiation of HVC regression. Neural progenitor cell proliferation in the vVZ peaked four days following HVC regression onset and two days after the peak in HVC cellular apoptosis. This increase in proliferation follows a time course similar to that observed in other species; new neuronal precursors are generated at highest levels two to seven days following lesion of different brain regions in the lizard, *Podarcis hispanica* [7], several rodent species (reviewed in [29]), and zebrafish (*Danio rerio*; [30]).

We found that the increased vVZ proliferation was functionally linked to HVC cell death: infusion of caspase inhibitor cocktail decreased HVC neuronal death and vVZ proliferation. These results are consistent with observations that 1) apoptosis is correlated with new HVC neuronal addition in canary [31], and 2) experimentally-induced neuronal death increased new HVC neuron number in zebra finch [32]. Moreover, Thompson & Brenowitz (2008 and 2009) demonstrated that infusion of the same caspase inhibitor cocktail into HVC decreased both HVC regression and HVC neuronal addition in white-crowned sparrows. Our observations suggests that both the reduced neuronal addition observed by Thompson and Brenowitz (2008, 2009) and the increase in HVC neuronal addition following ablation of RA-projecting neurons in HVC observed by Scharff et. al (2000) may have resulted from a decrease in vVZ neuronal precursor proliferation.

Given the physical proximity of the VZ and HVC, it is possible that caspase inhibitors diffused to and acted directly on the vVZ neural stem cells. This scenario seems unlikely to explain our results, however. Given the anti-apoptotic action of the inhibitors, one might expect them to increase the number of surviving daughter cells, rather than decrease the number of these cells in the vVZ. The alternative proposal that inhibitor infusion decreased stem cell proliferation by decreasing HVC cell death, therefore, seems more parsimonious.

It might seem puzzling that vVZ proliferation increases as total HVC neuron number is decreasing; most of the new neurons added to HVC in nonbreeding birds subsequently die (Larson, Thatra, and Brenowitz, unpublished observation). Why generate large numbers of new neurons only to have most of them die? It may be that the reactive neurogenesis observed in HVC did not evolve specifically to regenerate HVC, but rather reflects a generalized response to selection for brain repair following neurodegeneration. Thus, the linkage between HVC cell death and vVZ proliferation could be mediated by mechanisms that promote injury-induced reactive neurogenesis: neuroinflammation and activation of the innate immune response associated with apoptotic cells. Neuroinflammation and immune cells including microglia, regenerative astrocytes, and leukocytes have emerged as factors regulating injury-induced neurogenesis (reviewed in [33]). After neuronal death, microglia migrate to the injury site to remove cell debris, whereupon they release pro-inflammatory cytokines and pro-proliferative factors (reviewed in [34]; [35]) to promote regeneration [7, 36]. Thus, a neuroinflammatory response may mediate apoptotic neuron clean-up during HVC regression and may promote the vVZ proliferation increase we observed.

Alternatively, both HVC apoptosis and vVZ proliferation may be driven independently by a common factor such as reduced T levels. Direct regulation by T is unlikely, however. We found no significant correlations between T plasma levels and either apoptotic cell number or vVZ proliferation, and there was no significant difference in baseline vVZ proliferation between breeding and nonbreeding seasons. Moreover, treatment of canaries with T, estradiol, or sex steroid antagonists did not alter VZ proliferation [37]. These findings suggest that T and its metabolites do not directly regulate vVZ proliferation.

3.5.2 Persistence of neurons and neuroblasts

About half of newly generated neurons in both early development and the adult brain undergo apoptosis before functionally incorporating into neural circuits (reviewed in [28]). Although factors regulating death of excess neurons in early brain development are well understood to include electrical activity and trophic support [38], few studies have examined the causes and consequences of neuronal pruning in the adult brain. We found that nearly half of the new neurons survive through HVC regression. This observation suggests that whether a neuron survives is not determined by its age alone. Other factors, such as trophic support derived from target cells in RA, may influence the survival of HVC neurons [39].

We observed a small increase in HVC AC-3 positive cell number at 14D of SD. This increase may reflect the well-documented 50% die off of migrating neuroblasts [40]. A large number of new cells were born by 4D of SD and many presumably died over the next two weeks during migration or incorporation. New HVC neurons that do not establish functional connections with RA likely die due to lack of trophic support derived from their target neurons. Thus, lower trophic support (eg. from T and BDNF) during nonbreeding conditions not only initiates regression of HVC [41], but also may account for the second peak in cell death observed at 14D of SD.

3.5.3 Reduction of neuronal turnover and regenerative rate with age

Adult neurogenesis is ongoing and assumed to continue throughout the entire lifespan of an animal. Several studies, however, have demonstrated that neural stem cell proliferation decreases with age ([42], [43], [7], [44]). We too found that vVZ proliferation decreases with age in adult white-crowned sparrows. Unique to our study, we found that under baseline conditions apoptosis in HVC is also lower in older sparrows, suggesting that neuronal turnover declines with age. We observed, however, that regardless of age, birds have similar breeding and nonbreeding HVC volumes and neuronal number. Reduced neuronal turnover but similar

amounts of HVC cell death during regression in older birds suggest that older birds may take more time than younger birds to repopulate the neurons that die during HVC regression.

3.5.4 Effect of circuit regression on song behavior

As the seasonally shifting balance between neuronal birth and death favors neuronal birth and incorporation of new HVC neurons in breeding conditions, song quality improves across several songbird species. In Gambel's white-crowned sparrows, transition into breeding condition drives both an increase in HVC neuronal number and an increase in song duration, completeness, and stereotypy [10, 14]. Moreover, the number of new neurons incorporated into HVC is positively correlated with quantity of song in canaries [45, 46] and accuracy of song recovery following vocal muscle paralysis in zebra finches [26]. Conversely, song degrades as HVC regresses - as neuronal death exceeds neuronal addition. The manner in which each individual bird's song degraded varied: some birds' songs changed only in syntactical structure, whereas other birds' songs changed in stereotypy or spectral properties, or some combination of the two. Regardless of degradation manner and degree, the majority of birds stopped singing entirely by 28D of SD. Overall, song degradation matched the timing of HVC regression, corroborating song quality as a measurable output of the dynamic cellular composition and function of the song production pathway.

3.5.5 Conclusion

We have shown that a pallial nucleus in adult songbirds is capable of reactive neurogenesis in a context where neurodegeneration occurs naturally rather than as the result of an insult. This observation suggests that the ability to replace lost neurons may be a fundamental feature of the adult brain. The pronounced regenerative capacity of the white-crowned sparrow brain and the discrete singing behavior that represents the functional output of the song production circuit provides an excellent system in which to examine the mechanisms driving natural neuronal replacement and how neuronal loss and replacement contributes to the maintenance of normal behavior.

3.6 References

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Table 1. Systemic plasma testosterone levels

	LD+T	SD						ANOVA
	28 D	**1 D**	**2 D**	**4 D**	**7 D**	**14 D**	**28 D**	
	(n=52)	*(n=8)*	*(n=7)*	*(n=9)*	*(n=10)*	*(n=10)*	*(n=8)*	
Testosterone (ng/mL)	16.38 ± 5.25[a]	2.05 ± 0.41[b]	1.31 ± 0.38[b]	1.36 ± 0.25[b]	1.49 ± 0.26[b]	1.04 ± 0.18[b]	0.78 ± 0.14[b]	**$F_{(6,97)}$=24.4997 p <0.0001**

All values are mean ± S.E.M. Superscript letters denote significant differences across treatment groups.

Table 2. Morphometrics of HVC and RA

	LD+T	SD						ANOVA
	28 D	**1 D**	**2 D**	**4 D**	**7 D**	**14 D**	**28 D**	
HVC	*(n=8)*	*(n=6)*	*(n=8)*	*(n=8)*	*(n=9)*	*(n=10)*	*(n=8)*	
Unilateral Volume (mm^3)	0.60 ± 0.03[a]	0.49 ± 0.05[a,b]	0.52 ± 0.05[a,b]	0.46 ± 0.03[a,b]	0.45 ± 0.04[a,b]	0.40 ± 0.04[b]	0.45 ± 0.02[a,b]	**$F_{(6,51)}$=3.0252 p=0.0133**
	(n=6)	*(n=5)*	*(n=4)*	*(n=5)*	*(n=6)*	*(n=4)*	*(n=5)*	
Neuron Density ($x10^3/mm^3$)	93 ± 7[a]	67 ± 2[b]	65 ± 3[b]	69 ± 6[b]	57 ± 4[b]	61 ± 5[b]	66 ± 3[b]	**$F_{(6,29)}$=5.8862 p=0.0013**
Neuron Number ($x10^3$)	50 ± 4[a]	33 ± 7[a,b]	29 ± 8[a,b]	31 ± 2[a,b]	25 ± 3[b]	20 ± 3[b]	28 ± 3[a,b]	**$F_{(6,29)}$=3.6745 p=0.0136**
RA	*(n=8)*	*(n=6)*	*(n=8)*	*(n=8)*	*(n=9)*	*(n=10)*	*(n=8)*	
Unilateral Volume (mm^3)	0.25 ± 0.02	0.20 ± 0.04	0.27 ± 0.02	0.20 ± 0.02	0.25 ± 0.04	0.18 ± 0.02	0.17 ± 0.02	**$F_{(6,51)}$=2.9037 p=0.0158**

All values are mean ± S.E.M. Superscript letters denote significant differences across treatment groups.

Table 3. Cellular changes in HVC and the vVZ

	LD+T	SD					ANOVA
	28 D	**1 D**	**2 D**	**4 D**	**7 D**	**14 D**	**28 D**	
HVC	*(n=6)*	*(n=7)*	*(n=8)*	*(n=7)*	*(n=7)*	*(n=5)*	*(n=6)*	
AC-3 Cell Number	836 ± 142[a]	1351 ± 309[a,b]	1681 ± 222[b]	1062 ± 68[a,b]	865 ± 91[a]	1313 ± 131[a,b]	1081 ± 151[a,b]	**$F_{(6,39)}$=2.8600; p=0.0207**
	(n=9)	*(n=5)*	*(n=7)*	*(n=8)*	*(n=7)*	*(n=6)*	*(n=7)*	
BrdU Positive Neuron Number	162 ± 30	103 ± 31	113 ± 29	120 ± 20	103 ± 17	61 ± 15	76 ± 11	$F_{(6,42)}$=2.0819; p=0.0758
vVZ	*(n=5)*	*(n=5)*	*(n=6)*	*(n=9)*	*(n=5)*	*(n=6)*	*(n=6)*	
BrdU Positive Cell Number	631 ± 51[a]	1116 ± 73[a,b]	1680 ± 225[a,b]	2246 ± 303[b]	1018 ± 235[a,b]	893 ± 185[a,b]	1187 ± 149[a,b]	**$F_{(6,35)}$=6.6854; p<0.0001**
Coefficient of determination	*(n=6)*	*(n=5)*	*(n=6)*	*(n=8)*	*(n=5)*	*(n=5)*	*(n=4)*	
vVZ Proliferation to HVC AC-3 Number	**R^2=0.8696; p=0.0208**	R^2=0.4469; p=0.1466	R^2=0.0028; p=0.9212	R^2=0.4009; p=0.5544	R^2=0.0352; p=0.8124	R^2=0.8641; p=0.2404	**R^2=0.9067; p=0.0479**	

All values are mean ± S.E.M. Superscript letters denote significant differences across treatment groups.

Table 4. Physiological and morphological changes in birds infused with vehicle or caspase inhibitor cocktail

	Vehicle	Caspase Inhibitors	*t-test*
	SD 3D	**SD 3D**	
	(*n=5*)	(*n=6*)	
Testosterone			
Plasma levels (ng/mL)	0.39 ± 0.17	0.83 ± 0.37	$t_{(4.27)}$=1.0798 p=0.3374
HVC			
Cannula Distance (mm)	0.17 ± 0.07	0.27 ± 0.04	$t_{(6.91)}$=1.8701 p=0.1042
Unilateral Volume (mm^3)	0.69 ± 0.02	0.60 ± 0.04	$t_{(7.79)}$=1.5342 p=0.1645
Neuron Density ($x10^3/mm^3$)	97 ± 7	144 ± 8	**$t_{(8.94)}$=4.3868 p=0.0018**
Neuron Number ($x10^3$)	70 ±7	90 ± 5	**$t_{(7.72)}$=2.4514 p=0.0409**
AC-3 Cell Number	5370 ± 681	1844 ± 290	**$t_{(6.76)}$=4.7617 p=0.0022**
RA			
Unilateral Volume (mm^3)	0.33 ± 0.03	0.28 ± 0.04	$t_{(7.08)}$=0.8746 p=0.4105
vVZ			
BrdU Positive Cell Number	2308 ± 223	934 ± 160	**$t_{(7.56)}$=5.0000 p=0.0012**

All values are mean ± S.E.M.

Table 5. Quantification of song degradation

	LD+T	SD						ANOVA
	21 D	**1 D**	**2 D**	**4 D**	**7 D**	**14 D**	**28 D**	
Song Rate	*(n=18 birds)*	*(n=10)*	*(n=8)*	*(n=8)*	*(n=8)*	*(n=9)*	*(n=8)*	
Max # Songs/ Hour	152 ± 33[a]	73 ± 29[a,b]	93 ± 46[a,b]	104 ± 46[a,b]	32 ± 9[b]	33 ± 21[b]	17 ± 12[b]	**$F_{(6,62)}$=2.5623 p=0.0279**
Spectral Feature	*(n=11 birds*)*	*(n=8)*	*(n=4)*	*(n=6)*	*(n=7)*	*(n=4)*	*(n=2)*	
Duration (sec)	1.68 ± 0.07[a]	1.65 ± 0.09[a]	1.42 ± 0.14[a,b]	1.16 ± 0.19[b]	1.16 ± 0.09[b]	1.33 ± 0.18[a,b]	1.09 ± 0.31[b]	**$F_{(6,38)}$=3.9942 p=0.0042**
Aggregate Entropy	3.51 ± 0.11[a]	3.48 ± 0.14[a,b]	3.27 ± 0.12[a,b,c]	3.07 ± 0.14[b,c]	3.14 ± 0.16[b,c]	3.37 ± 0.16[a,b]	2.63 ± 0.02[c]	**$F_{(6,38)}$=2.5117 p=0.0381**
Average Power (dB)	64.60 ± 1.89[a,b]	66.01 ± 3.38[a,b]	61.06 6.83[b]	64.64 ± 6.05[a,b]	71.35 ± 4.84[a,b]	75.72 ± 3.43[a]	56.70 ± 6.59[b]	$F_{(6,38)}$=1.4236 p=0.2311
Coefficient of Variation								
High Frequency	0.05 ± 0.01[a]	0.05 ± 0.02[a]	0.09 ± 0.05[a]	0.08 ± 0.02[a]	0.08 ± 0.02[a]	0.05 ± 0.02[a]	0.19 ± 0.05[b]	**$F_{(6,38)}$=2.4167 p=0.0446**
Low Frequency	0.12 ± 0.02	0.14 ± 0.05	0.23 ± 0.05	0.12 ± 0.03	0.11 ± 0.03	0.08 ± 0.04	0.08 ± 0.04	$F_{(6,38)}$=0.2938 p=0.9361
Cross Correlation								
To LD+T 21D	21D: 0.48 ± 0.02[a] 23D: 0.43 ± 0.02[a,b]	0.36 ± 0.04[b,c]	0.37 ± 0.02[b,c]	0.37 ± 0.04[b,c]	0.34 ± 0.04[c]	0.32 ± 0.03[c]	N/A	**$F_{(6,62)}$=4.0390 p=0.0018**
Percent Complete								
Relative to LD+T	-	0.943 ± 0.04[a]	0.89 ± 0.06[a,b]	0.64 ± 0.07[c]	0.71 ± 0.04[c]	0.87 ± 0.09[a,b]	0.73 ± 0.02[b,c]	**$F_{(6,35)}$=9.8902 p<0.001**

*For spectral features, CV, cross correlation, and % complete, only birds that sang at least 30 songs for the given day were analyzed.

All values are mean ± S.E.M. Superscript letters denote significant differences across treatment groups. Lack of letters indicates no differences.

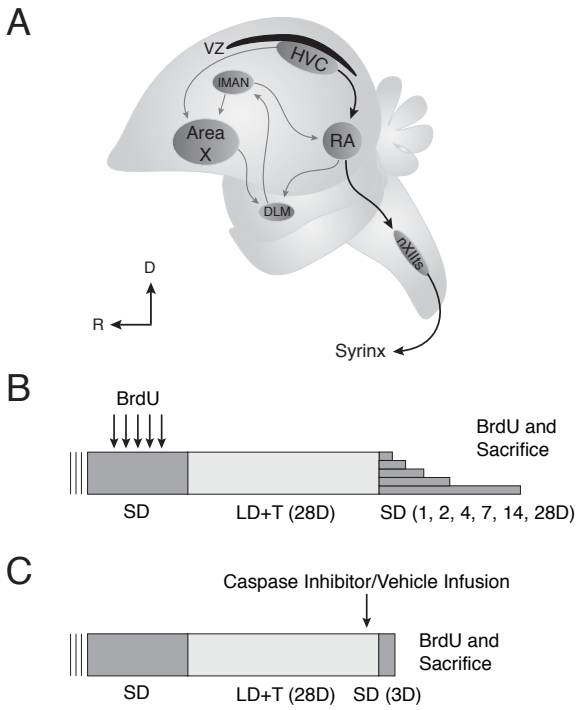

Figure 1. *(A)* Schematic illustrating the major projections within the song control circuits. Black arrows indicate the song production pathway, grey arrows show the anterior forebrain pathway. Note the ventricular zone (VZ) directly dorsal to HVC. RA=robust nucleus of the arcopallium, nXII=tracheosyringeal portion of hypoglossal nucleus, DLM=dorso-lateral division of the medial thalamus, lMAN=lateral subdivision of the magnocellular nucleus of the anterior nidopallium, D=dorsal, R=rostral. *(B)* Experimental design for natural rapid regression study. *(C)* Experimental design for caspase inhibitor cocktail infusion study.

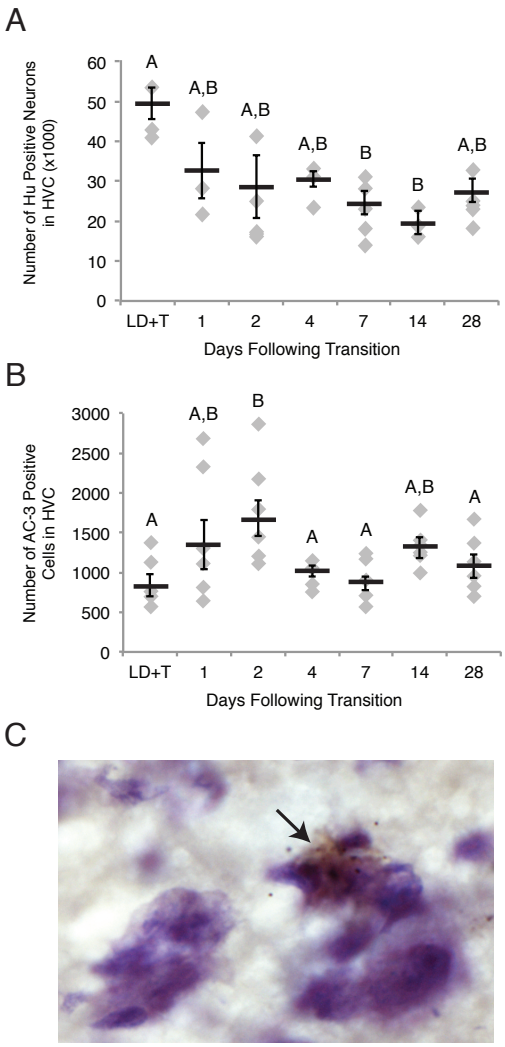

Figure 2. Apoptosis in HVC occurs rapidly after transition from breeding to nonbreeding conditions. *(A)* Number of Hu positive neurons in one hemisphere of HVC across the time course of regression. HVC neuron number drops by 7D following transition to nonbreeding conditions. *(B)* Number of apoptotic cells in HVC on one side of the brain over the time course of regression. Number of activated caspase-3 (AC-3) positive cells peaks at 2 days following transition from breeding to nonbreeding conditions. Letters above bars indicate significant differences among groups (One-way ANOVA, Tukey post-hoc pairwise comparisons). All data plotted as Mean ± S.E.M. *(C)* Representative image of AC-3 immunohistochemistry in fresh frozen tissue counter-stained for Nissl (cytoplasm stains light purple, nuceli stain dark purple) illustrating apoptotic cells in HVC. The arrow indicates an AC-3 positive cell (brown). Note the condensed chromatin (a distinctive feature of apoptotic cells; shown in dark purple) contained within the AC3 positive cell.

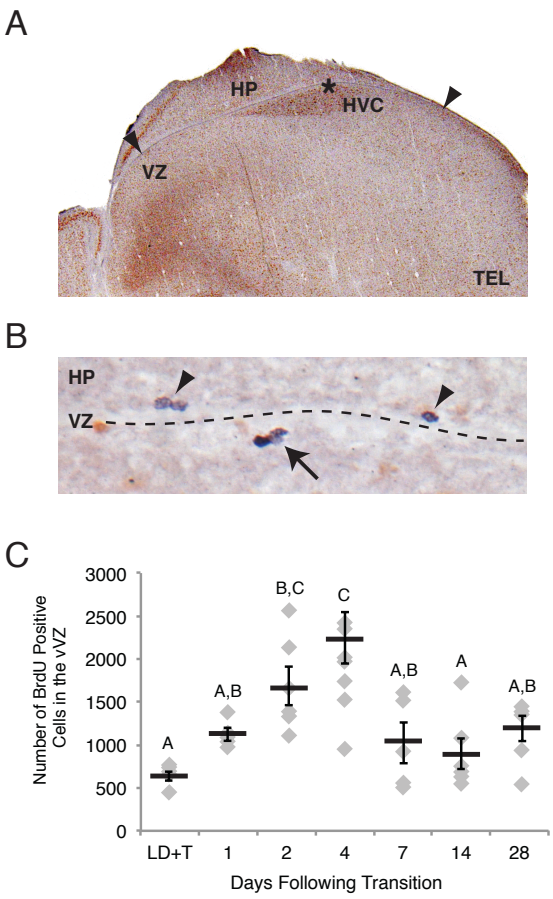

Figure 3. Proliferation in the ventral ventricular zone (vVZ) changes rapidly during regression of the song control circuits. *(A)* An image of Hu staining in the brain illustrating the method for counting BrdU positive cells in the VZ. Arrow heads demonstrate the medial and lateral-most extents within which BrdU positive cells were counted on the ventral side of the VZ. The star indicates the location of the image shown in *(B).* HP=hippocampus, TEL=telencephalon. *(B)* A representative image of BrdU immunohistochemistry showing proliferative cells and their progeny in the VZ. BrdU labeling in the nucleus is stained dark purple whereas Hu labeling in the cytoplasm is stained brown. The arrow indicates BrdU positive cells (i.e. neural progenitor cells or daughter cells) labeled at the time of BrdU injections. The arrow heads point to BrdU positive cells on the dorsal side of the VZ (ventral to HP) that were not included in counts of BrdU positive cells in the vVZ due to their inability to migrate across the ventricle to HVC. *(C)* Number of BrdU positive cells in the vVZ of one hemisphere over the experimental time course. Proliferation in the vVZ peaked at 4 days following transition from breeding to nonbreeding conditions. Letters above bars indicate significant differences among groups. All data plotted as Mean ± S.E.M.

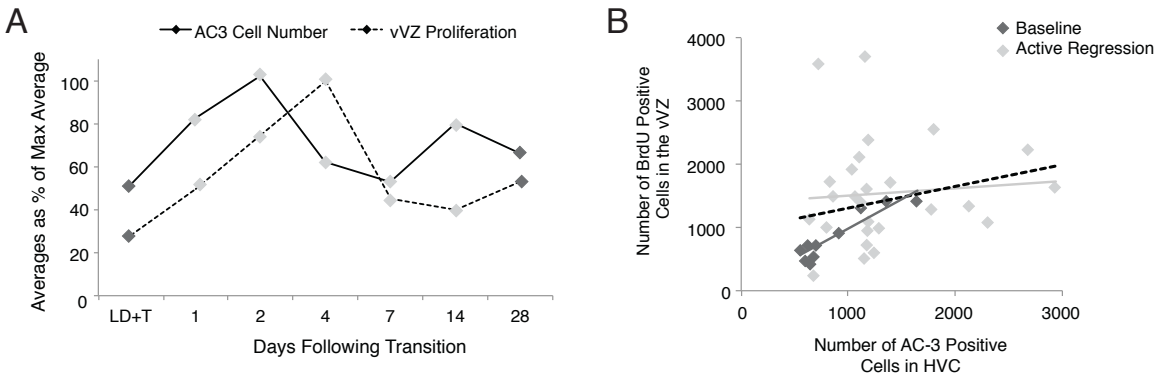

Figure 4. The relationship between proliferation in the vVZ and the number of apoptotic cells in HVC during rapid regression of the song control system. *(A)* The lag in time of peak vVZ proliferation compared to HVC cell death. The levels of cell death in HVC and proliferation in the vVZ are plotted relative to the maximum value of each measure to facilitate comparison of the time course of both measures. Proliferation in the vVZ peaks at 4D following transition from breeding to nonbreeding conditions and 2 - 3D following the peak of HVC cell death. *(B)* The correlation between proliferation in the vVZ and the density of cell death in HVC during rapid regression of the song control system. All data pooled is indicated by the dashed line (R^2=0.0464, p=0.2140, n=30 birds), pooled baseline condition data (i.e. 28D of LD+T and SD; n=9 birds) by the dark grey diamonds (individual data points) and dark grey solid line, and pooled active regression data (i.e. 1D, 2D, 4D, 7D, and 14D of SD; n=21 birds) by the light grey diamonds and solid line. See Table 3 for correlations between vVZ proliferation and AC-3 cell number within individual time points.

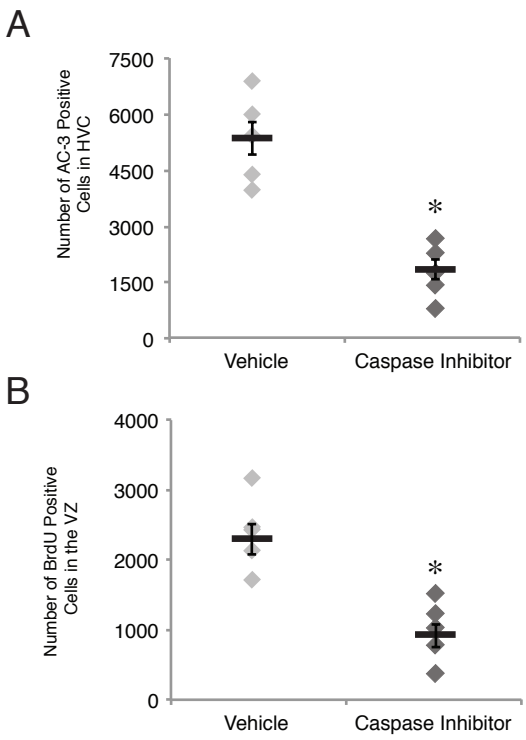

Figure 5. Cell death in HVC is functionally linked to stem cell proliferation in the vVZ. *(A)* Number of apoptotic cells in HVC of birds infused with vehicle or caspase inhibitor cocktail infusion near HVC for three days into the seasonally-induced regression of HVC. The number of AC3 positive cells was significantly lower in birds infused with the caspase inhibitor cocktail, confirming that the infusion prevented HVC cell death. *(B)* Number of BrdU positive cells in the vVZ of birds infused with vehicle (n=5) or caspase inhibitor cocktail (n=6) near HVC after 3D of SD. Proliferation in the vVZ was significantly reduced in birds receiving the caspase inhibitor cocktail compared to those receiving vehicle following transition from breeding to nonbreeding conditions. Asterisk above bars indicate significant differences among groups. All data plotted as Mean ± S.E.M.

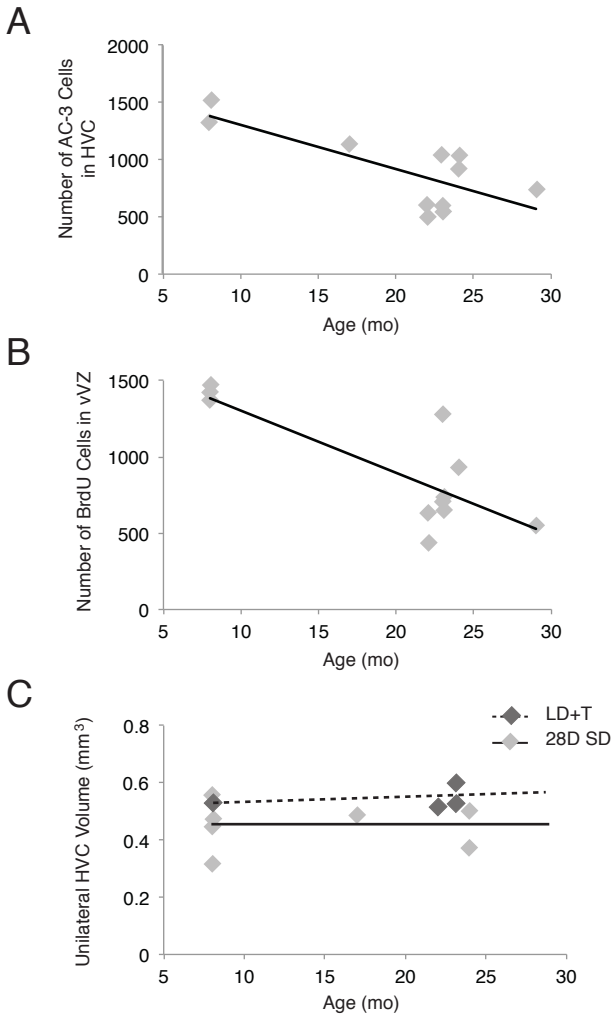

Figure 6. The effect of age on neuronal turnover in male white-crowned sparrows following HVC regression. *(A)* HVC cell death negatively correlates with sparrow age at stable baseline breeding and nonbreeding conditions (i.e. LD+T and SD 28D). Solid line is the regression line from pooled LD+T and SD 28D birds, while grey dots represent the value for each bird (n=11). *(B)* Proliferation in the vVZ negatively correlates with sparrow age in stable baseline conditions (n=11). *(C)* HVC volume does not change with age. Maximal HVC growth (i.e. LD+T volume; n=4) correlation with age is represented by the dotted line and complete HVC regression (i.e. 28D of SD volume; n=7) correlation with age is indicated by the solid line. For all scatter plots individual birds' values and age are represented by one dot: light grey for LD+T and dark grey for 28D of SD.

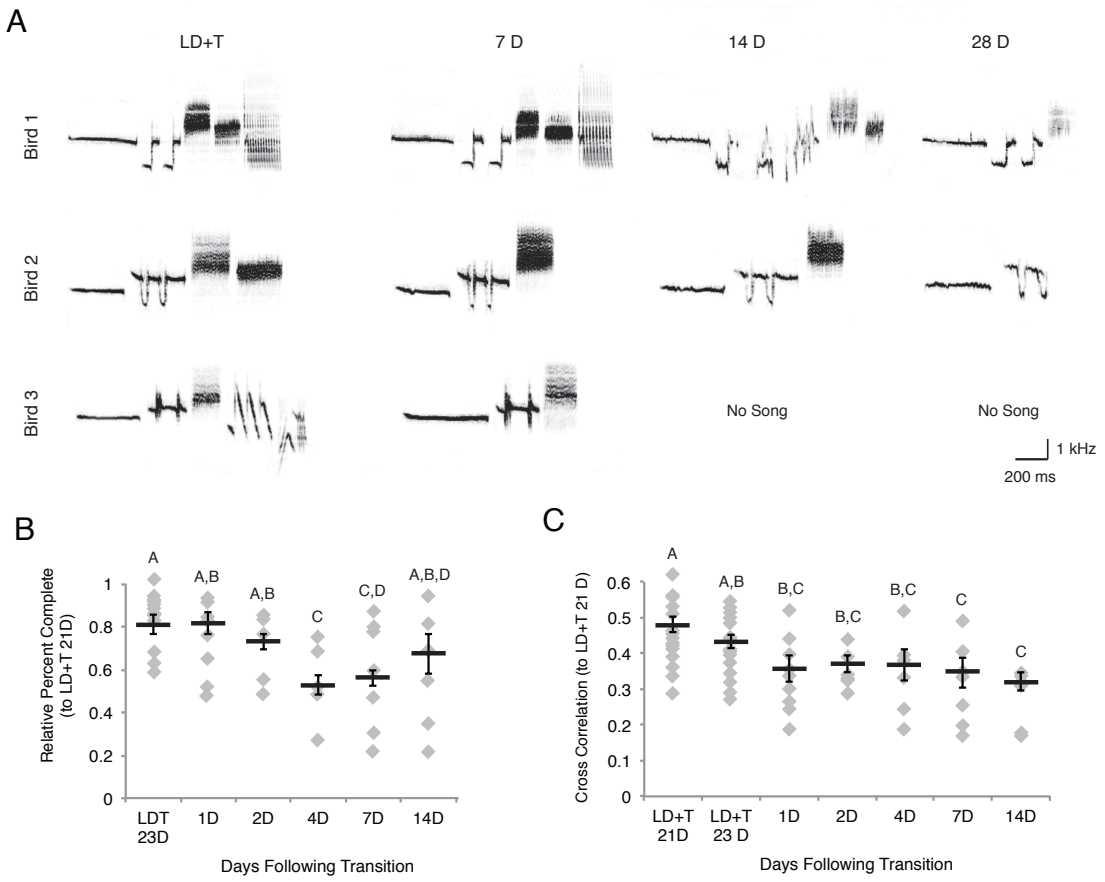

Figure 7. Song degrades as Gambel's white-crowned sparrows are transferred from breeding to nonbreeding conditions. *(A)* Spectrograms of songs produced by three different individuals prior to and after the birds were transferred to nonbreeding conditions. The manner in which song degraded varied between birds. "Bird 1" song illustrates degradation of the spectral properties within individual syllables. "Bird 2" and "Bird 3" continued to sing individual syllables similar in spectral properties to those sung under breeding condition, but sang fewer syllables over time. "Bird 3" stopped singing prior to 14D of SD, as did 40% of the birds remaining in the experiment through at least 14 D of SD. *(B)* Song completeness across the time-course of regression plotted as percent of songs that contained each of the syllable types produced under breeding conditions. Song completeness decreased significantly by 4D into nonbreeding conditions. *(C)* Cross correlation, a measure of similarity, of song from 21D and 23D of LD+T and days 1-14 of SD compared to 21D of LD+T song. Song similarity decreased significantly by 7D of SD relative to 23D of LD+T. Letters above bars indicate significant differences among groups. All data plotted as Mean ± S.E.M.

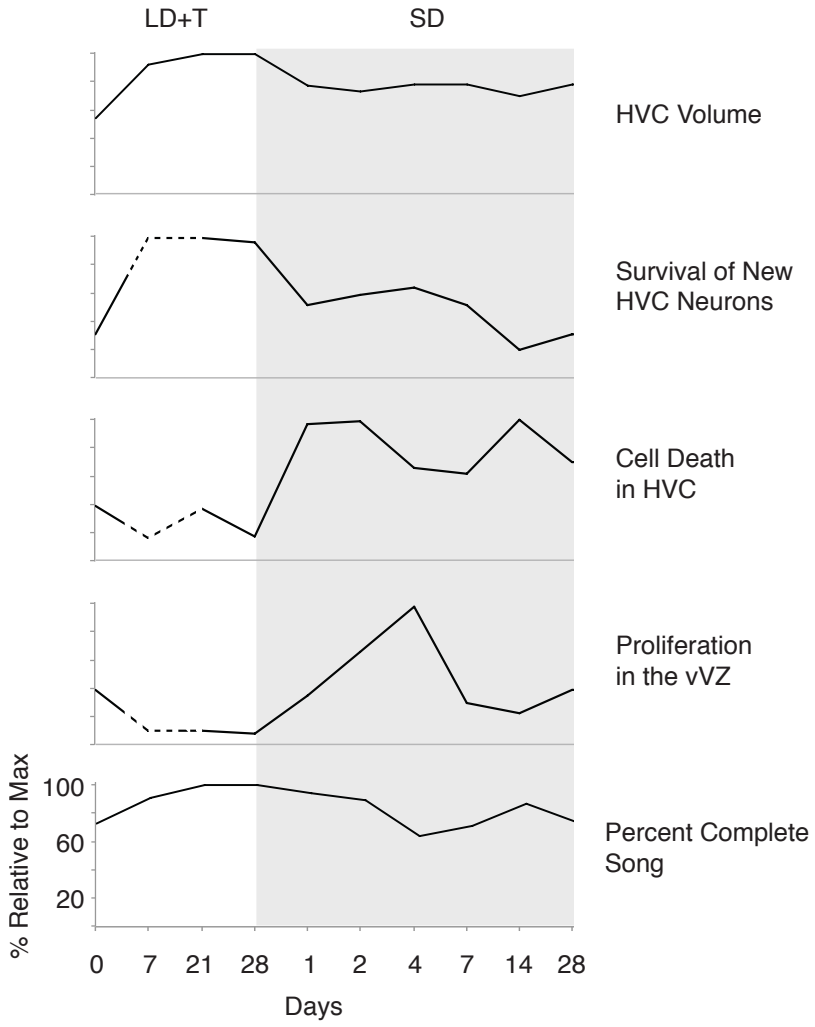

Figure 8. Summary of the morphological and behavioral changes in Gambel's white-crowned sparrows occurring throughout breeding and nonbreeding conditions and transitions between the two. Data from previous publications [64, 108-110] were pooled with our data to construct the time-course of growth and regression of HVC and the associated song behavior. Each property is presented as a percent relative to the maximum value regardless of seasonal condition for that property. Solid lines represent existing data, dashed lines represent interpolated values. Grey background indicates nonbreeding condition, and white background indicates breeding condition.

Chapter 4. Post-Synaptic Neural Activity Regulates Neuronal Addition in the Adult Avian Song Control System

4.1 Abstract

A striking feature of the nervous system is that it shows extensive plasticity of structure and function that allows animals to adjust to changes in their environment. Neural activity plays a key role in mediating experience-dependent neural plasticity, and thus, creates a link between the external environment, the nervous system, and behavior. One dramatic example of neural plasticity is ongoing neurogenesis in the adult brain. The role of neural activity in modulating neuronal addition, however, has not been well studied at the level of neural circuits. The avian song control system allows us to investigate how activity influences neuronal addition to a neural circuit that regulates song, a learned sensorimotor social behavior. In adult white-crowned sparrows new neurons are added continually to the song nucleus HVC (proper name), and project their axons to its target nucleus, the robust nucleus of the arcopallium (RA). We report here that electrical activity in RA regulates neuronal addition to HVC. Decreasing neural activity in RA by intracerebral infusion of the $GABA_A$ receptor agonist muscimol decreased the number of new HVC neurons by 56%. Our results suggest that post-synaptic electrical activity influences the addition of new neurons into a functional neural circuit in adult birds.

4.2 Significance Statement

Neural activity in the adult brain plays a key role in mediating experience-dependent neural plasticity. We show that inhibiting electrical activity in the song nucleus RA in adult bird brain decreases the number new projection neurons added to the afferent nucleus HVC. Our results are consistent with the general principle of activity-based target selection of newborn neurons during nervous system development, and support the idea that developmental and adult plasticity exploit similar mechanisms. Understanding the mechanisms influencing the incorporation of new neurons into established neural circuits in adult brains is critical both for our basic understanding of neural plasticity and for exploiting the clinical potential of neuronal replacement to repair damage associated with injury and neurodegenerative diseases.

4.3 Introduction

The ongoing birth and incorporation of neurons into functional neural circuits in the central nervous system of higher vertebrates was first demonstrated conclusively in songbirds and subsequently in rodents, nonhuman primates, and humans (reviewed in [1]). Because new

neuron generation continues throughout adulthood, the fundamental importance and the clinical implications of neurogenesis are clear. The mechanisms by which new neurons integrate into functional neural circuits, however, are not well understood.

Songbirds provide a tractable model for understanding the mechanisms that regulate new neuron addition into functional circuits. Song is a learned sensorimotor behavior that is important for songbird reproduction. Song learning and production are regulated by a discrete, well-characterized neural circuit that includes HVC (proper name) and its target nucleus, the robust nucleus of the arcopallium (RA), both located in the avian forebrain (Fig. 1A) [2]. In the adult Gambel's white-crowned sparrow (WCS) the song control system shows extreme seasonal neuroplasticity (reviewed in [3]). Early in the breeding season HVC and RA of WCS nearly double in volume. The increase in HVC volume results largely from an increase in new neuron incorporation, while the increase in RA volume results from increases in neuron size and spacing, but not number. RA neurons also show increased spontaneous electrical activity in the breeding season [4, 5]. WCS typically produce only one song-type that is longer and more stereotyped in structure during the breeding season [6, 7].

HVC contains three types of neurons: HVC→RA and HVC→Area X projection neurons, and interneurons. During seasonal growth most, if not all, neurons incorporated into HVC project to RA ([8], but see [9]). Neural progenitor cells are born at the dorsal and ventral portion of the lateral ventricle and migrate from the ventricular zone (VZ) to HVC within a week after birth [10]. Over the next two to three weeks, new neurons send axonal projections to RA [11]. These new HVC→RA projection neurons are functional; they can fire action potentials in response to sound stimuli [12], and express the immediate-early gene *EGR-1* during bouts of song production [13].

Environment and experience play important roles in both brain development and adult neurogenesis. For example, during embryonic development, neural activity in the visual cortex is required for target selection by axons from the lateral geniculate nucleus [14]. In adult rodents, voluntary exercise and hippocampal-dependent learning enhance neurogenesis in the dentate gyrus (reviewed in [1]). In adult songbirds, auditory experience and song production influence neuronal turnover in HVC [15-17]. This literature suggests that adult neurogenesis in the vertebrate brain is activity-dependent. *In vitro* studies show that excitatory stimuli act directly on hippocampal neural progenitor cells and promote survival of new neurons (reviewed in [18]).

Thus, activity–dependent mechanisms likely influence neuronal recruitment *in vivo* in both developing and adult brains.

One factor that may influence the recruitment to and survival of new neurons in HVC is the electrical activity of their post-synaptic targets in RA [5]. We hypothesized that inhibiting the electrical activity in RA neurons *in vivo* would reduce neuronal addition to adult HVC. We show that decreasing RA electrical activity does indeed reduce neuronal addition to HVC, indicating that target activity is essential for appropriate neuronal addition to HVC.

4.4 Results

We tested our hypothesis by inhibiting neural activity within RA in freely behaving adult Gambel's WCS and assaying the addition of new neurons to HVC.

RA contains two populations of neurons: 1) neurons that project to motor neurons in nXIIts in the hindbrain and are spontaneously active *in vivo,* and 2) inhibitory GABAergic interneurons, which are not spontaneously active *in vitro* [19, 20]. To inhibit electrical activity in RA, we infused the $GABA_A$ receptor agonist, muscimol, near RA [21, 22]. To label the greatest number of newborn cells, WCS were injected with the thymidine analog BrdU while in non-breeding conditions (i.e., short days, SD, when neuronal addition to HVC is highest) [23, 24]. Two days after the last BrdU injection, WCS were shifted from SD to breeding conditions (i.e., long days with testosterone (T) pellets, LD+T), which normally increases survival of new neurons in HVC and increases spontaneous activity in RA [5, 24]. Simultaneously, birds were implanted unilaterally with a cannula near RA, alternating between the left and right hemispheres in different birds (Fig. 1A, B). The cannula was immediately connected to a micro-osmotic pump containing muscimol, which pumped continuously for the duration of the experiment. In control birds RA was infused with saline. Vehicle and muscimol cannulae were placed 1250 ±100 μm from RA (Supporting Information (SI) Fig. 1). WCS were housed on LD +T for two to four weeks after infusion onset (Fig. 1B).

4.4.1 Muscimol effectively diffused to and inhibited neural activity in RA but not HVC

To confirm the diffusion of muscimol to RA neurons but not HVC neurons, we visualized spread of fluorophore-conjugated muscimol (FCM; BODIPY muscimol-TMR-X, Sigma-Aldrich) and recorded *in vivo* spontaneous firing rates in RA and HVC following muscimol infusion for 2 weeks. FCM diffused to RA, but not to nearby HVC or VZ (n=4 birds; Fig. 2A & B). Muscimol completely inhibited spontaneous activity of RA neurons (n=2 birds; Fig. 2D and SI Table 1) [25-27], but did not alter spontaneous activity of HVC neurons (ipsilateral v. contralateral HVC

firing rate; p>0.5; n=28 cells in 2 birds; Fig. 2C, SI Table 1) . Together these results demonstrate that muscimol infusion effectively inhibited RA neural activity, but did not diffuse to HVC or inhibit its spontaneous neural activity.

4.4.2 Reducing the activity of RA neurons decreased the addition of new HVC neurons

The number of newborn neurons (i.e. cells co-labeled with antibodies against BrdU and the neuron-specific antigen Hu) and single labeled BrdU cells (presumably glial or ependymal cells) was counted throughout the full extent of HVC. The number of BrdU positive neurons was lower in HVC ipsilateral to muscimol infused in RA (74±19 [mean±SEM]) when compared to HVC contralateral to muscimol infusion (167±19, n=8; p=0.01), HVC ipsilateral to vehicle infusion (170±25, n=5; p<0.05), and HVC contralateral to vehicle infusion (164±24, n=5; p<0.05; Fig. 1C). We found no significant difference in new neuron number between HVC contralateral to muscimol infusion and HVC either ipsilateral or contralateral to saline infusion (both p>0.1). We found no effect of hemisphere in birds infused with muscimol on the number of new neurons added to ipsilateral and contralateral HVC (right hemisphere infusion, 112±21; left, 135±26; p=0.5). The number of non-neuronal BrdU positive cells was not significantly different between HVC ipsilateral to muscimol infusion (105±22) and HVC contralateral to muscimol infusion (88±22, n=8; p>0.5), HVC ipsilateral to vehicle infusion (120±24, n=5; p>0.5) or HVC contralateral to vehicle infusion (137±23, n=5; p>0.5). These results demonstrate that inhibiting RA activity unilaterally and selectively reduced the addition of new neurons to the ipsilateral HVC.

Although HVC neuronal addition was reduced with muscimol infusion in RA, neither total HVC neuron number nor HVC volume changed significantly (SI Table 2). Furthermore, muscimol infusion did not significantly alter RA volume, neuron density or neuron soma size (SI Table 2). These results suggest that RA neural activity is required for new HVC neuron addition, but may not be necessary for mature HVC neuronal survival or for neuronal growth in RA.

4.4.3 Unilateral disruption of the song production circuit transiently altered song behavior

We recorded and analyzed song production prior to and during muscimol or vehicle infusion. Birds did not differ in the number of songs produced per hour (i.e. rate) or the number of syllables, but did differ in some spectral properties with muscimol infusion (SI Table 3). Muscimol infused near RA transiently increased the variability and entropy of specific temporal and spectral properties of song during the first 14 days of treatment. Song recovered, however, by 21 days (Fig. 3 and SI Table 3). Vehicle infusion did not alter song, which demonstrates that cannula placement alone did not cause the transient changes in song attributes found in

muscimol-infused birds (SI Table 4). We found no differences in any song attributes of birds infused with muscimol in the right versus left hemisphere and no correlation between the degree of song degradation and the number of new or total number of neurons either ipsilateral or contralateral to muscimol infusion. The mechanism underlying song recovery during muscimol infusion and its effect, if any, on neuronal addition remains to be identified.

4.5 Discussion

We found that inhibiting electrical activity in RA by infusing muscimol significantly reduced neuronal addition to the ipsilateral but not contralateral HVC. These results support the hypothesis that post-synaptic neuronal electrical activity modulates afferent neuronal addition. Our findings are consistent with the general theory that neural activity shapes and refines neural circuits during nervous system development ([14] but see [28]), and support the idea that developmental and adult plasticity exploit similar mechanisms. For example, in rodents, odor deprivation caused by naris closure reduces neurogenesis in the olfactory bulb and survival of newborn olfactory interneurons [29], while odor enrichment promotes the survival of newborn neurons and enhances odor memory [30]. Similarly, NMDA receptor expression by new neurons in adult mouse dentate gyrus is required for neuronal incorporation into an existing circuit [31]. In addition, dysfunctional adult hippocampal neurogenesis has been proposed as a possible biological mechanism of depression in humans. Decreased neural activity in the hippocampus in response to positive social stimuli was found in depressed patients. Electroconvulsive therapy (ECT), used to treat severe depression, indiscriminately increases neural activity and enhances hippocampal neurogenesis (reviewed in [32]). These studies indicate that neural activity plays an important role in regulating adult neurogenesis. Few studies however have tested the role of neural activity in a target nucleus on neuron addition in an afferent nucleus within functional neural circuits. Our study is unique in that it shows *in vivo* the effects of neural activity in one nucleus on neuron addition in another nucleus of an adult neural circuit that regulates a learned sensorimotor behavior.

Muscimol might influence neuron addition by inhibiting activity of new HVC neurons via direct or indirect projections from RA to HVC (Fig. 1A). If such inhibition were mediated by the weak unilateral direct projection from RA to HVC [25], then we might expect to find lower spontaneous firing of HVC neurons ipsilateral to the muscimol infusion, given the positive relationship between activity and survival of new neurons observed in other models. We did not, however, observe a decrease in spontaneous firing rate in ipsilateral HVC in the muscimol-

treated birds. The indirect connection between RA and HVC via the thalamic nucleus DMP and the cortical nucleus mMAN is bilateral [27, 33]. We therefore might expect ipsilateral muscimol infusion in RA to decrease electrical activity and neuronal addition in both sides of HVC. We did not observe differences in the number of new neurons or total number of neurons between contralateral HVC in muscimol-infused birds and either side of HVC in vehicle-infused birds. These observations suggest that there was neither a direct or indirect effect of muscimol infused in RA on HVC neural activity, though they do not absolutely rule out this possibility.

Another mechanism by which reduced activity in RA could affect HVC neuronal addition is through muscimol-induced alteration of song production. This behavioral impairment could indirectly affect neuronal addition in HVC through altered sensory feedback (eg. proprioceptive or auditory). Proprioceptive respiratory and syringeal feedback is conveyed through the rostral ventrolateral medulla, which indirectly projects to HVC via direct bilateral projections to thalamic nucleus uvaeformis (Fig. 1A) [34]. Wilbrecht et al. (2002) reported that in zebra finches, unilaterally denervating the vocal production organ, the syrinx, impaired song production and increased neuronal addition to the contralateral HVC. Deafening birds prevented this compensatory increase in neuronal recruitment. Their results suggest that motor activity and/or auditory feedback from song can influence neuronal recruitment. One would expect that altered auditory and/or proprioceptive feedback during song impairment would disrupt neuronal addition in both sides of HVC. We found, however, that muscimol infusion only impaired neuronal addition to the ipsilateral HVC. Our results suggest that decreased neuronal addition to HVC in WCS in this experimental context cannot be explained by altered song behavior in a straight-forward way. This observation does not rule out the possibility that sensory feedback might modulate neuronal addition.

A possible mechanism through which post-synaptic activity can influence neuronal addition is by activity-induced regulation of genes encoding molecules that promote survival of adult-born HVC neurons, axon path finding, and/or synapse formation [35, 36]. Interestingly, activity-induced guidance molecules are seasonally regulated in RA neurons of WCS; microarray analysis of cDNA extracted from RA revealed that the expression of guidance cue genes including netrin 4 and galectin are increased in breeding-condition birds [37]. Activity in RA may modulate recruitment via activity-induced trophic factors produced by target neurons that are transported retrogradely to influence the survival of new neurons. In canaries expression levels of brain-derived neurotrophic factor (BDNF) mRNA in HVC are higher in singing birds compared to non-singing birds, and infusion of BDNF protein near HVC in non-

singing birds increases neuronal addition in HVC [17, 38]. Microarray analysis also revealed that the expression of the pro-neurogenic genes including insulin-like growth factor 1 and neuromodulin is increased in breeding-condition WCS [37]. In HVC the mRNA expression of sex steroid receptors, which facilitate the retrograde transport of trophic factors bound to their receptor towards the neuronal soma, also increases during breeding conditions [39, 40]. Once transported back to the soma, trophic factors likely activate signaling cascades that promote the growth and survival of new neurons [41, 42]. Muscimol-mediated reduction of neural activity in RA may result in a failure of new HVC neurons to form synapses on RA neurons and/or a decreased production of activity-induced trophic factors in RA. The consequence would be a lack of retrograde transport of the trophic signals, and thus, a decrease in addition of adult-born neurons to HVC.

We found that RA neural activity is not required to maintain total HVC neuron number (mature plus new) under breeding conditions. The comparatively small decrease in new HVC neuron number caused by muscimol infused near RA is likely not sufficient to be reflected as a significant decrease in total HVC neuron number. Steroid-induced autocrine signals such as neurotrophic factors produced within HVC itself may be sufficient to maintain mature neurons when RA neural activity is abolished [39, 43].

Local neuronal activity is important in regulating neurogenesis during development [44] and adult neurogenesis (reviewed in [45]). Our results show in an *in vivo* neural circuit that neuronal activity in a target nucleus is important for the addition of new neurons in an afferent nucleus in the adult brain. Understanding the mechanisms underlying the relationship between neuronal activity and adult neurogenesis may provide insight to the etiology of and treatments for many neurological disorders.

4.6 Material and Methods

4.6.1 Animals

We used adult male Gambel's white-crowned sparrows (WCS), *Zonotrichia leucophrys gambelii* captured in eastern Washington during their pre- or post-breeding season migration. Prior to experiment onset, all birds were housed in indoor group aviaries exposed to short days (SD; 8 hr light, 16 hr dark) for 12 weeks. All experiments followed National Institutes of Health animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee.

4.6.2 Experimental Procedures

We injected birds housed on SD with BrdU (50 mg/kg in 15% DMSO; Sigma) intramuscularly once a day for 5 days. Two days following the final BrdU injection, unilateral intracerebral cannulae were implanted stereotaxically adjacent to RA. Briefly, birds were anesthetized with isoflurane (2%) in a non-rebreathing system. We randomly chose a hemisphere and lowered the cannula (Alzet 3-5 mm, Durect) 2.4 mm into the telencephalon caudal to RA (lateral, 3.4 mm; posterior, 1.25 mm using the intersection of the midsagittal and transverse sinuses as the reference point). We fixed the cannula to the skull with dental cement and attached a micro-osmotic pump (Alzet model 1002) to the cannula. Seventeen birds received osmotic pumps containing the inhibitory $GABA_A$ receptor agonist, muscimol (2.8 mg/ml in 0.09% NaCl; Sigma), while 7 control birds received only vehicle. Of the 17 birds infused with muscimol, four received osmotic pumps filled with BoDipy muscimol-TMR (2.8 mg/ml in 15% DMSO, 60% PEG, 25% dH_2O; Invitrogen). We placed osmotic pumps into sealed microcentrifuge tubes filled with saline and mounted the tubes into custom-made “backpacks” that allowed the birds to fly freely. Two days after surgery, each bird was implanted subcutaneously with a 12 mm Silastic capsule (1.47 mm inner diameter, 1.96 mm outer diameter) filled with crystalline T. We kept all of the birds on LD+T in individual sound isolation recording chambers until sacrifice two to four weeks after T implantation to determine whether the effect of inhibiting post-synaptic neurons in RA was most pronounced at a particular stage between cell proliferation and integration of fully differentiated neurons into a functional circuit. Because we found no significant differences in the number of new and pre-existing neurons between birds infused with muscimol for two to four weeks, we pooled these data. To ensure that all birds in the experiment longer than 14 days received continuous infusion, we exchanged the first osmotic pump with a second pump filled with the same solution at 14 days post surgery.

4.6.3 Tissue collection and processing

After we deeply anesthetized birds with isoflurane inhalation, we removed and immediately froze the brain on dry ice. We sectioned the brains in the coronal plane at 40 μm on a cryostat and thaw-mounted each section. We Nissl stained every third section and stored remaining sections at -80°C.

4.6.4 Immunohistochemistry

To visualize BrdU-labeled neurons, brain sections were fixed in 4% paraformaldehyde for 15 minutes, rinsed in phosphate buffered saline with 0.5% DMSO and 0.5% Triton-X (PDTX; pH 7.4), incubated with 0.1 mg/mL proteinase K for 5 minutes, and post-fixed for 15 minutes.

Slides were dipped in distilled water, incubated with 2N HCl at 37°C for 30 minutes, and rinsed with PDTX. After blocking for 1 h in 5% goat serum, slides were incubated with rat anti-BrdU (1:200; Accurate) and mouse anti-Hu (1:2000; Molecular Probes) antibodies [46]. Labeling was visualized with Alexa Fluor 594- or 488- conjugated goat anti-rat and anti-mouse IgG secondary antibodies (1:200; Invitrogen). A confocal laser-scanning microscope (Leica SPE5) was used to verify double-labeling. All single and double labeled cells in each HVC section were counted. All tissue processing was performed blind to treatment.

4.6.5 Morphometric measurements in HVC and RA

Volumes were determined by tracing the borders of the nuclei onto paper using a microprojector. These drawings were scanned into a microcomputer, and the area was determined using NIH Image J software. The total volume was calculated from the areas of the tracings using the formula for a truncated cone [47]. We used a random, systematic sampling protocol to measure neuron size and density [48]. We measured neuronal density visually by counting all neurons, distinguished by their single, densely staining nucleolus and uniform, nongranular cytoplasm, that fell within an ocular grid (1.9×10^{-3} mm^2 at 1000X) at each sampling site.

4.6.6. Electrophysiological recordings and analysis

Following a two-week continuous infusion of muscimol as discussed above, birds were anesthetized with three intramuscular injections of 25% urethane 30 min apart for a total volume of 6 μL/g. Birds were placed in a stereotaxic apparatus in a sound isolation chamber. Single and multi-unit extracellular recordings were made using glass electrodes filled with 0.9% NaCl. We alternated recordings between the two hemispheres and used the same electrode for multiple passes though each hemisphere. Small, ionophoretic injections of the tracer 10 kDa dextran amine conjugated to Alexa 488 or 568 or 10% fluororuby (10,000 MW tetramethylrhodamine dextran, Invitrogen; loaded into the recording electrode solution prior to recordings) were made to mark recording locations.

RA was targeted through a separate guide cannula that was implanted with the cannula attached to the osmotic pump. HVC was targeted through a small craniotomy just dorsal to the nucleus. Voltage signals from each structure were amplified 10X with an Axoclamp-2B amplifier (Molecular Devices, Sunnyvale, CA) in bridge mode and then filtered (0.1 kHz high-pass, 7 kHz low-pass) and amplified a further 100X with a Model 440 amplifier (Brownlee Precision, San Jose, CA). Signals were digitized at 25 kHz with a Micro 1401 data acquisition unit and Spike2 software (Cambridge Electronic Design, Cambridge, England, UK). We measured spontaneous

rates of single units in RA and both single and spike-sorted units (Spike 2 software) in HVC that had a stable firing rate for at least two minutes.

Each bird was transcardially perfused with 4% paraformaldehyde. The brains were removed and post-fixed in 20% sucrose + 4% paraformaldehyde solution before slicing 40 μm thick on freezing microtome. Sections were counterstained and examined to verify that recording locations were within RA or HVC.

4.6.7 Song recording and analysis

We individually recorded song of all birds housed in sound isolation chambers using Syrinx (John Burt, www.syrinxpc.com). Baseline song was obtained just as experimental song in a prior exposure to LD+T conditions. We measured song rate and the variability of 16 different song attributes using the protocol described in [6]. To determine the stereotypy of the song attributes, we compared coefficients of variation (CV=SD/mean). We also calculated song completeness from the number of syllables produced in 20 randomly selected songs and divided by the maximum number of different syllables produced by the bird - typically five syllables in Gambel's WCS. We analyzed the spectral properties of individual syllables using Sound Analysis Pro software (SAP; ofer.sci.ccny.cuny.edu) [49]. For each syllable, we used SAP to automate measurement of four spectral properties: pitch (indicates the period of oscillation, or mean frequency, of the sound), frequency modulation (FM; the mean slope of frequency contours), entropy (a measure of randomness) and pitch goodness (a measure of periodicity) [49]. Using the same 20 digitized songs, we calculated each property's mean value per syllable and averaged syllable values for each bird before statistical analysis [6]. We measured song rate as the average number of songs produced during a one hour period beginning two hours after lights on for each bird between 13 and 22 days. This is the time of maximal daily singing in WCS [6].

4.6.8 Statistical analysis

Spike rates between ipsilateral and contralateral HVC and RA were analyzed with non-parametric *t*-tests. All other comparisons were made with a two- or three-way mixed model ANOVA and post-hoc Tukey tests using JMP 8 (SAS).

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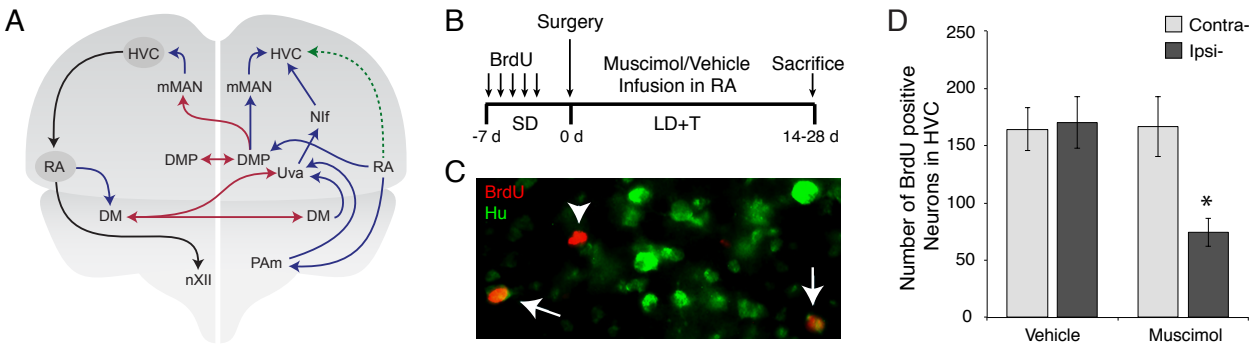


Figure 1. Inhibition of RA neural activity by muscimol infusion decreases HVC neuronal addition. Birds were injected with BrdU to label adult-born neurons, and muscimol, a $GABA_A$ receptor agonist, was infused unilaterally near RA to inhibit its neural activity. *A)* A coronal schematic of the song control system showing major song system projections (modified from [29]). Black arrows show projections in the motor output circuit, red arrows show bilateral projections, blue arrows show recursive projections, and the green dashed arrow is a weak projection from HVC to RA. *B)* Experimental timeline. *C)* A representative image of BrdU and Hu immunolabeling in HVC. BrdU is shown in red, and Hu, a neuronal marker, is shown in green. Arrows show BrdU positive neurons. The arrowhead indicates a BrdU positive cell that does not co-label with HU. *D)* The number of BrdU positive neurons in HVC at time of sacrifice. The number of Hu and BrdU co-labeled neurons in HVC is significantly lower in the hemisphere ipsilateral to muscimol infusion than in the contralateral uninfused hemisphere, and lower than either hemisphere in vehicle-infused controls (* $p \leq 0.05$). Contralateral hemispheres shown in light grey, ipsilateral in black. All data presented as mean±SEM.

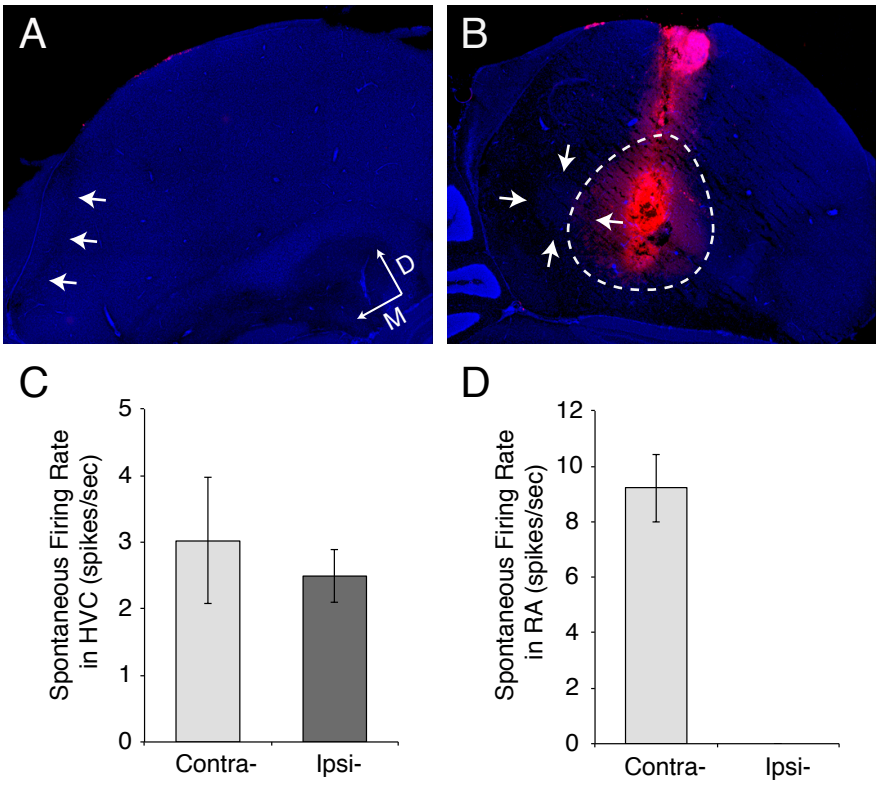


Figure 2. Reduction in HVC neuronal addition is not an effect of cannula placement or muscimol diffusion. *A)* Fluorescently conjugated muscimol (FCM) diffusion in HVC. D=dorsal, M=medial. White arrows outline HVC. FCM is shown in red, Hoechst 33342, a nuclear marker, shown in blue. Muscimol did not diffuse to HVC. *B)* The diffusion of muscimol-TMR in RA. White arrows outline RA. FCM area of diffusion outlined with dotted line. The cannula did not damage RA and muscimol diffused to most of RA. *C)* Representative raw electrophysiological trace recordings from ipsilateral and contralateral HVC and RA. Electrical activity was suppressed by muscimol infusion in ipsilateral RA (i.e. lack of spike trace), but not ipsilateral HVC. *D)* The spontaneous firing rates in HVC and RA both ipsilateral and contralateral to muscimol infusion. Spontaneous firing in HVC does not differ between HVC ispilateral and contralateral to muscimol infusion (see SI Table 1 for values), further suggesting muscimol did not diffuse to HVC. Muscimol completely suppressed activity in ipsilateral but not contralateral RA (SI Table 1).

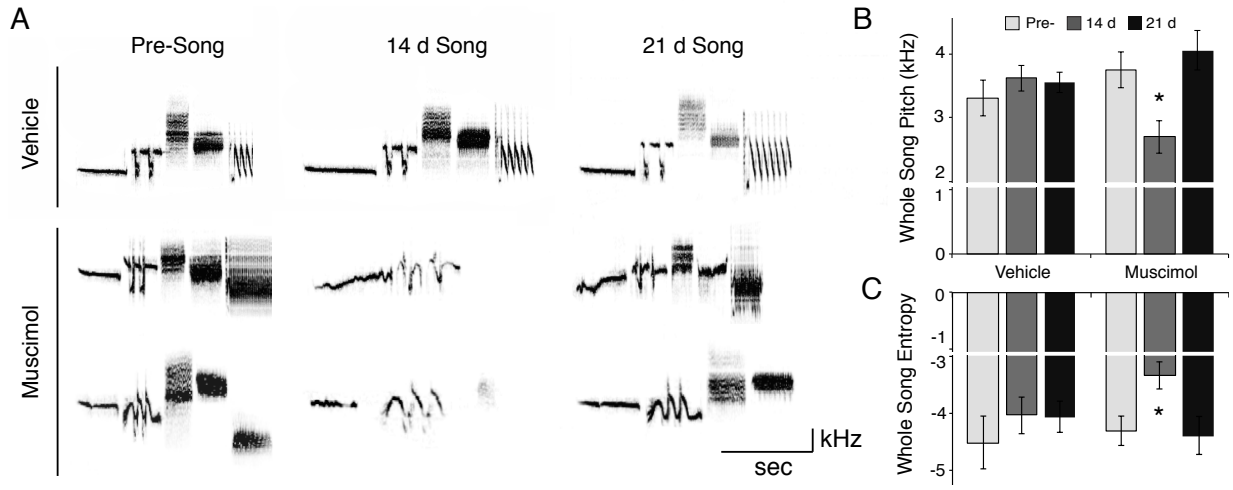


Figure 3. Muscimol infusion transiently alters song behavior. *A)* Representative sonograms of songs produced by the same birds before and during unilateral infusion of muscimol or vehicle into RA. *B)* Whole song pitch of birds infused with vehicle and muscimol. *C)* Whole song entropy of birds infused with vehicle and muscimol. Pitch and entropy of whole songs produced 14 D after onset of muscimol infusion are significantly different when compared to songs produced prior to and 21 D after infusion of muscimol (all comparisons, n=7, $p<0.05$; see SI Table 3 for values). Pitch and entropy of songs recorded prior to experiment and 21 D after infusion onset is not significantly different between vehicle and muscimol infused birds (all comparisons, n=10, $p>0.1$; SI Tables 3 and 4). Pre-experiment song shown in light grey, song recorded 14 D after infusion onset in dark grey, and 21D after infusion onset in black. * $p < 0.05$.

SI Table 1. Firing rates in HVC and RA of muscimol-infused birds

	Contralateral	Ipsilateral	# of Units (Contra-; Ipsi-)	# of Birds (Contra-; Ipsi-)	p-Value (Contra v. Ipsi)
Spontaneous Firing Rate (spike/sec)					
HVC	3.02 ± 0.95	2.49 ± 0.40	12; 16	2; 2	**0.5630**
RA	9.21 ± 1.23	N/A	12; 0	2; 2	N/A

SI Table 2. Morphometrics of HVC and RA

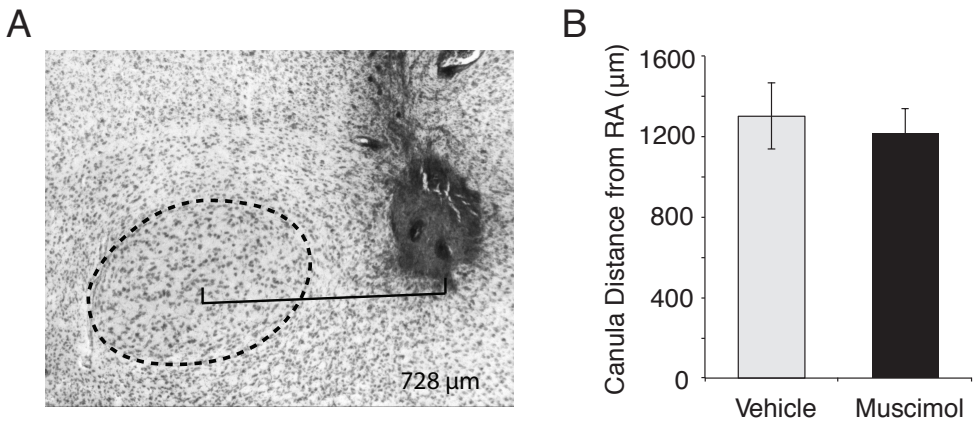
	Vehicle		p Value	Muscimol		p Value	p Value
	Contralateral	Ispilateral	Vehicle	Contralateral	Ispilateral	Muscimol	M v. V (Ipsi)
HVC			(n=5)			(n=8)	
Volume (mm^3)	0.587 ± 0.042	0.587 ± 0.050	1.0000	0.734 ± 0.065	0.710 ± 0.056	0.9877	0.4857
% Volume of Telencephalon	0.125 ± 0.003	0.121 ± 0.003	0.9151	0.193 ± 0.003	0.167 ± 0.003	0.9995	0.6467
Neuron Density ($x10^3/mm^3$)	250.152 ± 71.779	252.713 ± 76.910	1.0000	241.981 ± 26.320	242.521 ± 35.953	1.0000	0.9988
Neuron Number ($x10^3$)	155.485 ± 48.780	172.212 ± 68.673	0.9912	233.574 ± 43.528	216.256 ± 37.671	0.9965	0.9212
Neuronal Soma Area (um^2)	28.630 ± 5.092	30.950 ± 6.723	0.9916	37.650 ± 4.8705	39.712 ± 5.8570	0.9944	0.7284
RA							
Volume (mm^3)	0.222 ± 0.016	0.227 ± 0.015	0.9997	0.278 ± 0.017	0.276 ± 0.015	0.9981	0.2022
% Volume of Telencephalon	0.054 ± 0.005	0.053 ± 0.005	0.7799	0.059 ± 0.005	0.065 ± 0.005	0.9994	0.2800
Neuron Density ($x10^3/mm^3$)	35.668 ± 3.900	41.782 ± 6.000	0.9998	41.225 ± 2.741	40.868 ± 3.135	0.7678	0.9983
Neuron Number ($x10^3$)	7.979 ± 0.572	10.104 ± 1.560	0.9834	10.987 ± 0.662	11.333 ± 0.515	0.4110	0.7218
Neuronal Soma Area (um^2)	128.636 ± 12.894	124.082 ± 15.219	0.9955	134.541 ± 8.605	131.212 ± 11.629	0.9959	0.9770

SI Table 3. Quantification of song attributes produced by muscimol infused birds

	Muscimol (n=4)			p Value		
	Pre-Song	14d Song	21d Song	Pre- v. 14d Song	Pre- v. 21d Song	21d v. 14d Song
Pitch (Hz)						
Whole Song	3753.66 ± 281.38	2702.38 ± 256.86	4057.23 ± 314.59	**0.0425**	0.7571	**0.0152**
Whistle	3528.63 ± 414.22	2284.33 ± 370.49	4098.07 ± 414.22	0.0652	0.5858	**0.0098**
Warble	3558.29 ± 334.38	2547.85 ± 273.02	3770.79 ± 334.38	0.0915	0.8957	**0.0399**
First Buzz	3973.94 ± 321.51	3300.13 ± 293.50	4514.90 ± 359.46	0.3362	0.6266	0.0686
Second Buzz	4204.49 ± 339.61	2962.94 ± 277.29	4308.22 ± 339.61	**0.0400**	0.9747	**0.0266**
Third Buzz	2799.43 ± 416.07	2601.38 ± 263.15	2781.98 ± 339.72	0.9157	0.9994	0.9085
Pitch Goodness (dB)						
Whole Song	82.81 ± 16.35	132.24 ± 14.90	91.55 ± 18.27	0.1055	0.2364	0.9327
Whistle	50.45 ± 20.78	86.13 ± 18.59	30.33 ± 20.78	0.2921	0.7711	0.0905
Warble	75.02 ± 20.85	121.65 ± 17.02	64.07 ± 20.85	0.2371	0.9272	0.1270
First Buzz	89.12 ± 15.65	152.12 ± 14.28	89.87 ± 17.49	**0.0317**	0.9667	0.0504
Second Buzz	112.24 ± 16.78	148.89 ± 13.70	110.52 ± 16.78	0.5871	0.9045	0.8542
Third Buzz	169.10 ± 58.84	211.00 ± 37.21	263.88 ± 48.04	0.8237	0.4652	0.6746
Entropy						
Whole Song	-5.31 ± 0.26	-4.34 ± 0.24	-5.40 ± 0.29	**0.0423**	0.9732	**0.0381**
Whistle	-5.76 ± 0.39	-4.98 ± 0.35	-5.95 ± 0.39	0.1868	0.9426	0.1058
Warble	-5.44 ± 0.35	-4.36 ± 0.28	-5.52 ± 0.35	0.0818	0.9842	0.0598
First Buzz	-4.45 ± 0.30	-3.83 ± 0.27	-5.01 ± 0.34	0.2057	0.6826	**0.0454**
Second Buzz	-5.46 ± 0.27	-4.43 ± 0.22	-5.62 ± 0.27	0.2515	0.9971	0.2239
Third Buzz	-4.35 ± 0.40	-3.97 ± 0.25	-4.38 ± 0.33	0.7151	0.9979	0.6034
Frequency Modulation						
Whole Song	22.49 ± 2.89	28.13 ± 2.64	25.95 ± 3.23	0.3522	0.7113	0.8620
Whistle	2.95 ± 1.18	5.73 ± 0.96	3.45 ± 1.18	0.2066	0.9525	0.3286
Warble	18.99 ± 4.27	22.32 ± 3.49	16.82 ± 4.27	0.8206	0.9316	0.5928
First Buzz	25.79 ± 2.91	41.29 ± 2.38	31.64 ± 2.91	**0.0044**	0.3645	0.0627
Second Buzz	46.67 ± 4.12	41.68 ± 3.36	42.77 ± 4.12	0.6620	0.8135	0.9771
Third Buzz	46.82 ± 9.90	54.08 ± 6.26	60.96 ± 8.08	0.8140	0.5401	0.7860
Coefficient of Variance Duration (sec)						
Whole Song	20.47 ± 3.43	23.35 ± 4.20	14.42 ± 3.43	0.8575	0.4486	0.2624
Whistle	5.97 ± 1.79	13.90 ± 2.19	4.98 ± 1.79	**0.0368**	0.9208	**0.0194**
Warble	3.96 ± 4.93	25.73 ± 6.04	2.43 ± 4.93	**0.0379**	0.9738	**0.0264**
First Buzz	6.68 ± 3.88	18.36 ± 4.75	5.50 ± 3.88	0.1763	0.9742	0.1283
Second Buzz	9.09 ± 6.87	27.16 ± 8.41	9.53 ± 6.87	0.2556	0.9989	0.2710
Third Buzz	10.30 ± 11.89	32.77 ± 13.73	11.62 ± 11.89	0.4663	0.9966	0.5050
Frequency Difference (Hz)						
Whistle	26.12 ± 4.52	19.96 ± 5.53	19.50 ± 4.52	0.6731	0.5686	0.9977
Warble	14.44 ± 4.90	23.38 ± 6.00	9.20 ± 4.90	0.4993	0.7341	0.1977
First Buzz	14.85 ± 2.43	17.33 ± 2.97	7.72 ± 2.43	0.7971	0.1336	0.0638
Second Buzz	18.39 ± 3.70	21.61 ± 4.54	12.85 ± 3.70	0.8482	0.5547	0.3240
Third Buzz	13.99 ± 2.36	10.23 ± 3.05	8.31 ± 2.64	0.6106	0.2936	0.8838
Percent Complete						
Whole Song (%)	78.50 ± 6.29	76.72 ± 7.71	83.72 ± 6.29	0.6728	0.6804	0.9970
Song Rate						
Songs/Hour	56 ± 33	53 ± 19	62 ± 21	0.9999	0.9999	0.9999

SI Table 4. Quantification of song attributes produced by vehicle infused birds

	Vehicle (n=3)			p Value		
	Pre-Song	14d Song	21d Song	Pre- v. 14d Song	Pre- v. 21d Song	21d v. 14d Song
Pitch (Hz)						
Whole Song	3315.84 ± 284.64	3621.70 ± 201.27	3557.11 ± 164.34	0.6886	0.7629	0.9669
Whistle	2949.36 ± 309.18	2561.33 ± 252.45	2930.46 ± 252.45	0.9970	0.9968	0.9878
Warble	3087.16 ± 263.60	3506.34 ± 263.60	3358.65 ± 215.22	0.5507	0.7241	0.9038
First Buzz	4365.97 ± 423.01	4456.86 ± 423.01	4424.43 ± 345.39	0.9874	0.9937	0.9981
Second Buzz	4116.25 ± 110.40	4041.47 ± 110.40	3896.87 ± 90.14	0.8846	0.3672	0.6975
Third Buzz	2967.40 ± 198.59	3211.40 ± 198.59	2917.97 ± 162.15	0.6857	0.9798	0.6857
Pitch Goodness (dB)						
Whole Song	83.67 ± 33.23	122.74 ± 23.64	114.57 ± 19.30	0.6717	0.7301	0.9756
Whistle	42.88 ± 22.75	76.33 ± 1858	48.10 ± 18.58	0.8927	0.9620	0.9694
Warble	80.06 ± 15.96	80.40 ± 15.96	83.33 ± 13.03	0.9999	0.9862	0.9890
First Buzz	88.95 ± 18.12	107.59 ± 18.12	116.16 ± 14.79	0.7618	0.5311	0.9298
Second Buzz	146.01 ± 59.66	220.52 ± 59.66	180.73 ± 48.71	0.6779	0.8968	0.8677
Third Buzz	200.33 ± 46.08	178.28 ± 46.08	160.89 ± 37.62	0.8237	0.4652	0.6746
Entropy						
Whole Song	-5.52 ± 0.46	-5.04 ± 0.33	-5.07 ± 0.27	0.7060	0.7013	0.9984
Whistle	-5.90 ± 0.59	-4.98 ± 0.48	-5.86 ± 0.48	0.8344	0.9929	0.8621
Warble	-5.31 ± 0.20	-5.22 ± 0.20	-5.15 ± 0.16	0.9395	0.8220	0.9690
First Buzz	-5.18 ± 0.54	-4.39 ± 0.54	-4.50 ± 0.44	0.5953	0.6256	0.9866
Second Buzz	-5.71 ± 0.39	-5.27 ± 0.39	-4.91 ± 0.32	0.7324	0.3561	0.7700
Third Buzz	-4.75 ± 0.36	-4.57 ± 0.36	-4.56 ± 0.29	0.7151	0.6034	0.9979
Frequency Modulation						
Whole Song	24.95 ± 5.98	30.02 ± 4.23	28.27 ± 3.45	0.7846	0.8853	0.9459
Whistle	2.34 ± 0.45	23.72 ± 0.45	3.07 ± 0.37	0.1953	0.4934	0.5580
Warble	18.61 ± 1.77	19.09 ± 1.77	17.72 ± 1.44	0.9799	0.9225	0.8291
First Buzz	34.45 ± 2.27	32.16 ± 2.27	34.83 ± 1.85	0.7707	0.9905	0.6630
Second Buzz	58.42 ± 9.50	58.09 ± 9.50	49.90 ± 7.76	0.9996	0.7793	0.7937
Third Buzz	53.66 ± 7.65	44.97 ± 7.65	42.59 ± 6.24	0.7212	0.5526	0.9688
Coefficient of Variance Duration (sec)						
Whole Song	19.42 ± 8.40	31.58 ± 5.93	26.89 ± 4.20	0.4103	0.4792	0.9540
Whistle	7.70 ± 7.21	23.10 ± 5.10	7.36 ± 3.60	0.6131	0.7344	0.2583
Warble	3.51 ± 4.76	9.30 ± 3.37	5.06 ± 2.38	0.5770	0.9782	0.6286
First Buzz	4.92 ± 6.16	10.75 ± 4.36	10.26 ± 3.08	0.9538	0.9765	0.9928
Second Buzz	7.42 ± 4.48	6.68 ± 3.17	5.90 ± 2.59	0.4598	0.2331	0.7509
Frequency Difference (Hz)						
Whistle	12.68 ± 2.68	17.92 ± 1.90	26.35 ± 1.34	0.8176	0.6774	0.3582
Warble	7.28 ± 1.95	7.03 ± 1.38	6.85 ± 0.98	0.4202	0.2600	0.9417
First Buzz	7.52 ± 3.83	11.13 ± 2.71	12.44 ± 1.91	0.6795	0.8062	0.9454
Second Buzz	39.43 ± 8.63	8.80 ± 8.63	15.62 ± 4.98	0.3275	0.6129	0.6184
Percent Complete						
Whole Song (%)	80.00 ± 13.25	80.15 ± 10.97	63.50 ± 10.97	0.5492	0.6137	0.9646
Song Rate						
Songs/Hour	109 ± 37	52 ± 37	93 ± 37	0.3431	0.7636	0.7636



SI Figure 1. *A)* A representative image of Nissl staining showing RA and the cannula track as used to measure cannula distances from RA. Dotted line outlines the border of RA. *B)* The mean distance of the canulae tips from the center of RA (vehicle n=10, muscimol n=15; p>0.5).

Chapter 5. Network analysis of microRNA and mRNA seasonal dynamics in a highly neurogenic sensorimotor neural circuit

5.1 Abstract

Seasonal plasticity of the avian song control system requires temporally coordinated changes across molecular networks controlling different cellular processes, including neurogenesis, apoptosis, neurotrophin regulation, and electrical activity. MicroRNAs (miRs) – short, non-coding RNA sequences that alter gene expression by post-transcriptional inhibition or degradation of mRNA sequences – are likely involved in the global coordination of such diverse biological processes. To test the hypothesis that miRs related to neurogenesis and neural plasticity are expressed and seasonally regulated in the song control system, we measured changes in expression of miRs in the song circuit nuclei HVC, RA, and Area X of breeding and nonbreeding Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelli*) using microarray analyses. We found forty-six different miRs seasonally expressed in HVC, RA, and Area X. Of these miRs, miR-132 and miR-210 were the most highly differentially expressed. Cross correlating predicted targets of miR-132 with seasonally expressed genes identified 114 candidate mRNA targets regulating processes including cell cycle control, calcium signaling, and neuregulin signaling in HVC. Likewise, miR-210 was predicted to target fourteen seasonally expressed mRNAs that regulate serotonin, GABA, and dopamine receptor signaling and inflammation. Our results begin to identify the dynamic miR–mRNA regulatory networks related to seasonal neurogenesis and neural plasticity and provide opportunities to discover novel genetic and epigenetic control of the diverse biological processes involved in the functional incorporation of new neurons to the adult brain.

5.2 Background

Since the discovery of adult neurogenesis, explosive growth in the field has revealed complex but yet precise temporal and spatial coordination of the processes and mechanisms regulating functional incorporation of adult-born neurons. For example, neural stem cells residing in specific niches throughout the adult brain [13 - 16] proliferate and give rise to new neurons and glia [17]. The new immature neurons depart the neurogenic niche, migrate to their final destinations [17, 18], and integrate into functional circuits [17, 19 - 21]. Once fully integrated mature adult-born neurons persist for periods ranging from days to years [17, 22 - 24]. Each of these processes are regulated by a plethora of interacting autonomous and non-

autonomous factors. Some of these factors include but are not limited to sex steroid hormones secreted by the gonads and synthesized *de novo* in the brain, local neurotrophins, neural use and activity, cell death and inflammation, social interactions, seasonal changes, and stress (for reviews see [17, 25]).

One candidate for globally regulating the biological processes and mechanisms of adult neurogenesis are microRNAs (miRs). miRs are short, non-coding RNA sequences that alter gene expression by translational repression and mRNA target degradation (for review see [26]). Individual miRs have many mRNA targets, and thus can act as global regulators of complex temporal and spatial patterns of gene or protein expression changes underlying neural plasticity [26]. Moreover, miR expression is highly enriched in the brain [27] and has been implicated as involved in a variety of neurological disorders and diseases including Amyotrophic Lateral Sclerosis [28], Fragile X mental retardation [29], mood and mental disorders [30, 31], and Alzheimer's Disease [32]. Specific miRs play major roles in the normal processes of neural plasticity including fate specification [33], arborization and synapse formation [34, 35], adult-born neuronal addition and survival [36], and apoptosis [37]. The gene regulatory networks of brain expressed miRs has been studied little, however, in the context of adult neurogenesis specifically.

One prominent model for adult neurogenesis is that which occurs in the avain song control cirtuit (Figure 1A) of songbirds. Several nuclei that control the learning and production of song including HVC and Area X exhibit pronounced seasonal cycles of neuronal number [38, 39]. For example, during the breeding season neuronal number in HVC of Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*) increases by 25% (nearly 68,000 neurons), due to increased addition of adult-born neurons [40, 41]. As white-crowned sparrows transition back into nonbreeding conditions, HVC neuronal number decreases due to caspase-mediated apoptosis [42-44]. Most, if not all, of the new neurons added to the adult HVC have long axons that project four millimeters or more to synapse on target cells in RA [45, 46]. The seasonal incorporation new neurons in HVC of adult birds, corresponds to changes in song production – a sensorimotor behavior; each breeding season as HVC increases in neuronal number song production rate and song stereotypy increase [45, 47]. The quantitative and plastic changes in song control nuclei neuronal number, the incorporation of adult-born neurons into long-range neural circuits, and the relationship between adult neurogenesis and a biologically relevant and learned sensorimotor behavior make songbirds, and specifically the HVC–RA–AreaX circuits, a

unique model for investigating the spatiotemporal pattern of genetic networks that regulate the processes related to adult neurogenesis and circuit plasticity.

5.3 Methods

5.3.1 Animals

All of the following animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. Sixty adult male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii)* were collected in eastern Washington during their spring and autumnal migration under State of WA Scientific Collecting permit #10-162 and U.S. Fish and Wildlife Permit #MB708576-0. Birds were housed in outdoor aviaries for up to 30 weeks prior to transitioning into indoor aviaries. Once indoors, birds were exposed to a short-day photoperiod (SD; 8 h light: 16 h dark) for at least 10 weeks prior to experiment onset to ensure that they were photosensitive and responsive to circulating plasma T. Food and water were available ad libitum throughout the experiment. We castrated all birds by anesthetizing them with isoflurane, making a small incision on the left side anterior to the caudal-most rib and dorsal to the uncinate process, and aspirating the testes [1].

To synchronize the seasonal cycles, birds were implanted with a subcutaneous Silastic pellet (i.d. 1.0 mm; o.d. 2.0 mm; length: 12 mm; VWR) filled with crystaline T (Sigma), and shifted to long day photoperiod (LD; 20 h light: 4 h dark) for 21 d (see Figure 1B for experimental design). On day 21 we removed the subcutaneous T capsules of the birds, and shifted them back onto SD photoperiod for 10 weeks. On the final day of SD we we quickly decapitated nine birds and removed the brain for processing detailed below. The SD group of birds represent the steady-state of the regressed song control system and served as a baseline of comparison for all other groups. Another group of 45 birds were transitioned back to LD photoperiods following T pellet implantation (LD+T). On days 3, 7, and 21 of LD+T exposure, we quickly decapitated nine birds from each group and removed the brain. Of the nine remaining birds in LD+T for 21 d, all had T pellets removed and were transitioned back to SD overnight. After one day in SD, all remaining birds were killed for tissue collection.

5.3.2 Tissue harvesting

The brains of all birds were dissected rapidly and Vibratome sectioned at 300 µm in ice-cold, oxygenated artificial cerebral spinal fluid (ACSF; 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1 mM NaH2PO4, 16.2 mM NaHCO3, 11 mM D-glucose, and 10 mM HEPES). From these slices, punches of tissue containing only HVC, RA, or Area X were

collected from both hemispheres. Tissue punch location was verified post-hoc by Nissl-staining of re-sectioned, fixed tissue, as in (Figure 1C; [48]). HVC punches included the proliferative ventricular zone just dorsal to HVC. All of tissue punches from one nucleus of one bird were pooled and flash frozen in a dry ice/ethanol slurry, and stored at -80 degrees C until processed for microarray hybridization.

5.3.3 RNA isolation, microRNA and mRNA array processing

Total RNA was extracted from individual snap frozen tissue samples using the mirVana Paris Kit according to the manufacturer's protocol (Life Technologies, PN 1556M Rev C) to generate samples of isolated small (<200 nucleotides) and large (>200 nucleotides) RNAs. Total RNA concentration was determined measuring OD26, and the integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Only RNA samples with appropriate size distribution, quantity, and OD260/280 and OD 260/230 ratios of 1.8 - 2.1 were used for further array analysis. Total RNA samples were used for microRNA and mRNA arrays analysis

For microRNA analysis, samples were processed and hybridized to Affymetrix miRNA 3.0 arrays (Affymetrix, Santa Clara, CA). The Affymetrix miRNA 3.0 array was designed based on the miRBase v17 database that contained microRNAs from 153 organisms. Samples isolated form three individual birds belonging to the same experimental groups were pooled. Three such pools of samples from each experimental group were processed and hybridized to Affymetrix miRNA 3.0 arrays according to the manusfacturer's recommended protocol. Arrays were scanned with an Affymetrix GeneChip® 3000 scanner.

The hybridized arrays were scanned with an Affymetrix GeneChip 3000 scanner.

For mRNA analysis, samples were processed and hybridized to Agilent Zebrafinch Oligoarrays v2.2 (Agilent, Santa Clara, CA). The RNA isolation from the brain punches resulted in a lower yield than is required for Agilent Zebrafinch Oligoarrays v2.2. Therefore, RNA amplification was performed using the Nugen Ovation PicoSL WTA kit according to the manufacturer's protocol. Amplified RNA was used for further processing and hybridization to the Agilent arrays using the manufacturere's established one color protocol. Hybridization and washing of these arrays was accomplished using HS 400 Pro hybridization and wash stations (Tecan Systems, Inc., San Jose, CA). Arrays were scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc. Santa Clara, CA), according to the manufacturer's established standard protocol.

Both the microRNA and mRNA data will be submitted to NCBI's Gene Expression Omnibus database.

5.3.4 miRNA and mRNA array data analyses

Raw mRNA array data Agilent Zebrafinch Oligoarrays was generated with the Agilent Feature Extraction image analysis software (Agilent, Santa Clara, CA). The data was normalized using a variance-stabilization procedure (VSN) [49]. Image generation and feature extraction for Affymetrix microRNA 3.0 arrays was performed using Affymetrix GeneChip Command Console Software. The raw microarray microRNA array data was normalized using quantile normalization, followed by a robust multi-array average (RMA; [50]) with Bioconductor [51]. Several quality control steps were followed to insure that the data were of high quality: (1) visual inspection of the GCOS chip images, (2) visual inspection of the chip pseudoimages generated by the Bioconductor affyPLM package, (3) generation of percent present calls and average background signals with the Bioconductor simpleaffy package, (4) generation and inspection of histograms of raw signal intensities, and (5) generation and comparison of the Relative Log Expression and Normalized Unscaled Standard Errors using the Bioconductor affyPLM package. MiRs and mRNAs with significant differential expression were identified using the limma package [52] in Bioconductor. Data were fit with a weighted analysis of variance (ANOVA) and p-values were calculated with an empirical Bayes method. The weighted ANOVA model assigned array weights that smoothly up or down-weight the importance of a particular array, based on how similar that array is to others of the same type [53]. The empirical Bayes adjustment was based on computing an array-wide variance estimate and then using this value to adjust the estimates used in each contrast [52].

5.3.5 Real-time quantitative PCR confirmation of miR expression

Differentially expressed genes of interest were selected for confirmation of the microarray results by TaqMan based real-time quantitative reverse transcription (qRT)-PCR. Four micro-liters of remaining cRNA not used for microarray hybridization was added to each 25 µl PCR mixture consisting of primers (0.16 µM each) specific to one miR of interest, buffers, salts, and SYBR Green PCR master mix. Fluorescence detection was measured using the 7900HT FAST Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following PCR reaction profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 60 s, followed by a melt curve. DNA amplification was quantified from the C (T) value based on

standard curves to ensure quantification was within a linear range. All signals were normalized against U6, and fold-change ratios were calculated for experimental samples compared to SD controls using R software (http://www.r-project.org/). Pearson's correlations were used to correlate PCR data with array data. P-values <0.05 were considered significant.

5.3.6 MiR sequencing and analysis

In order to cross correlate seasonally regulated mRNAs to the differentially regulated miRs in IPA (Ingenuity, http://www.ingenuity.com) as described below, the miR sequence, specifically the mature and seed region sequences, were confirmed to be 100% homologous to the equivalent human miR. Briefly, primers for PCR were designed to amplify the full coding sequence of miRs of interest using the sequence of the miR of interest in closely related bird species and a broad range of vertebrates. From genomic DNA, full miR sequences were amplified with PCR using Quick Load Master Mix (New England Biolabs) as per the manufacturer's protocol. MiR sequence was verified (via GeneWiz, Seattle, WA) from independent PCR product of five individual birds after isolation from a 1.5% electrophoresis gel using the Qiagen Gel Extraction Kit. All miRs of interest were confirmed to have 100% homology within the mature and seed regions. For miR-132 and miR-210, rooted phylogenetic trees with branch lengths were constructed in ClustalW (www.genome.jp/tools/clustalw/) using the white-crowned sparrow sequence and the respective miR sequences of other vertebrates obtain through miRBase (mirbase.org, University of Manchester).

5.3.7 Canonical signaling pathway and miR target predictions

Conserved canonical signaling pathways were predicted for both mRNAs and miRs using the core analysis module in IPA software (Ingenuity, http://www.ingenuity.com). Canonical pathways with $p<0.05$ were considered significant. Potential targets of miR-132 and miR-210 were predicted using the miR target filter module in IPA (Ingenuity) with the filter criteria of moderate or greater likelihood of being a target from a manually curated database of functional interactions based on previous publications. Targets were additionally filtered by the seasonally regulated mRNAs (log_2-fold >2, $p<0.001$) from the mRNA microarray.Targets of miRs were identified using matching experimental time points and nuclei during mRNA target filtering. For generating the target network plots, we used the microRNA Target Filter in IPA and limited miR targets to mRNAs whose expression were inversely correlated with those of the miRs.

5.4 Results and discussion

5.4.1 Seasonal expression of mRNAs in the song control nuclei

We identified the differential expression of genes between breeding and nonbreeding condition using microarray analyses of tissue harvested from HVC, RA, and Area X, in SD, LD +T at 3D, 7D, and 21D, and LDW conditions. We obtained 155, 61, 125, and 148 of differentially expressed (log_2-fold >2.0, $p<0.0001$) mRNAs in HVC at LD+T 3D, 7D, 21D, and LDW, respectively (Figure 2A). In RA 17, 24, 19, and 29 and in Area X 31, 31, 42, and 146 genes varied by log_2-fold >2.0 with $p<0.001$ in LD+T 3D, 7D, 21D, and LDW, respectively (Figure 2A). We found in the neurogenic nuclei HVC and Area X that 8.6% to 13.9% and 31.0% to 41.9% of the genes that were differentially expressed across all time points were down regulated (Figure 2A). In the non-neurogenic nucleus RA we found a higher percentage of genes down-regulated and greater variability in this percentage across experimental groups compared to the neurogenic nuclei; the percentage of down regulated genes in RA varied from 50% to 97% across all time points (Figure 2A). Seasonally regulated genes included those of canonical signaling pathways including ERK5 and NGF signaling in HVC and Wnt/Ca+ and axonal guidance signaling in RA (Table 1). Upon transition from breeding to nonbreeding conditions, we found that genes in the canonical signaling pathways for cell death (tumorcidal) and inflammation (LPS/IL-1) were differentially expressed compared to SD in HVC and Area X (Table 1).

Gene regulatory networks that control patterning and plasticity within song control nuclei have been previously examined with the sequencing and analysis of the zebra finch genome and microarray analyses *[49, 54]*. More specifically, Thompson et. al. (2012) identified 132 genes in HVC cells that changed in expression between breeding and nonbreeding conditions when compared to gene expression in RA, a non-neurogenic region of the song bird brain *[1]*. In comparison, we identified 265 genes differentially expressed between HVC and RA in breeding conditions and 239 in nonbreeding conditions. In both studies, genes that promoted proliferation, angiogenesis, and neurite extension were up-regulated, whereas genes that support programmed cell death were down-regulated in HVC under breeding conditions *[1]*. Specific genes that encode for neurotrophins known to promote neuronal migration, recruitment, and survival, including brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF1) were differentially regulated in HVC under breeding conditions in both studies *[1]*.

5.4.2 miRs are differentially expressed in song control nuclei between breeding and nonbreeding conditions

To test whether miRs were differentially expressed between breeding and nonbreeding conditions, we quantified via microarray the fold-change relative to SD of over 150 unique miRs from over 153 different species in tissue from HVC, RA and Area X of birds in LD+T and LDW. Of the three miRs that were significantly differentially expressed across condition (log_2-fold change >2.0 and false discovery rate <0.05), miR-132, specifically within HVC, had the highest fold changes in expression (Table 2). Using a less stringent threshold of log_2-fold change >2.0 and $p<0.0005$, we obtained four, thirteen, nineteen, and twenty-nine differentially expressed miRs in HVC, at LD+T 3D, 7D, 21D, and LDW, respectively (Table 3 and Figure 2B). We found in RA thirty miRs were differentially expressed in one or more experimental groups, whereas in Area X only five miRs were differentially expressed (Table 3 and Figure 2B) with the less stringent threshold. The expression landscapes in HVC, RA, and Area X suggest that the majority of miRs are generally up-regulated with time into breeding condition (Figure 2B). Concomitantly, large clusters of mRNAs became down-regulated with progression into breeding condition in all three nuclei (Figure 2A). Alternatively, in HVC during LDW thirteen miRs decreased significantly in expression compared to SD, while large clusters of mRNAs related to macrophage function (e.g. MPEG1), cell arrest (e.g. ZAR1), and sirtuin signaling (e.g. SIRT3) increased in expression.

Real-time quantitative PCR confirmed consistent seasonal expression of select miRs of interest. The directional trends for miR-132 showed consistent up-regulation in PCR expression across all experimental groups for which miR-132 was significantly up-regulated in the microarray analyses (Figure 3A). Other miRs of interests from HVC (Figure 3B) and RA (Figure 3C) including miR-181b, miR-30, and miR-124 also showed directional trends consistent with the array data. In HVC Pearson's *r* correlation found a significant positive correlation between the log_2-fold changes of miRs assayed with rt-Q-PCR and the respective log_2-fold change from the microarray ($R^2=0.30702$, $p=0.0138$). The equivalent correlation with data from RA revealed no significant correlation ($R^2=0.1465$, $p=0.2453$). Area X did not differentially express any of the miRs of interest that were assayed with rt-Q-PCR except miR-132, and thus were not included in the analyses.

Previous Illumina sequencing of auditory regions in zebra finches in response to song exposure identified 121 miRs conserved in other vertebrates and 34 novel miR sequences specific to zebra finches [55]. Consistent with this previous work we find both conserved and

bird-specific miRs are seasonally expressed in the song control circuit. Some of the bird-specific miRs differentially expressed in HVC included miR-1562, -1782, -1915, -2962, -1700, -1575, and -1704. Of the conserved miRs identified in our microarray, several are known to play roles in neural plasticity. For example, miR-295, a miR that suppresses autophagic cell death [56], was down-regulated during LDW in HVC, a period in which cell death peaks as HVC volume collapses [43, 44]. Previous studies also found that miR-184 regulates the translation of Numb [57], a protein necessary for the survival of neural progenitor cells [58] and neuronal differentiation in the mammalian cortex [59]. Interestingly, we found high expression of miR-184, which could confer low levels of Numb, in the non-neurogenic nucleus RA. Another miR of interest up-regulated in RA during breeding conditions is miR-135. miR-135 inhibits the expression of the serotonin transporter and the inhibitory serotonin receptor thereby promoting an increase in serotonin signaling [60]. The up-regulation of miR-135 in RA coincides with increases in spontaneous firing activity [61] and probable increased serotonin signaling in RA [62, 63]. Finally, miR-129, a miR down-regulated in RA during breeding conditions, has been previously shown to inhibit Foxp2 translation [64], a protein necessary for proper human speech [65], vocal learning in songbirds [66], and neurite outgrowth [67]. Our data are not only consistent with these and other previous reports but also go one step further to suggest that sex steroids may modulate the aforementioned processes via alterations in miR–mRNA expression networks.

5.4.3 The seasonal interaction network of miR-132 in HVC

miR-132 mediates the integration of adult-born neurons in dentate gyrus [36], the arborization of new neurons in the hippocampus [35], and radial migration of neurons via Foxp2 [68]. Based on the previously documented role of miR-132 in mammalian adult neurogenesis and synaptic formation and plasticity and the fact that miR-132 was the most significantly up-regulated miR in HVC, we examined the miR–mRNA regulatory network of miR-132 more thoroughly. Prior to such analyses we first confirmed that the sequence of white-crowned sparrow miR-132 (i.e. zlg-mir-132) mature and seed regions was the same as the human miR-132 (hsa-mir-132) sequence. One-hundred percent homology is necessary because IPA software predicts targets based on the human miR seed region and mRNA 3‘UTR sequences. The zlg-mir-132 sequence was indeed 100% homologous to hsa-mir-132 in both the seed regions and the mature sequence. The full sequence of zlg-mir-132 was 62% homologous to the full sequence of hsa-mir-132, 64% to rno-mir-132 (rat), and 96% to tgu-mir-132 (zebra finch). Generating a rooted phylogenetic tree with various vertebrate miR-132 sequences

represented, grouped the zlg-miR-132 sequence with tgu-mir-132, further confirming correct full white-crowned sparrow sequence (Figure 4A and SI Table 1).

Through IPA software, miR-132 was predicted to target a total of 767 mRNAs. We filtered the predicted mRNA targets by mRNAs that were differentially expressed (log_2-fold >2.0, $p<0.001$) between breeding and nonbreeding conditions in HVC to generate a total of 114 mRNA seasonally expressed miR-132 targets (Figure 4B). Of the one-hundred-fourteen miR-132 targets 55, 32, 59, and 22 targets were differentially expressed during LD+T at 3D, 7D, 21D, and LDW, respectively (Figure 4B). All of the predicted seasonally-expressed targets of miR-132 were investigated for functional relatedness, from which we obtained the top significant canonical pathways represented by the filtered targets across all experimental groups (Table 4 and Figure 4C). Independent analyses of functional relatedness of miR-132 targets from LD+T 3D, 7D, 21D, and LDW identified top canonical pathways including cell cycle control, PTEN signaling, calcium signaling, neuregulin signaling, and retinoic acid signaling (Table 5 and Figure 4C).

High levels of miR-132 expression in HVC are consistent with previous reports of high miR-132 expression in the brains of zebra finches [69]. Moreover, high expression of miR-132 in breeding condition HVC corroborates previous studies on the interactions between this miR, neurotrophins, and the ERK/MAPK signaling cascade. Transcription of miR-132 is controled directly by the transcription factor cAMP-response element binding protein (CREB; [70]). Activation of CREB by phosphorylation occurs with BDNF binding its receptor [71], circadian gene oscillation [72], and synaptic activity via nuclear calcium signaling [73]. Activated CREB in combination with BDNF activation of the ERK/MAPK pathway in turn increase expression of miR-132 [74]. In HVC during breeding conditions, BDNF expression is enhanced by the presence of T [75] via the expansion of vasculature endothelial cells [2]. Concomitantly, CREB is co-expressed seasonally in HVC with androgen receptors [76], which trans-activate CREB when T binds [77]. Thus, in breeding conditions T level increases likely promote miR-132 expression via the enhanced activation of CREB and production and signaling of BDNF in HVC. In turn, miR-132 represses the translation of a variety of repressor genes to promote cell cycle entry [78], neuronal addition [36], and arborization [35], and even the proliferation of endothelial cells [79] that secrete BDNF [2].

5.4.4 The seasonal interaction network of miR-210 in Area X

Of the significantly seasonally regulated miRs in Area X – another neurogenic and seasonally plastic song circuit nucleus – miR-210 stood out as an miR of interest. Given the role

of miR-210 in promoting neural repair through angiogenesis [80-82], and that miR-210 was significantly down regulated during periods after which Area X would require angiogenesis for growth (i.e. 7D and 21D of LD+T; [38, 39, 83]), we investigated the miR–mRNA network of miR-210 more thoroughly. We first confirmed that the sequence of white-crowned sparrow miR-210 (i.e. zlg-mir-210) mature and seed region was 100% homologous to hsa-mir-210 sequence. The full sequence of zlg-mir-210 was 67% homologous to the full sequence of hsa-mir-210, 67% to rno-mir-210, and 82% to cli-mir-210 (pigeon). A rooted phylogenetic tree placed the zlg-miR-210 sequence in the same clade as several other bird species including the collard-fly catcher (*Ficedula albicollis*) and the pigeon (*Columbia livia*; Figure 5A and SI Table 2).

Using IPA we identified 1100 total targets of mir-210, fourteen of which were differentially expressed (log2-fold >2.0, $p<0.001$) on the mRNA microarray in Area X. Nine predicted target mRNAs were seasonally expressed in Area X at LD+T at 7D, while twelve and seventeen were seasonally expressed in LD+T 21D and LDW, respectively. Examining the filtered targets across experimental groups for functional relatedness identified top significant canonical pathways related to dopamine signaling, phospholipase signaling, and calcium signaling (Table 4 and Figure 5C). Independent analyses of functional relatedness of miR-210 targets from LD+T 7D, 21D, and LDW identified top canonical pathways including serotonin, GABA, and dopamine receptor signaling, calcium transport, and lymphocyte signaling (Table 6 and Figure 5C).

Although the mechanism through with T or its metabolites reduces miR-210 expression is unclear, our data suggests that miR-210 is indeed under sex steroid control. No previous reports find evidence of direct control of miR-210 expression by sex steroids. Alternatively, miR-210 regulation by T may be indirect: miR-210 expression is induced by HIFα [84], which is suppressed by estrogen [85], a metabolite of T. Thus, increased systemic T, and resultantly, estrogen, in Area X could prevent expression of HIF1α thereby also preventing mir-210 expression.

5.5 Conclusions

We identified several seasonal miR–mRNA interaction networks that likely coordinate the various processes and mechanisms required for successful integration of new neurons in neural circuits and for the seasonal deconstruction of the HVC to RA neural pathway. Highly differentially expressed miRs including miR-132 and miR-210 likely target many gene products that are also seasonally regulated and shed light on the complex regulatory networks of seasonal plasticity. Our results provide opportunities to test novel genetic and epigenetic

regulatory networks that control the diverse biological processes involved in the functional incorporation of new neurons to the adult brain.

5.6 References

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Table 1. Top canonical pathways of genes seasonally regulated in HVC, RA, and Area X

	Top Canonical Pathways	p-value	Ratio	# of Genes
HVC				
3D	RAN Signaling	0.000899	0.214	3
	Cell Cycle Control	0.023500	0.118	2
7D	Galactose Degradation	0.028000	0.200	1
	UDP-N-acetyl-D-galactosamine Biosynthesis	0.005660	0.143	1
21D	ERK5 Signaling	0.000098	0.103	4
	NGF Signaling	0.006400	0.062	5
LDW	ATM Signaling	0.016800	0.075	3
	Tumoricidal Function	0.017100	0.133	2
RA				
3D	Wnt/Ca+ Pathway	0.050000	0.214	1
	Axonal Guidance Signaling	0.050000	0.008	2
7D	Myo-inositol Signaling	0.008130	0.086	3
	T Lymphcyte Signaling	0.047800	0.042	1
21D	Cancer Signaling	0.002660	0.056	2
	Synaptic Long Term Depression	0.010500	0.023	2
LDW	Sphingosine Metabolism	0.005430	0.5	1
	Ceramide Degradation	0.005430	0.5	1
Area X				
3D	Galactose Degradation	0.015200	0.200	1
	RAN Signaling	0.042000	0.071	1
7D	Flavin Biosynthesis	0.002940	1.000	1
	Salvage Pathway	0.011000	0.025	1
21D	Granulocyte Adhesion	0.013000	0.045	2
	Gylcerol Degradation	0.019700	0.200	1
LDW	LPS/ IL-1 Mediated Function	0.006040	0.056	5
	MIF-mediated Glucocorticoid Regulation	0.010000	0.167	2

Table 2. Significantly differentially expressed miRNAs with log2 >2 and empirical Bayes FDR<0.05

miRNA (# hits)	Log2 fold	FDR
HVC LD+T 3D		
miR-132 (1)	5.899313681	0.027848468
HVC LD+T 7D		
miR-132 (6)	7.13 ± 0.14	0.0002 ± 0.0001
HVC LD+T 21D		
miR-132 (13)	8.03 ± 0.40	0.0021 ± 0.0021
miR-1356 (1)	6.36	0.0005
HVC LDW 1D		
miR-4516 (1)	-5.28	0.0141
miR-132 (13)	8.33 ± 0.32	0.0000 ± 0.0000
RA		
No significant hits		
Area X		
No significant hits		

***Mean ± SEM**

Table 3. Significantly differentially expressed miRNAs with log2 >2 and p<0.0005

	3D			7D			21D			LDW		
HVC	**miRNA (# hits)**	**Log2 fold**	**p-value**	**miRNA (# hits)**	**Log2 fold**	**p-value**	**miRNA (# hits)**	**Log2 fold**	**p-value**	**miRNA (# hits)**	**Log2 fold**	**p-value**
	miR-132 (4)	1.88 ± 0.18*	0.0002000	miR-132 (12)	2.83 ± 0.06	0.0000060	miR-132 (13)	3.63 ± 0.07	0.0000010	miR-132 (13)	3.65 ± 0.10	0.0001000
							miR-1356	2.00	0.0000003	miR-134	-1.12	0.0001375
							miR-212	1.96	0.0000820	miR-1182	-1.81	0.0004752
							miR-2840	1.69	0.0003796	miR-404	-1.83	0.0004679
										miR-1915	-1.98	0.0002142
										miR-395f	-2.11	0.0002592
										miR-2455	-2.23	0.0002717
										miR-1562	-2.39	0.0001816
										miR-4516	-2.44	0.0000073
										miR-574	-2.45	0.0004553
										miR-295	-2.53	0.0003721
										miR-395b	-2.57	0.0001199
										miR-1782	-2.68	0.0004008
										miR-1362	-2.79	0.0000570
RA												
	miR-281	4.47	0.0000813	miR-34a	5.00	0.0000164	miR-132 (4)	1.88 ± 0.05	0.0000100	miR-132 (4)	1.90 ± 0.11	0.0001000
	miR-1192	3.98	0.0003389	miR-2111n	4.84	0.0000271	miR-2525	-2.40	0.0003735			
	miR-184	3.94	0.0003805	miR-2571	4.47	0.0000798						
	miR-77	3.86	0.0004735	miR-311b	4.29	0.0001382						
	miR-4197	-3.92	0.0003983	miR-4142	4.02	0.0003034						
	miR-236	-3.94	0.0003769	mir-135a	3.98	0.0003372						
	miR-2182	-4.03	0.0002950	miR-8	3.97	0.0003425						
	miR-758	-4.04	0.0002797	miR-199b	3.85	0.0004929						
	miR-982	-4.33	0.0001228	miR-837	-3.90	0.0004224						
	miR-34	-4.62	0.0000521	miR-528	-4.03	0.0002895						
	mir-4478	-4.73	0.0000371	miR-441a	-4.15	0.0002040						
	miR-3479	-5.23	0.0000084	miR-395g	-4.17	0.0001938						
				mir-23a	-4.22	0.0001688						
				miR-2357	-4.23	0.0001631						
				mir-3673	-4.35	0.0001137						
				mir-129	-4.64	0.0000488						
Area X												
	miR-2491	2.00	0.0001818	miR-210	-1.96	0.0000343	miR-3751	1.35	0.0000110	miR-132 (6)	1.69 ± 0.06	0.0001849
	miR-132 (2)	-1.79 ± 0.16	0.0001526				miR-210 (4)	-1.71 ± 0.09	0.0002311	miR-1713	-1.55	0.0000029

***Mean ± S.E.M. Where more than one hit, p-value listed as average.**

Table 4. Top pathways for miR-132 and miR-210 across all time points

Top Canonical Pathways	p-value	Ratio	Genes
miR-132 HVC			
Molecular Mechanisms of Cancer	0.000007	0.0301	E2F5, CFLAR, ADCY3, ARHGEF10, CDKN1A, MAPK1, TGFB2, PRKD1, RAP2B, PRKAG2, FOXO1
RAR Activation	0.000062	0.0398	ADCY3, MAPK1, TGFB2, PRKD1, PRKAG2, NCOR1, GTF2H1
VDR/RXR Activation	0.000078	0.0641	CDKN1A, TGFB2, PRKD1, NCOR1, FOXO1
Breast Cancer Regulation by Stathmin1	0.000102	0.0366	E2F5, ADCY3, ARHGEF10, CDKN1A, MAPK1, PRKD1, PRKAG2
ErbB Signaling	0.000123	0.0581	HBEGF, MAPK1, PRKD1, NRG2, FOXO1
Pancreatic Adenocarcinoma Signaling	0.000331	0.0472	E2F5, HBEGF, CDKN1A, MAPK1, TGFB2
Cell Cycle: G1/S Checkpoint Regulation	0.000468	0.0625	E2F5, CDKN1A, TGFB2, FOXO1
PPARα/RXRα Activation	0.000525	0.0335	ADCY3, MAPK1, TGFB2, CAND1, PRKAG2, NCOR1
Leptin Signaling in Obesity	0.000813	0.0541	ADCY3, MAPK1, PRKAG2, FOXO1
Acute Myeloid Leukemia Signaling	0.000933	0.0519	KITLG, MAPK1, TCF7L2, TCF7L1
miR-210 Area X			
Phospholipase C Signaling	0.002239	0.0126	ADCY5, BTK, PPP1CB
Dopamine Receptor Signaling	0.003311	0.0256	ADCY5, PPP1CB
CDK5 Signaling	0.005248	0.0202	ADCY5, PPP1CB
NAD Biosynthesis III	0.006607	0.1670	NMNAT2
Phosphatidylcholine Biosynthesis I	0.007762	0.1430	PCYT1B
NAD Salvage Pathway III	0.007762	0.1430	NMNAT2
Cellular Effects of Sildenafil (Viagra)	0.008913	0.0155	ADCY5, PPP1CB
β-adrenergic Signaling	0.009333	0.0150	ADCY5, PPP1CB
Calcium Transport I	0.010000	0.1110	ATP2B3
Dopamine-DARPP32 Feedback in cAMP Signaling	0.013490	0.0124	ADCY5, PPP1CB

Table 5. Top pathways for miR-132 in HVC at each time point sampled

Top Canonical Pathways	p-Value	Ratio	# Genes
LD+T 3D			
Breast Cancer Regulation	0.001660	0.0209	4
Estrogen-mediated S-phase Entry	0.001850	0.0833	2
Molecular Mechanisms of Cancer	0.002800	0.0137	5
Cell Cycle Checkpoint Control	0.009420	0.0364	2
Wnt/β-catenin Sginaling	0.010300	0.0178	3
LD+T 7D			
PTEN Signaling	0.000886	0.0254	3
AMPK Signaling	0.001280	0.0224	3
Melanoma Signaling	0.002970	0.0476	2
Calcium Signaling	0.002870	0.0169	3
Antiproliferative Role	0.004590	0.0317	2
LD+T 21D			
ErbB Signaling	0.000005	0.0581	5
Neuregulin Signaling	0.000117	0.0455	4
ErbB2/3 Signaling	0.000582	0.0526	3
Molecular Mechanisms of Cancer	0.000599	0.0164	6
ErbB4 Signaling	0.000677	0.0500	3
LDW			
Melanocyte Development, Pigmentation Signaling	0.000105	0.0357	3
CDK5 Signaling	0.000171	0.0303	3
RAR Activation	0.000922	0.0170	3
PPARα/RXRα Activation	0.000968	0.0168	3
Pyridoxal 5'-phosphate Salvage Pathway	0.002250	0.0313	2

Table 6. Top pathways for miR-210 in Area X at each time point sampled

Top Canonical Pathways	p-Value	Ratio	# Genes
LD+T 7D			
Phospholipase C Signaling	0.004880	0.0084	2
B Lymphocyte Signaling	0.018400	0.0244	1
Serotonin Receptor Signaling	0.019700	0.0227	1
Primary Immunodeficiency Signaling	0.023200	0.0192	1
GABA Receptor Signaling	0.029900	0.0149	1
LD+T 21D			
Phosphatidylcholine Biosynthesis	0.003890	0.1430	1
Calcium Transport I	0.005012	0.1110	1
Choline Biosynthesis III	0.007079	0.0769	1
RAN Signaling	0.009333	0.0588	1
Chemokine Signaling	0.038905	0.0141	1
LDW			
Phospholipase C Signaling	0.000692	0.0126	3
Dopamine Receptor Signaling	0.001549	0.0256	2
CDK5 Signaling	0.002455	0.0202	2
Cellular Effects of Sildenafil	0.004169	0.0155	2
Cardiac β-adrenergic Signaling	0.004365	0.0150	2

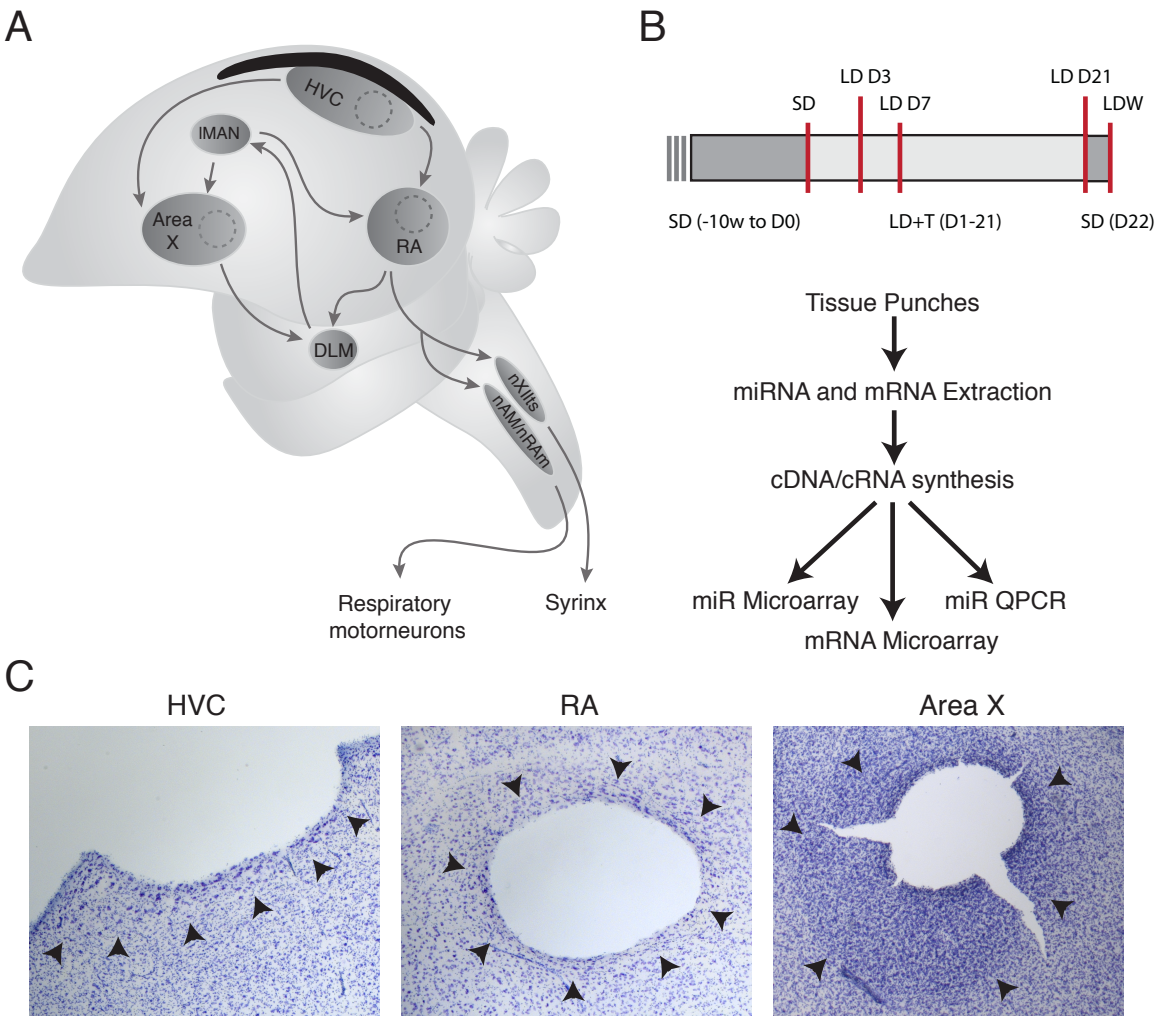

Figure 1. Experimental design. *A)* A schematic of the song nuclei sampled for microarray analysis. The dotted line indicates where tissue samples were obtained. *B)* Experimental time-line for all experimental groups. Red lines indicate termination of the experiment for the given group.*C)* Representative images of Nissl-stained brain sections showing confirmation of tissue punch locations in HVC, RA and Area X. The arrow heads indicate the borders of the respective nuclei as determined by cell morphology and density.

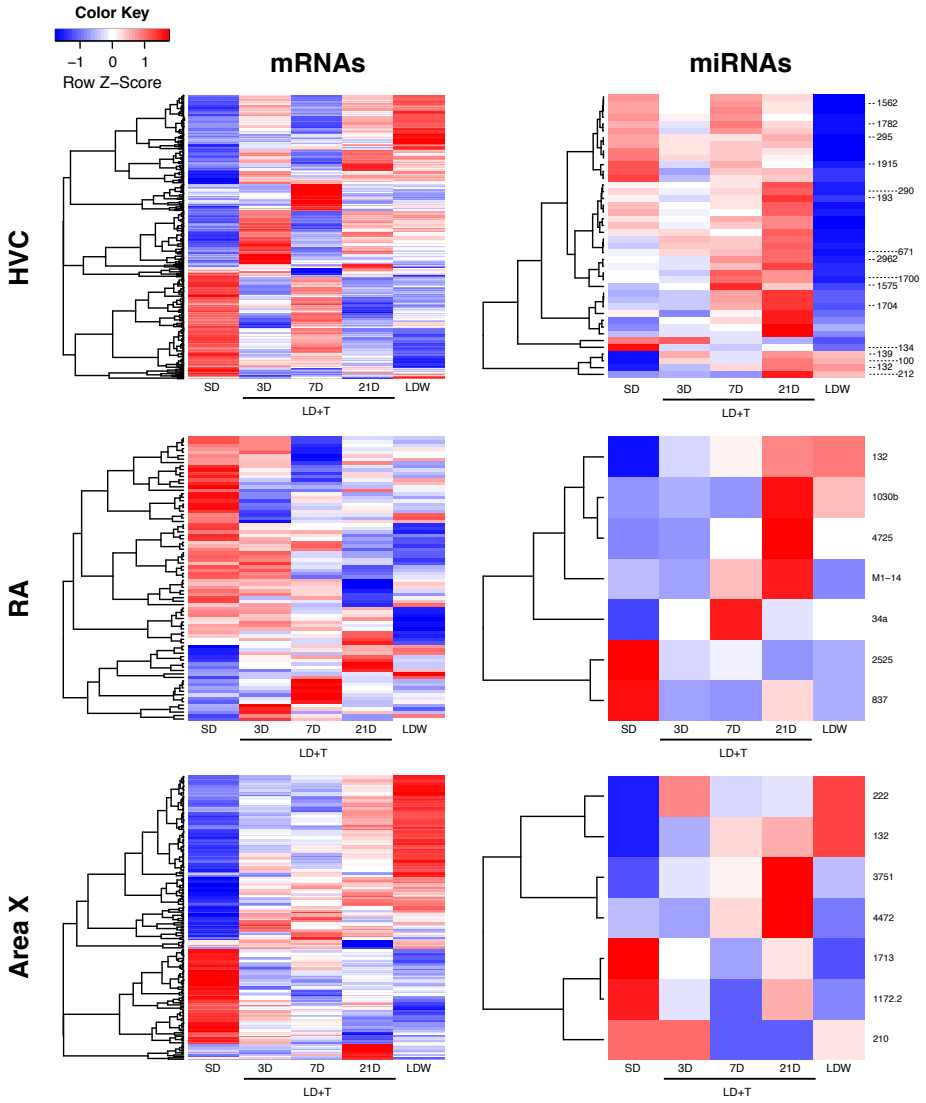

Figure 2. microRNA and gene microarray expression across breeding and nonbreeding conditions. *A)* The expression fold changes of mRNAs that were differentially regulated in at least one experimental group (i.e. LD+T 3D, 7D, 21, or LDW) compared to SD. A p-value of <0.0005 and $\log_2$-fold change >2.0 were used as the threshold for inclusion. *B)* miRs that were differentially regulated in at least experimental group with $\log_2$-fold change >2.0 and p<0.0005 compared to SD. miRs of interest based on predicted function or miRs specific to birds are denoted. Red indicates increases in expression compared to SD, whereas blue indicates decreases in expression. SD expression patterns were generated by computing log-fold changes of individual samples from the mean of the SD group. All mRNAs and miRs were allowed to cluster by temporal expression pattern.

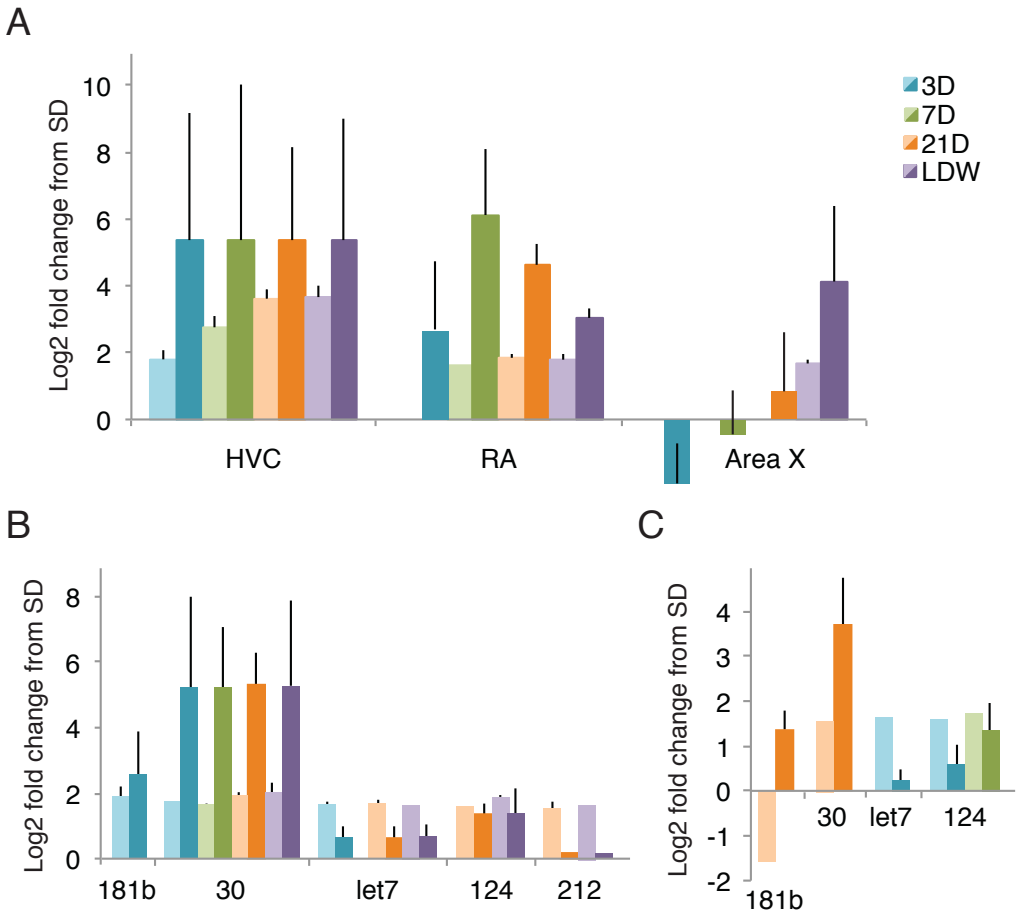

Figure 3. Real-time Q-PCR verification of miRs of interest from microarray data. *A)* Log_2-fold change in expression of mir-132 from the microarray (shown in light colors) compared to rt-Q-PCR (dark colors). All fold-changes are relative to SD. Real-time Q-PCR confirms that miR-132 expression is consistent with significant differential expression obtained with the microarray. Different base colors indicate the various experimental time points independent of nucleus. *B)* Log_2-fold expression changes in HVC of other miRs of interest from the microarray (shown in light colors) compared to rt-Q-PCR (dark colors). Log_2-fold changes in rt-Q-PCR correlated significantly and positively with microarray fold-changes. *C)* Log_2-fold expression changes in RA of miRs of interest from the microarray (shown in light colors) compared to rt-Q-PCR (dark colors). Expression trends are consistent between rt-Q-PCR and the microarray data in all miRs except miR-181b.

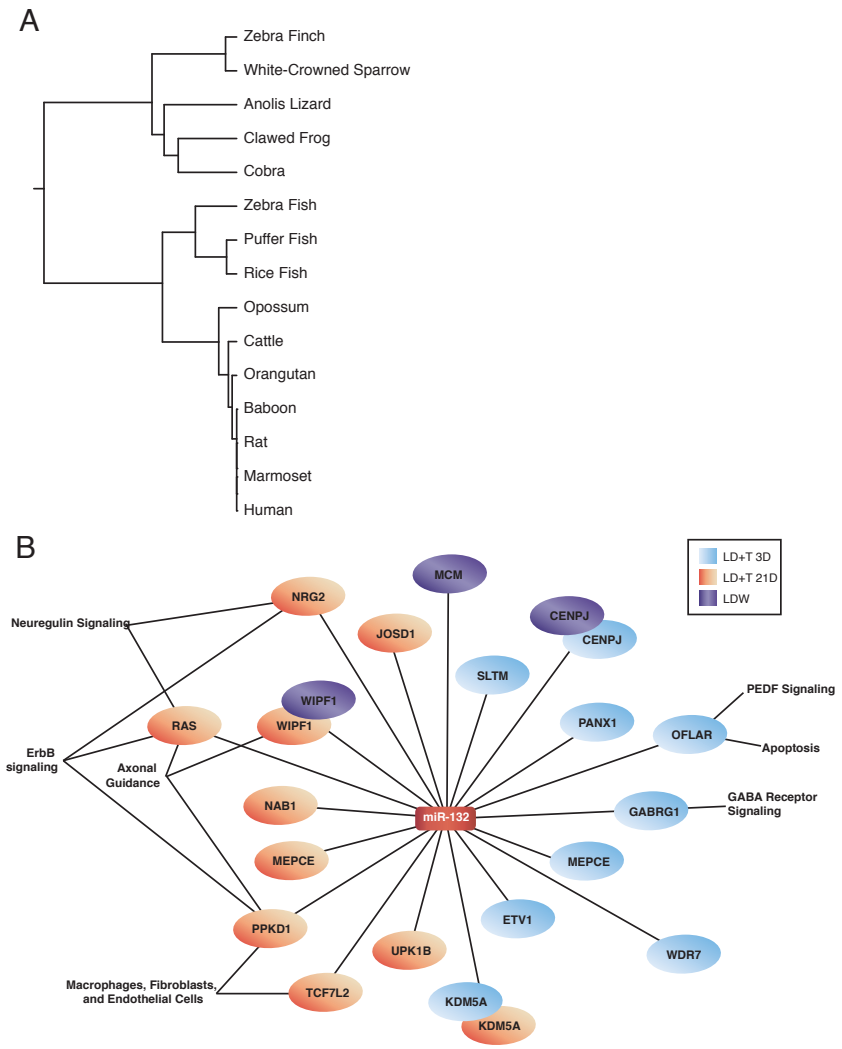

Figure 4. The seasonal miR-132 – mRNA regulatory network in HVC. *A)* Confirmation of miR-132 sequence and phylogenetic relationship based on miR-132 sequence to other closely related and distantly-related vertebrates. The full miR-132 sequence was most similar to zebra finch pre-miR-132 and more distantly related to mammalian miR-132 sequences. The mature region of all miR-132 sequences had 100% homology. *B)* An interaction network of mRNAs that were differentially anti-expressed with a $\log_2$-fold change > -1.5 and $p<0.05$ from conditions in which miR-132 was also differentially expressed (i.e. $\log_2$-fold change > +2.0). IPA network analyses revealed several key pathways were down-regulated during periods of HVC new neuronal addition and functional incorporation.

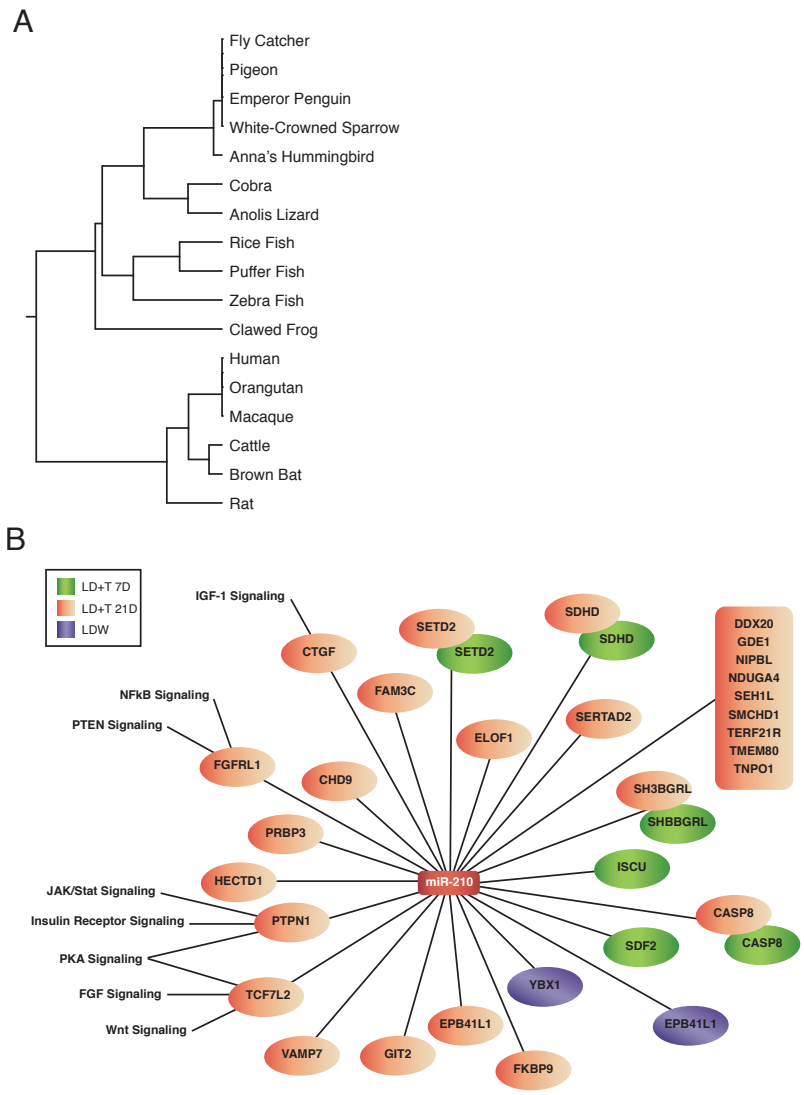

Figure 5. The seasonal miR-210 – mRNA regulatory network in HVC. *A)* Confirmation of miR-210 sequence and phylogenetic relationship of miR-210 sequence to other closely related and distantly-related vertebrates. The full miR-210 sequence was most similar to other bird's pre-miR-210 and more distantly related to mammalian miR-132 sequences. The mature region of all miR-210 sequences had 100% homology. *B)* An interaction network of mRNAs that were differentially anti-expressed with a $\log_2$-fold change > +1.5 and $p<0.05$ from conditions in which miR-210 was also differentially expressed (i.e. $\log_2$-fold change > -2.0). IPA network analyses revealed several key pathways were up-regulated in Area X during periods of volume expansion.

SI Table 1. Organisms included in phylogenetic analyses for miR-132

Common Name	Genus species	Sequence
miR-132		
Anolis Lizard	*Anolis carolinensis*	CACGGCAACCGTCTCCAGGGCAACCGTGGCTTTAGAT TGTTACTGTGCGGACGTGGGTTTGGTAACAGTCTACAG CCATGGTTGGTCCGGCGGACGCAA
Baboon	*Papio anubis*	GCGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCTCCA GTTCCCACAGTAACAATCGAAAGCCACG
Cattle	*Bos taurus*	GCGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCTCCG GTTCCCACAGTAACAATCGAAAGCCACG
Clawed Frog	*Xenopus tropicalis*	CTGTCTCCAGGGCAACCGTGGCTTTAGATTGTTACTGT AGTTCTGCATTGGTAACAGTCTACAGCCATGGTCGCTC GGGCAAGATGC
Cobra	*Ophiophagus hannah*	TCATCCTGCGTCTCCAGGGCAACCGTGGCTTTAGATTG TTACTGTGTGGATGTGGGCTGGTAACAGTCTACAGCCA TGGTTGCCCGGACT
Human	*Homo sapiens*	CCGCCCCCGCGTCTCCAGGGCAACCGTGGCTTTCGAT TGTTACTGTGGGAACTGGAGGTAACAGTCTACAGCCAT GGTCGCCCCGCAGCACGCCCACGCGC
Marmoset	*Callithrix jacchus*	GCGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCTCCA GTTCCCACAGTAACAATCGAAAGCCACG
Opossum	*Monodelphis domestica*	CCGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCCCTG GTTCCCACAGTAACAATCGAAAGCCACG
Orangutan	*Pongo pygmaeus*	GCGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCTCCA GTTCCCACAGTAACAATCGAAAGCCACG
Puffer Fish	*Fugu rubripes*	TGCCCCCTAGCGACCATGGCTGTAGACTGTTACCACTG GTGCAGTTGCTACAGTAACAATCTAATGCCACG
Rat	*Rattus norvegicus*	GAGCGTGGGCGTGCTGCGGGGCGACCATGGCTGTAG ACTGTTACCTCCG GTTCCCACAGTAACAATCGAAAGCCACG
Rice Fish (Medaka)	*Oryzias latipes*	TGCCCCCTAGCGACCATGGCTGTAGACTGTTACCAGTG GTGCTGTTGCTACAGTAACAATCTAATGCCACG
White crowned sparrow	*Zonotrichia leucophyrus gambelli*	GGCGACCGTGGCTTTAGATAGTTACTGTGCCGCTGGG GGAGTAACAGTCTACAGCCATGGTCGC
Zebra Finch	Taeniopygia guttata	ATCCGACGCCGCCCGGGCGACCGTGGCTTTAGATAGT TACTGTTCCACTGGGGGAGTAACAGTCTACAGCCATGG TCGCCGGGACAGGCAGACTCG
Zebrafish	*Danio rerio*	TGCCCCTTGGCGACCATGGCTGTAGACTGTTACCACGT GCCAACGCTACAGTAACAATCTAAAGCCACG

SI Table 2. Organisms included in phylogenetic analyses for miR-210

Common Name	Genus species	Sequence
miR-210		
Anna's Hummingbird	*Calypte anna*	CCTCCAGGAGCAGGTGAGCCACTGACTAACGCACATTGTGCTCTCGGTGACTCCACTGTGCGTGTGACAGCGGCT
Anolis Lizard	*Anolis carolinensis*	CCAGTTCTCCAGGAGCAGATGAGCCACTGACTAACGCACATTGTGCTTCTCGTGTCCCCACTGTGCGTGTGACAGCGGCTAACCTGCTTTTCGGAC
Brown Bat	*Eptesicus fuscus*	CCTCCAGGCGCAGGGCAGCCACTGCCCACCGCACACTGCGCTGCTCCGGACCCACTGTGCGTGTGACAGCGGCTGATCTGCTCCTGGGCAGCGCGACCGCCCCGGCCCCT
Cattle	*Bos taurus*	CCTCCAGGCGCAGGGCAGCCACTGCCCACCGCACACTGCGCTGCTCCGGACCCACTGTGCGTGTGACAGCGGCTGATCTGTCCCTGGGCAGCGCGACC
Clawed Frog	*Xenopus tropicalis*	TCCAGATGCAGGTCAGCCACTGACTAACGCACATTGCGCTGCTCCTAAAATGCCACTGTGCGTGTGACAGCGGCTAACCAGCATCTAGGAA
Cobra	*Ophiophagus hannah*	CAGAAGCAGGTGAGCCACTGACTAACGCACATTGTGCTGCTATGTCCCCACTGTGCGTGTGACAGCGGCTAACCTGC
Collared Fly Catcher	*Ficedula albicollis*	CCTCCAGGAGCAGGTGAGCCACTGACTAACGCACATTGTGCTCTCGGCGACTCCACTGTGCGTGTGACAGCGGCT
Emperor Penguin	*Aptenodytes forsteri*	CCTCCAGGAGCAGGTGAGCCACTGACTAACGCACATTGTGCTCTCGGCGACTCCACTGTGCGTGTGACAGCGGCT
Human	*Homo sapiens*	CCTCCAGGCGCAGGGCAGCCCCTGCCCACCGCACACTGCGCTGCCCCAGACCCACTGTGCGTGTGACAGCGGCTGATCTGTGCCTGGGCAGCGCGACCC
Orangutan	*Pongo pygmaeus*	CCTCCAGGCGCAGGGCCAGCCCCTGCCCCACCGCACACTGCGCTGCCCCCAAGACCACACGTGTGCCGTGTGACAGCGGCTGATCTGTGCCTGAGGCAGCGCGACCC
Pigeon	*Columba livia*	CCTCCAGGAGCAGGTGAGCCACTGACTAACGCACATTGTGCTCTCGGCGACTCCACTGTGCGTGTGACAGCGGCTA
Puffer Fish	*Fugu rubripes*	TCTAAAAGCAGGTGAGCCACTGACTAACGCACATTGCGCCAGTTGACAGTTCCACTGTGCGTGTGACAGCGGCTAACCTG
Rat	*Rattus norvegicus*	CCTCCAGGCTCAGGACAGCCACTGCCCACAGCACACTGCGTTGCTCCGGACCCACTGTGCGTGTGACAGCGGCTGATCTGTCCCTGGGCAGCGCGAACC
Rhesus macaque	*Macaca mulatta*	CCTCCAGGCGCAGGGCAGCCCCTGCCCACCGCACACTGCGCTGCCCCAGACCCACTGTGCGTGTGACAGCGGCTGATCTGTGCCTGGGCAGCGCGACCC
Rice Fish (Medaka)	*Oryzias latipes*	TCCGAGTTCTAAAAGCAGGTAAGCCACTGACTAACGCACATTGTGCGTGTGACAGATCCACTGTGCGTGTGACAGCGGCTAACCTG
White crowned sparrow	*Zonotrichia leucophyrus gambelli*	CCTCCAGGAGCAGGTGAGCCACTGACTAACGCACATTGTGCTCTCGGCGACTCCACTGTGCGTGTGACAGCGGCT
Zebrafish	*Danio rerio*	GCAGGTAAGCCACTGACTAACGCACATTGCGCCTATTCTCCACTCCACTGTGCGTGTGACAGCGGCTAACCAG

Chapter 6. A discussion of the dissertation contribution and function of adult neurogenesis[3]

6.1 Dissertation contributions to the field of neurogenesis

6.1.1 Seasonality

The number of new neurons that functionally incorporate and survive in HVC changes dramatically depending on seasonal condition (i.e, breeding vs. nonbreeding; Figure 1). Plasma T levels are high throughout the breeding season and decrease to basal levels at the end of breeding season [1]. Underlying the seasonal changes in song behavior and T levels, are changes in morphology, electrophysiology, and gene expression of the song nuclei (reviewed by [2, 3]). The volumes of HVC, RA, X, and nXIIts increase by up to 200% during the breeding season [4]. The increase in HVC volume is primarily driven by an increase in the incorporation of new neurons; neuron number in HVC increases by nearly 25% (around 60,000 neurons) each spring leading into the breeding season [5]. At the end of each breeding season, circulating T levels decrease, prompting the death of mature HVC neurons by caspase-mediated apoptosis [6, 7].

Although neuronal incorporation in HVC is low in nonbreeding conditions, neural stem cell proliferation and the migration of new neurons to HVC does not cease entirely. For example, when two cohorts of new neurons are labeled in nonbreeding conditions by injections of BrdU and EdU at a four to sixteen week interval, more than 50% of the adult-born neurons present four weeks following the final injection belong to the younger 4 week old cohort (Larson et al., unpublished observation; Chapter 2). The absence of the trophic support that permits the incorporation of new HVC neurons in breeding conditions likely prevents new HVC neurons from persisting in the song control circuit in nonbreeding conditions. Conversely, when two cohorts of new neurons are labeled at a four to eight week interval in breeding conditions, more than 50% of the adult-born neurons present four weeks following the final injection belong to the initially-labeled cohort (see Chapter 2). These results suggest that the majority of new neurons incorporated in HVC during nonbreeding conditions are transient and replaced by younger neurons, whereas the initial cohort of new neurons incorporating into HVC during the onset of breeding condition survive long-term. We found, however, that number of four-week old neurons

[3] Excerpted and modified from: Brenowitz EA & Larson TA. Neurogenesis in the Adult Avian Song Control System. “Adult Neurogenesis.” 2nd ed. Gage, Kemperman, and Song. Cold Spring Harbor Press. To be published 2015

is similar in both breeding and nonbreeding conditions. All together, these data demonstrate that breeding conditions promote the survival of an initial population of new neurons entering HVC, but that once HVC attains its fully grown or regresses state, neuronal turnover also reaches a steady-state that is not dependent on T or other factors differentially regulated between breeding and nonbreeding conditions.

6.1.2 Behavioral Use and Neural activity

The survival of adult-born neurons is influenced by the neural activity within the circuit to which these new neurons are added [8]. For example, singing behavior increases neuronal survival in HVC via increased BDNF mRNA and protein expression in the population of HVC_{RA} neurons to which new neurons are added [9]. The number of new neurons added to the adult HVC correlates with individual differences in the average amount of song produced [10] and breeding condition [7, 11]. In Gambel's white-crowned sparrows, transition into breeding condition drives both an increase in HVC neuronal number and an increase in song duration, completeness, and stereotypy [4, 11]. Conversely, in Larson et. al. (2014) we found that transition into nonbreeding condition drives neuronal death in HVC and a decrease in song production rate and similarity [7].

The addition of HVC_{RA} projections is also directly influenced by the electrical activity of target neurons in RA. The spontaneous activity of RA neurons is high in breeding sparrows when neuronal addition to HVC is high, and low in non-breeding birds when neuronal addition is low [12, 13]. In Larson et. al. (2013) we found that inhibiting activity in breeding-condition birds by infusing RA with the $GABA_A$ receptor agonist muscimol decreased the number of new neurons in HVC of adult white-crowned sparrows by 56% [14]. Increasing RA activity in non-breeding condition birds by infusing KCl also increased the number of new HVC neurons by 93% (Figure 2; Larson, Wang, and Brenowitz, unpublished observations). These results suggest that neural activity at the target of new projection neurons is necessary and sufficient for new neuronal survival. These results are consistent with the observation that activity is required for the survival of new born interneurons in the mammalian olfactory bulb [15, 16] and dentate gyrus [17]. The precise mechanisms through which neural activity influences neuronal addition to HVC remains unclear, and so is a question requiring further exploration.

6.1.3 Cell Death and Inflammation

The addition of new neurons to the vertebrate brain is linked with the death of mature neurons. Broadly across taxa, neuronal addition increases after *artificially* inducing cell death [18 - 20]. For example, laser photo-ablation of both HVC_X and HVC_{RA} projection neurons in adult

male zebra finches increases the addition of new HVC_{RA} neurons, but not of new HVC_X neurons [21]. We expanded on the descriptions of this general phenomenon, by demonstrating in Larson et. al. (2014) that neuronal birth in response to cell death occurs with *natural* neural degradation in the HVC of white-crowned sparrows. Seasonally occurring cell death had previously been reported to correlate with HVC neuronal addition in canary [22] and to be necessary for neuronal addition in Gambel's white-crowned sparrows [23]. None of these previous studies, however, examined the effects of cell death on the proliferation of new cells. In Larson et. al (2014) we found that cell death in HVC was necessary for proliferation of neural stem cells. Thus, the changes in neuronal addition to HVC observed in these previous studies is likely mediated specifically by changes in vVZ proliferation.

The direct demonstration that vVZ proliferation is affected by altering levels of cell death provides a focal point for investigating and uncovering the mechanisms guiding proliferation of neural stem cells and neuronal addition in the avian brain. One mechanism that could mediate interaction between cell death in HVC and vVZ proliferation may be neuroinflammation and the activation of an innate immune response. Neuroinflammatory cells including microglia, regenerative astrocytes, and leukocytes recently emerged as factors that regulate neurogenesis (reviewed in [24]). After neuronal death, microglia and leukocytes migrate to the site of injury to remove cell debris, whereupon they release pro-inflammatory cytokines to recruit more immune cells to the site of injury (reviewed in [25]). Thus, the innate and adaptive neuroinflammatory responses may mediate the clean-up of apoptotic neurons during HVC regression in white-crowned sparrows and may promote an increase in neural stem cell proliferation. We tested the necessity of activation of microglia and inflammation to regulate vVZ neural stem cell proliferation by inhibiting inflammation during rapid regression of HVC with the anti-inflammatory drug minocycline (see SI.1, SI.1 Table 2, SI.1 Figure 3). We found an oral dose of minocylcine significantly reduced the increase in proliferation associated with natural neuronal loss in HVC. We also tested the sufficiency of local inflammation to promote an increase in neural stem cell proliferation by injecting the inflammatory-causing agent, lipopolysaccharide (LPS), into HVC in both breeding (LD+T 28D) and nonbreeding (SD >10 wk) condition birds (see SI.1, SI.1 Table 1, SI.1 Figure 1). We found regardless of condition (i.e. LD+T or SD) LPS and it's resulting inflammation caused a significant increase in vVZ proliferation. Together these results demonstrate that inflammation is necessary and sufficient to promote an increase in neural stem cell proliferation in the absence of cell death, and that this increase in neural stem cell

proliferation is independent of breeding condition and the factors associated with breeding condition (e.g. high T and BDNF levels).

6.1.4 Age

Age-related decline in neurogenesis occurs broadly across taxa [18; 26-28]. In avians, mature neuronal replacement by new neurons begins in the juvenile canary as early as four months after hatching [29] and decreases steadily with age across avian species [30-32]. In white-crowned sparrows, we too found a significant decrease in neural stem cell proliferation with age [7]. Unique to our study, we found that: 1) HVC cell death and proliferation both decrease with age in baseline breeding and nonbreeding conditions, suggesting that turnover under steady state conditions decreases with age; 2) the amount of cell death during regression and total neuronal loss (ie. total neuronal counts at end of breeding season compared to end of nonbreeding season) do not change with age, suggesting that all birds regardless of age have the same amount of neuronal loss in HVC during regression; and, 3) vVZ proliferation decreases with age during active regression (i.e. when cell death does not change with age). Together these results suggest that it likely takes older birds longer to replace the neurons lost during regression [7]. Consistent with previous reports of low levels of neural stem cell proliferation and new neuronal incorporation in aged birds [33], we found that across all studies neural stem cell proliferation or neuronal addition to HVC never ceased entirely in any white-crowned sparrow regardless of age or condition.

6.1.5 Genomics

The ability of a neuron to mature, functionally incorporate into a neural circuit, interact with other cells, and undergo apoptosis requires precise coordination of genes expression. MicroRNAs (miRs), short non-coding RNA sequences, have many mRNA targets and thus can act as global post-transcriptional regulators of complex temporal and spatial patterns of gene or protein expression changes underlying neural plasticity. We tested the hypothesis that miRs related adult neurogenesis, plasticity, and apoptosis are expressed and seasonally regulated in HVC, RA, and Area X neurons. We found several miRs are indeed differentially expressed across seasons in all three nuclei. Most intriguing, the top two most significantly regulated miRs, miR-132 and miR-210, are known to regulate the integration of adult-born neurons in dentate gyrus [34], the arborization of new neurons in the hippocampus [35], and neural repair through angiogenesis [36-38]. Analyses of our microarray data identified predicted mRNA targets in the neurogenic song nuclei HVC and Area X that are seasonally regulated and predicted to regulate various signaling networks. Some predicted networks of interest include cell cycle control, PTEN

signaling, calcium signaling, neuregulin signaling, and retinoic acid signaling in HVC and serotonin GABA, and dopamine receptor signaling, calcium transport, and lymphocyte signaling in Area X. Our descriptions of the dynamic regulation of mRNAs related to neurogenesis and neural plasticity by miRs have begun to identify the complex regulatory networks of adult neurogenesis and plasticity, and provides opportunities to discover novel genetic and epigenetic control over adult neurogenesis.

6.2 Function of adult neurogenesis with suggested future directions

Does the replacement of mature neurons with newborn neurons (i.e., neuronal turnover) in adult avian brains serve an adaptive function, and if so, what is it? As discussed below, various functional hypotheses can be proposed [8, 39-41]. These hypotheses are not necessarily mutually exclusive, and neurogenesis may serve different functions in different taxa and different brain regions. A constraint on discussion of possible function(s) of adult neurogenesis is that no studies that experimentally manipulate levels of neurogenesis in avian brain regions have yet been successfully conducted, and existing tests of hypotheses are therefore largely limited to correlational observations. Correlations typically have weak explanatory power by their nature. It is easy to confound correlation and causation, and difficult to determine the direction of causal relationships. As a consequence, no single hypothesis has yet received unambiguous support [8]. Any evaluation of different functional hypotheses is limited by these considerations. There is an urgent need to develop pharmacological or genetic methods of manipulating neuronal addition to adult brain regions to allow experimental tests of functional hypotheses.

6.2.1 Neuronal addition is vestigial

The null hypothesis is that adult neurogenesis has no function in the avian brain. From this perspective, neurogenesis can be viewed as a vestige of developmental plasticity [39]. The observation that levels of neurogenesis seen in the adult brain is much lower than that seen in developing brains is consistent with this hypothesis. If developmental neurogenesis persists into adulthood, however, we might expect to observe uniformly low levels of new neurons present throughout the adult brain. Inconsistent with this logic, however, neurogenesis is widespread throughout the adult telencephalon, but is conspicuously absent from specific forebrain regions such as the song nuclei RA and LMAN. Furthermore, there is little adult neurogenesis outside the telencephalon [42]. These observations suggest that neurogenesis can be selectively

suppressed or absent in adult brains and, therefore, that the presence of neurogenesis is likely the consequence of active maintenance rather than passive persistence.

6.2.2 Neuronal turnover allows plasticity while keeping brain size small

Ongoing neuronal turnover in adult brains may represent a compromise between the need to continue to form and store new memories throughout the relatively long lifespans of songbirds (3 - 10+ years), and the pronounced size and energetic constraints on brain size imposed by the demands of flight [43]. Replacing mature neurons with more plastic new neurons may be an avian alternative to the encephalization widely observed among mammals, especially those with enhanced cognition or social organization [44, 45]. If individual neurons are viewed as the structural units of memory storage (e.g., [46]), then neuronal turnover may allow a bird to continually encode new information while keeping absolute brain size relatively small [41, 47]. As is often true of evolutionary scenarios, a definitive test of this hypothesis would be challenging to conduct.

6.2.3 Neuronal turnover enables adult birds to learn to produce new songs

The first demonstration of adult neurogenesis in songbirds was in the canary, a species that develops new motor patterns of song as an adult [48]. Nottebohm [49] hypothesized that the addition of new neurons to HVC provides plasticity for encoding the memory of new song programs. Consistent with this model, neurons are added to HVC of adult canaries at a higher rate in the fall, when changes to song structure are most pronounced, than in the spring breeding season, when song structure is stable [22]. Further support for this model comes from the observation that new neurons are added to HVC of juvenile zebra finches at a high level while they are actively learning song, and at a lower level when song learning is completed [50, 51]. This decrease in neuronal addition can be delayed by isolating juvenile birds from adult male song tutors, which extends the sensitive period for song learning [52].

Other observations, however, are inconsistent with this hypothesis. Even though the level of neuronal addition to HVC declines in zebra finches when song learning is completed, it does continue into adulthood after the birds have completed song learning. Like zebra finches, male song sparrows *(Melospiza melodia)* and white-crowned sparrows complete song learning in the first year of life, but continue to add new neurons to HVC and area X as adults [4, 53]. As in canaries, wild song sparrows show higher levels of neuronal addition to HVC in the fall than in the spring, even though they retain the same songs throughout adulthood [54]. The songs of both song sparrows and white-crowned sparrows, however, become variable in structure in the fall, as do those of canaries [5, 11, 55]. The loss of mature neurons which encode song, and

addition of many new neurons that have not yet been "programmed" to produce the previously learned song, may contribute to the increased variability of song that sparrows sing during the fall. Together these observations suggest that while neuronal turnover may be necessary for adult learning of new motor patterns of song, it is not sufficient to produce (or continue to allow) such learning.

6.2.4 Neuronal turnover enables adult birds to learn to recognize new songs

Alternatively, neuronal addition may be a mechanism for acquiring new perceptual memories of song. New neurons are added to the auditory region NCM [10, 56], as well as HVC and area X, all of which are necessary for proper auditory discrimination [57-59]. Both male and female birds learn to recognize the songs of individuals with whom they frequently interact; males can learn the individually distinctive songs of their immediate territorial neighbors (e.g., [60]) and females can recognize the songs of their mates (e.g., [61]). As birds may have a new neighbor or mate each year, the ability to update auditory song memories is advantageous. Neuronal turnover in auditory-responsive regions such as HVC, area X, and NCM may provide a cellular mechanism for this perceptual plasticity [51]. Support for this hypothesis comes from the observation that neuron number in HVC of juvenile swamp sparrows (*Melospiza georgiana*) increases more during the early sensory phase of song learning, when adult song is heard and memorized, than during the later sensorimotor phase, when a juvenile begins to sing [62]. If auditory experience influences neuronal addition, then deafening birds should disrupt this process. A decrease in neuronal addition to HVC was observed upon deafening in adult [63] but not juvenile [51] zebra finches. The disparity between these deafening studies is difficult to explain. A direct test of the hypothesis that neuronal addition enables perceptual learning has not yet been performed. Observation that a bird's ability to form new auditory memories is impaired when neuronal addition is experimentally suppressed in adults would support this hypothesis.

6.2.5 Neuronal turnover is necessary for song maintenance

Maintaining previously learned song in adults is an ongoing process and the addition of new neurons to HVC and area X may provide cellular plasticity for continual updating of motor programs for song [8, 39]. Disrupting auditory feedback can lead to degraded song structure in adult birds. Song begins to deteriorate within one week of deafening in Bengalese finches *(Lonchura striata domestica)*, and after 2-8 weeks in zebra finches, depending upon their age [64-66]. Exposing adult zebra finches to delayed auditory feedback similarly leads to song deterioration after several weeks [67]. Masking selected Bengalese finch song frequencies or

disrupting auditory feedback in real time results in rapid changes in song structure [68, 69]. These observations indicate that birds need to hear themselves sing in order to maintain stereotyped song production as adults. Adult birds may compare ongoing auditory feedback from their song with a previously memorized sensory model of song (i.e., a "song template"). Alternatively, the song control circuitry may generate an instructive signal that enables birds to adjust song as necessary to match a learned song production program [70].

An essential component of error correction models is that there is variability in song structure that can be exploited to allow birds to adjust song structure to more closely match a sensory template or learned motor program. The addition of many new HVC neurons that have not been programmed to produce a previously learned song may increase the variability of song and thus provide the raw material for error correction. The process of adjusting variable song to match a learned model may program new neurons to produce the appropriate pre-motor pattern. This function of adult neuronal turnover could be adaptive for the long-term maintenance of song in adults. A prediction of this hypothesis is that suppression of neuronal addition to HVC should result in a short-term decrease in song variability but a long-term progressive deviation of song structure away from the stereotyped version produced at the completion of juvenile song learning.

Alvarez-Borda and Nottebohm (2002) compared neuronal recruitment to HVC in gonadally intact and castrated male canaries that produced comparable amounts of song in the fall, when song becomes more variable. When matched for the amount of singing, the intact birds had about 4 times as many new RA-projecting neurons, and about 2.6 times as many total new HVC neurons as did the castrated birds. The authors observed no difference between the intact and castrated birds in either the diversity of song syllables or song stereotypy, however, despite the pronounced differences in the addition of new neurons to HVC. These results do not support the hypothesis, though the manipulation of circulating testosterone levels is a considerable potential confound. A test of the hypothesis involving a more direct manipulation of neuronal addition to HVC is warranted.

6.2.6 Neuronal turnover enables adult birds to replace over-excited neurons

Excessive activation of glutamate receptors by excitatory neurotransmission can result in cell death due to various factors including disrupted intracellular calcium homeostasis, impaired organelle function, increased production of nitric oxide and free radicals, prolonged activation of proteases and kinases, and increased expression of pro-apoptotic transcription factors [71].

Neuronal addition to adult HVC may be an adaptation for replacing cells that are damaged by excitotoxicity and high metabolic demand resulting from prolonged activity [39].

During the breeding season male birds can sing at extremely high rates and do so for several months consecutively. A territorial male red-eyed vireo (*Vireo olivaceus*), for example, may sing more than 20,000 times a day (www.birds.cornell.edu). Individual HVC_{RA} neurons fire at high rates in temporally sparse bursts of activity during singing [72, 73], and these bursts can propagate through the HVC_{RA} neuronal population via local excitatory synapses [74]. HVC_{RA} neurons stimulate excitatory post-synaptic potentials (EPSPs) via ionotropic glutamate receptors [74]. Excitatory synaptic transmission between HVC neurons, and between HVC and RA neurons, also involves the activation of both AMPA/kainate and NMDA-type glutamate receptors [75]. Given the extraordinarly high rate of song production, neuronal activation during the months-long breeding season should also be high. Supporting this assumption, the activity of cytochrome oxidase, an enzyme important in cellular oxidative metabolism, is higher in neurons of HVC, area X, and RA in breeding birds than nonbreeding birds, indicating that the metabolic demands of these neurons increase when birds sing at high rates [76].

This hypothesis is further supported by several other observations. About 50% of the HVC_{RA} neurons in adult male Gambel's white-crowned sparrows die rapidly when testosterone levels decrease at the end of the breeding season [6, 7, 77]. This burst of neuronal death coincides with a reduction in trophic support provided both by testosterone and its downstream effectors. HVC_{RA} neurons and HVC interneurons that have been highly active during the preceding months may be especially prone to apoptosis from the cumulative detrimental effects of excitotoxicity and metabolic challenge in the absence of trophic support.

Neuronal addition to HVC may be essential for reconstructing the motor circuit for song production by the start of the next breeding season, when males must once again sing stereotyped song to attract mates and defend territories. Most of the new neurons added to adult HVC after the breeding season project to RA, and these neurons reconstruct the HVC to RA circuit ([78, 79]; Larson et al. unpublished observation). As discussed above, there may be a direct functional linkage between the death of mature HVC neurons and increased neuronal proliferation and addition to HVC.

Other observations, however, do not support this hypothesis. Area X neurons also show high spontaneous and song-related activity, and receive glutamatergic inputs [80], but the rate of incorporation does not differ seasonally [81]. The neurons of RA, a non-neurogenic region of the brain, also show high levels of activity, both spontaneous and evoked, during the

breeding season[13, 72] and contain both AMPA/kainate and NMDA-type glutamate receptors [82]. Motor neurons in $nXII_{ts}$ that innervate the muscles of the vocal organ are presumably driven at a high rate during the months of frequent song production in the breeding season, and contain NMDA receptors [83], but this nucleus does not add neurons in adults. Together these observations suggest that the anatomical distribution of neuronal addition in adult brains is not related to either the presence of ionotropic glutamatergic receptors or metabolic demand in an obvious manner [84].

A prediction of this hypothesis is that the death of mature neurons, and the addition of new neurons, would be reduced by protecting HVC neurons from glutamate-mediated excitotoxicity. Thus, this hypothesis could be tested by manipulations that protect HVC from metabolic damage, such as infusing HVC with exogenous acetoacetate, an energy substrate [85], or overexpression of endogenous Sirt 3, a nicotinamide adenine nucleotide-dependent deacetylase [86], with a concomitant reduction in HVC neuronal death.

6.2.7 Adult born neurons replace mature neurons weakened by activity-related DNA damage

Neuronal activity, even at moderate levels such as those involved in exploratory activity of mice, can result in DNA double-strand breaks (DSBs) in neuronal genomes [87]. Activity-related DSBs are exacerbated in J20 mice that express human amyloid-β precursor protein, and blocking extrasynaptic NMDA-type glutamate receptors containing the NR2B subunit prevents amyloid-β DSBs in neuronal cultures [87]. Amyloid-β precursor protein is present in HVC neurons, and is expressed at higher levels in breeding-condition birds (Thompson, Meitzen, and Brenowitz, unpublished observations). As discussed above, birds sing at very high rates over the months of the breeding season and activity in HVC neurons is expected to be high during this period. Excitatory synaptic transmission between HVC neurons involves the activation of NMDA-type glutamate receptors [75], which contain the NR2B subunit [88, 89]. Together these observations raise the possibility that HVC neurons may experience high levels of DNA DSBs. Prolonged and recurrent DNA damage may make these neurons more susceptible to cell death when the trophic support provided by T and its downstream effectors is withdrawn at the end of the breeding season. Neuronal addition to adult HVC may be an adaptation to replace mature neurons that have experienced high levels of DNA damage during the prolonged period of high song-related activity in breeding birds. As with the activity-based hypothesis, this hypothesis predicts that there should be a relationship between song production rate, DNA DSBs in HVC neurons, and neuronal turnover in HVC.

6.2.8 Neuronal addition to HVC is an example of performance-associated hypertrophy

The sustained peak performance of a seasonally predictable behavioral or physiological task is often preceded by growth of the organs and/or tissues involved in performance of the task [90]. For example, the size of the gonads and other reproductive structures increases dramatically in preparation for the annual breeding season, and these organs regress when the breeding season is terminated. Preparatory changes such as these are stimulated by seasonal environmental cues and mediated by neural and endocrine signaling mechanisms. The maintenance of fully grown organ systems and tissues is energetically expensive and these systems therefore regress when peak performance is not required [91].

In applying the principle of performance-associated hypertrophy to seasonal cycles of neuronal turnover in HVC, one can make the following predictions:

1) Song performance should be maximal during the breeding season. This prediction is supported by data from canaries, white-crowned sparrows, song sparrows among many other species. As described above, males of these species sing more stereotyped songs, and sing more frequently, during the breeding season.
2) Growth of the song nuclei should precede behavioral changes. In the study of Tramontin et al. (2000), the increase in neuron number in HVC observed in male white-crowned sparrows in breeding condition occurred within seven days of exposure to a long day photoperiod and high T, while song stereotypy increased between seven and twenty days [4].
3) Energetic costs of maintaining a fully developed song system throughout the non-breeding season outweigh those associated with regrowing the song system each spring. The relative metabolic costs of maintaining and regrowing the song system each year are not yet known. The activity of cytochrome oxidase, an enzymatic marker of cellular metabolic capacity, increases considerably in HVC, RA, and area X of breeding-condition white-crowned sparrows [76]. This result suggests that the song system does impose a greater metabolic cost in its fully grown state than when regressed. Thus, changes in neuronal turnover in HVC, and plasticity of the song system in general, may be an adaptation to reduce the energetic costs imposed by the song system in the fall and winter. Outside the breeding season, males do not need to produce frequent, stereotyped song for mate attraction or territorial defense. In the fall and winter birds may experience the energetic stress of migration, increased thermoregulatory demands, and decreased food availability. Songbirds are relatively small animals with large surface area to volume ratios, and are therefore particularly subject to energetic constraints [43]. Given that the brain requires large amounts of energy to maintain

signaling activities (see [92]), regression of the song system outside the breeding season reduces the energetic costs imposed by the song nuclei. On balance, the reduced energy required by a regressed song system throughout the fall and winter may outweigh the energy required to regrow it the following spring.

6.2.9 Neuronal turnover in HVC occurs when trophic support is withdrawn

Most of the preceding hypotheses posit that neuronal turnover in HVC is functionally related to seasonal changes in motor or sensory aspects of song behavior. Plasticity in HVC may not be directly related to song, however. It may occur as a passive consequence of the sensitivity of HVC neurons to testosterone, its steroid metabolites, downstream trophic factors such as BDNF, and signaling cascades that regulate phosphorylation of proteins related to cell proliferation, growth, and survival ([93-97] and Brenowitz et. al., unpublished observations).

Increased circulating testosterone early in the breeding season initiates a trophic cascade that supports the survival of new neurons added to HVC [98, 99]. As HVC grows, it provides trans-synaptic trophic support to neurons in its efferent targets RA and area X [3, 12, 100, 101]. At the end of the breeding season, circulating T decreases to basal levels and the trophic cascade is terminated. As trophic support is withdrawn, both mature and new HVC neurons undergo apoptosis [6, 7, 23, 102]. As neurons are lost from the HVC→RA projection, song becomes shorter and more variable in structure. However, as noted above, birds do not need to produce stereotyped song to attract mates or defend territories during the fall and winter, and there is consequently no ecological cost to degradation of either the song circuits or song behavior. New neurons enter HVC in non-breeding birds, but most die within weeks as long as plasma T levels remain low ([7] and Larson et al., unpublished observations).

In this model, changes in song behavior are a passive consequence of, rather than the driving force behind, plasticity of the song control circuits. This model clarifies several inconsistencies with the above models:

1) It suggests that neuronal turnover in HVC could occur regardless of whether or not adult birds develop new songs. Seasonal patterns of neuronal addition to adult HVC are observed both in species that do and do not learn new songs.
2) This hypothesis permits seasonal patterns of neuronal turnover occurring only in select regions such HVC. HVC is immediately adjacent to the proliferative ventricular zone, which provides direct access to a pool of new cells that can be recruited into functional circuits in HVC. The addition of new neurons to HVC could thus be a passive process based on the

proximity of HVC to the neural progenitor pools and the presence of supporting factors such as VEGF and BDNF within HVC.

3) This model suggests that neuronal turnover could occur independently from neural activity. As discussed above, neurons in the song nuclei RA and $nXII_{ts}$ show high levels of activity when song is produced frequently during the breeding season, but new neurons are not added to these regions in adult birds.
4) This model also suggests that neuronal turnover is not just a consequence of cell death from excitotoxicity. HVC, RA, area X, and $nXII_{ts}$ all contain neurons with glutamate receptors, but adult-born neurons are only added to HVC and area X, and neuronal addition is only seasonal in HVC. To the extent that this model resolves some of the observations that are inconsistent with the other hypotheses presented above, it may provide the most parsimonious explanation of neuronal turnover in the adult HVC.

6.2.10 Neuronal turnover in the hippocampus enables adult birds to form new spatial memories

The hippocampal formation (HF) in birds is thought to play an important role in the formation of spatial memories in contexts including food hoarding, navigation, and migration (see [8] for review). Neuronal turnover in the adult HF may provide plasticity for encoding new spatial memories. New neurons added to the HF at different times may allow anatomical separation of memories formed at the different times (i.e., pattern separation [103]). Hoshooley and Sherry measured food caching behavior and neuronal addition to the HF in captive black-capped chickadees at four times of year [104]. They found that food caching was greatest in October, and that neuronal recruitment to HF one week after BrdU injection reached a peak in January. Using a six week post-BrdU survival time, Barnea and Nottebohm found neuronal addition to HF to be greatest in semi-captive black-capped chickadees in October [105]. Providing juvenile marsh tits the opportunity to cache food increased cell proliferation in the ventricular zone of the HF, compared with matched-age tits that were not allowed to cache food [106]. Captive adult mountain chickadees allowed to store food had more new doublecortin-expressing (DCX+) neurons in HF than birds deprived of this experience; both captive groups, however, had lower numbers of DCX+ neurons in HF than did wild chickadees [107]. Together these studies suggest that neuronal addition to HF varies seasonally, roughly correlates with the time of greatest food caching and retrieval, and is influenced by experience in storing food.

Birds in many taxonomic groups are able to navigate over long distances. In some species, such as geese, juveniles learn the migratory route from their parents. Some birds migrate to the same destination with great accuracy, as in males that return to the same territory

site year after year. Other birds such as homing pigeons are able to navigate over hundreds of miles to return to a home site. Successful navigation requires that birds learn the location of their home site with reference to various cues such as geographic landmarks, magnetic fields, polarization of sunlight, and olfactory cues (reviewed in [108, 109]). Hippocampal-dependent spatial learning is essential for such long-distance migration and navigation. Lesion of the HF in homing pigeons disrupts navigation based on landmarks and the formation of spatial maps [108, 109]. The role of the HF in migration and navigation raises the hypothesis that neuronal addition provides plasticity for encoding spatial memories necessary for long-distance orientation. Migration may take several weeks and ongoing neuronal addition may enable pattern separation of the different spatial memories that must be stored during the successive stages of these journeys. LaDage et al. reported that there are more DCX+ adult-born neurons in the HC of a migratory subspecies of white-crowned sparrow than a non-migratory subspecies [110]. A potential confound of this study, however, is that migratory birds experience intense and prolonged physical exercise, which could influence one or more components of neuronal addition to the HF [111].

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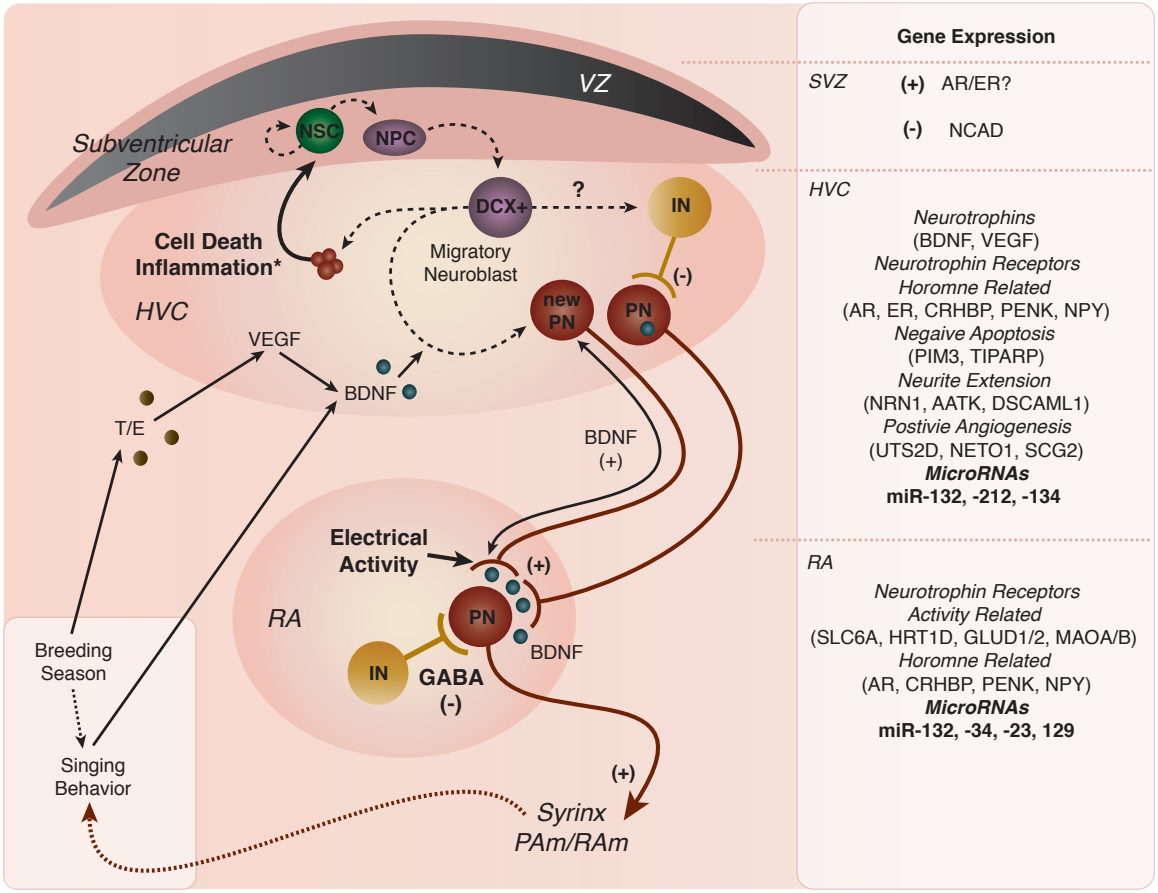

Figure 1. A schematic of the factors influencing avian adult neurogenesis in HVC from the findings of my dissertation. The solid black lines indicate factors that positively influence the given cell, factor, or process to which it points. The dashed lines represent changes in cell process indicated (e.g., cell divisions and migration). The dotted lines represent indirect routes of influence (i.e. through other genes, factors, etc.). The break-out panel to the right side summarizes patterns of gene expression in different brain regions that positively influence component processes of neurogenesis. In the VZ, the (+) indicates genes that promote proliferation, whereas the (-) indicates genes the promote exit from the cell cycle. The asterisk indicates unpublished research included in the supplemental information of this dissertation. Abbreviations: NSC, neural stem cell; NPC, neural progenitor cell; DCX+, doublecortin positive migratory neuroblast; IN, interneuron; PN, projection neuron.

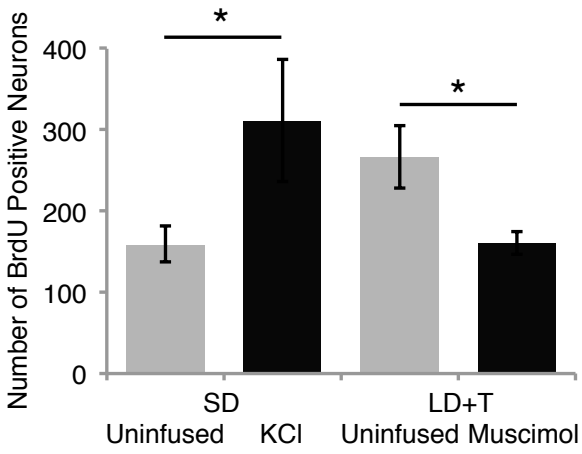

Figure 2. The effect of trans-synaptic activity on adult neurogenesis in HVC. KCl infusion near RA increased RA spontaneous neural activity and increased the addition of new neurons to HVC in nonbreeding-like conditions (contralateral to KCl infusion, 160 ± 23; ipsilateral to KCl infusion, 312 ± 75; p=0.0309). Muscimol infusion near RA increased RA spontaneous activity and decreased the addition of new neurons to HVC (Larson, et. al. 2013). Asterisks indicate $p<0.05$ in post-hoc *t*-test following two-way ANOVA ($F_{3,20}=3.0121$, p=0.0073).

SI.1 The role of inflammation in regulating natural reactive neurogenesis

SI.1.1 Background

Reactive neurogenesis, or the addition of new neurons to the vertebrate brain following the death of mature neurons, occurs broadly across taxa [1 - 3]. Prior to discovering that reactive neurogenesis occurs with the natural seasonal regression of the song control circuit in the songbird Gambel's white-crowned sparrow [4], reactive neurogenesis appeared to be limited to injury-induced neuronal loss. In Larson et. al. (2014) we found not only that neuronal birth in response to cell death occurs with natural neural degradation in HVC, but also that cell death is necessary for the increase in vVZ neural stem cell proliferation following rapid regression of HVC [4]. These results demonstrated that neuronal death could directly promote neural stem cell proliferation. We sought to identify the mechanism through which the dying HVC neurons prompt an increase in neural stem cell proliferation.

One mechanism that mediates reactive neurogenesis following injury-induced neuronal loss is neuroinflammation and the activation of an innate immune response [5]. After neuronal death, neuroinflammatory cells including microglia, regenerative astrocytes, and leukocytes migrate to the site of injury to remove cell debris, whereupon they release pro-inflammatory cytokines to recruit more immune cells to the site of injury (reviewed in [6]). In mammals this type of neuroinflammation typically forms a glial scar that hinders incorporation of new neurons into the injury site, while in zebrafish and reptiles neuroinflammation can promote a regenerative response [1, 7 - 9]. We tested whether the local neuroinflammatory responses mediate the clean-up of apoptotic neurons during HVC regression in white-crowned sparrows and promote an increase in vVZ neural stem cell proliferation.

SI.1.2 Material and methods

SI.1.2.1 Animals

Forty-seven adult male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*; white-crowned sparrows) were collected in eastern Washington during their pre- or post-breeding season migration. All birds were housed in indoor group aviaries exposed to short day photoperiods (SD; 8 hr light, 16 hr dark) for at least 10 weeks to ensure that the song and reproductive systems had regressed and would be sensitive to the stimulatory effects of transfer to long day photoperiods (LD) and testosterone (T) implant. All experiments followed

NIH animal use guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee.

SI.1.2.2 Experimental Procedures

To determine whether neuroinflammation promotes vVZ proliferation independent of HVC cell death, we used the lipopolysaccharides (LPS) of the outer membrane of Gram-negative bacteria to promote local inflammation [10]. To test whether breeding condition altered the neuroinflammatory response, we housed one group of birds in SD (SI Figure 2A; n=11) and another group of birds in long day photoperiods (SI Figure 2AI; LD; 20 hr light, 4 hr dark) with a subcutaneous implant of a 12-mm Silastic capsule (1.47 mm inner diameter, 1.96 mm outer diameter) filled with crystalline T (Sigma) above the scapula (LD+T, n=18). On the final day in SD or LD+T, birds were anesthetized with isoflurane (2%). We stereotaxically lowered a needle into HVC 0.7 mm ventral, 2.3 mm lateral to the intersection of the midsagittal and transverse sinuses. Fifteen birds had LPS (1 µg in 6 nL of phosphate buffered saline (PBS); SD n=6, LD+T n=9) pressured injected into HVC. To ensure that the needle tracks were within HVC, four birds were injected with LPS Alexa Fluor 568 Conjugate (Life Technologies, 1µg in 6 nL of 15% DMSO in PBS). Histological analysis demonstrated that the needle track were within HVC of all four birds. Experimental control birds had the needle lowered into HVC with no injection of fluid (SD n=5, LD+T n=9). We chose to perform control sham injections rather than vehicle injections because saline alone can promote an inflammatory response [11]. One hour following surgery and two hours prior to tissue collection, all birds were injected intraperitoneally with one dose of BrdU (50 mg/kg; 15 mg/ml in 15% DMSO in PBS) to label proliferating stem cells and their progeny in the VZ.

To determine whether neuroinflammation was necessary for the previously reported increase in vVZ proliferation that occurs as birds are transitioned from breeding to nonbreeding condition [4], we blocked microglia activation and neuroinflammation with an oral anti-inflammatory drug during the rapid regression of HVC. We housed all birds in LD for 28 days after T implantation to allow full breeding-like growth of the song control nuclei [12] (SI Figure 3A). All birds had T pellets removed after 28D of LD+T and were transferred overnight to SD. Two days prior to transfer (i.e. LD+T 26D), birds began receiving a twice daily oral dose of 30 µl of either minocycline (Sigma; 50 mg/mL in distilled water; n=10) or distilled water (n=8). Minocycline directly impairs the ability of T cells to activate microglia, and thus indirectly attenuates the innate inflammatory response through decreased TNF-alpha, cytokine, and interleukin production [14]. Following T pellet removal, all birds were housed in SD for three

days. On the third day of LD+T withdraw (LDW) and two hours prior to tissue collection, all birds were injected with one dose of BrdU (50 mg/ml) to label proliferation of neural stem cells in the VZ.

SI.1.2.3 Blood draw and hormone analysis

Blood samples were obtained from birds in LD+T at 28 D and upon euthanasia to measure circulating T concentrations. We drew 250 µl of blood from the alar vein in the wing into heparinized collection tubes, immediately centrifuged the tubes, and stored the plasma at -20°C until assay. We will measure plasma T concentration using the Testosterone Enzyme Immunoassay Kit (Enzo Life Sciences). Minimum and maximum detectable plasma T concentrations are 0.03 ng/ml and 40.00 ng/ml, respectively. Samples with undetectable levels of T will be treated as having concentrations at the lower detection limit for statistical analyses. Intra-and inter-assay coefficients of variation will be measured.

SI.1.2.4 Tissue collection and processing

Brains were removed quickly after birds were deeply anesthetized with isoflurane. Within 2 min, brains were bisected at the midline and one randomly chosen hemisphere was frozen on dry ice for histology as described below. The other half was immediately placed in ice cold artificial cerebral spinal fluid (19 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 2.5 mM NaH2PO4, 16 mM NaHCO3, 2.5 mM CaCl2 (2.5 mM), 11 mM D-glucose, and 10 mM Hepes) and sectioned on a vibratome at 500 µm. Under a dissecting scope, HVC and an equivalent sized piece of the optic tectum were dissected from the vibratome sections and stored at -80°C until processing for quantitative PCR. The other frozen half of each brain was sectioned in the coronal plane at 40 µm on a cryostat, and each section was thaw-mounted serially. Every third section was Nissl stained and the remaining slides were stored at -80°C until immunolabeling. Because only one hemisphere of each brain was sectioned, all histological measurements described below are reported as unilateral counts or densities.

SI.1.2.5 Immunohistochemistry and analysis

To visualize BrdU-labeled cells, we processed brain sections as follows. Briefly, sections were fixed in 4% paraformaldehyde, rinsed in phosphate buffered saline with 0.5% DMSO and 0.5% Triton-X (PDTX; pH 7.4), incubated with 0.1 mg/mL proteinase K for 5 minutes, and post-fixed. Slides were dipped in distilled water, incubated with 2N HCl at 37°C for 30 minutes, and rinsed with PDTX. After blocking in 5% heat inactivated goat serum (Vector), slides were incubated serially with rat anti-BrdU (1:200; ABD Serotec MCA2060) and mouse anti-HuC (1:100; Invitrogen) antibodies. Labeling was visualized with diaminobenzidine (DAB) staining

following incubation with biotinylated goat anti-rat and anti-mouse IgG secondary antibodies (1:200; Vector) and amplification with avidin-biotin peroxidase (ABC) complex (Vector). To obtain a purple precipitate for the BrdU antigen, we used DAB-nickel stain (0.05% DAB, 0.05% Nickel Ammonium Sulfate and 0.015% H2O2 in PBS). BrdU-positive cells in the ventral side of the VZ (vVZ) were counted from the hillock of the VZ medial to HVC, out to the most lateral extension of the vVZ in all sections that exhibited arching of the VZ [4, 16].

Activated caspase-3 (AC-3) immunolabeling will be used to identify cells undergoing apoptosis [17]. Sections will be blocked with 5% heat inactivated goat serum, incubated with rabbit anti-AC-3 antibody (1:400; Santa Cruz), and then incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector) [18]. DAB staining will be performed following amplification of the secondary antibody with ABC. Sections will be lightly counterstained with thionin, a Nissl stain, as previously described [19]. AC-3 positive cells will be counted from four HVC sections using a random, systematic sampling protocol as described below [20].

All IHC was and will be performed in batches of up to 28 slides with tissue from randomly assigned birds. Each batch included positive and negative controls. All measurements were and will be made blind to treatment group.

SI.1.2.6 Morphometric measurements in HVC and RA

Volumes of HVC and RA in the randomly chosen hemisphere will be determined by tracing the borders of the nuclei identified in Nissl stained tissue onto paper, these drawings will be scanned, and the area will be determined using Image J Software (Version 1.46; NIH; http://rsb.info.nih.gov/ij/). Total volume will be calculated from areas of the tracings using the formula for a truncated cone [20]. We will use a random, systematic sampling protocol to measure neuron density in HVC from Hu immuno-labeled tissue; this protocol yields estimates that do not differ from stereological methods [20]. Samples will be collected from sections through the full extent of each brain region. All Hu positive neurons that fall within an ocular grid (ca. 1.9 X 103 mm2 at 1000X) randomly positioned in each section of HVC will be counted to calculate neuronal density. Neuronal number will be obtained by multiplying neuronal density for each bird by that bird's unilateral HVC volume.

SI.1.2.7 Real-time quantitative PCR

Tissue dissected from HVC and the optic tectum will be placed in 150 µl of ice cold Cell Disruption Buffer (mirVana Paris Kit, Life Technologies) and sonicated gently 5 times for 5 sec each time. Tissue will be further digested in 150 µl of Denaturing Solution and run through the manufacturer's filter cartridge. Washes and elution for both small and large RNAs will be

performed according to the manufacturer's protocol (mirVana Paris Kit, Life Technologies, PN 1556M Rev C). cDNA will be synthesized from 100 ng of RNA using the miScript II RT kit with the optional High Flex Buffer (Qiagen, miScript PCR System Handbook, 4th edition). Real-time quantitative PCR will be performed according to the manufacturer's protocol using SYBR Green PCR Master Mix (Life Technologies). All primers will be validated as amplifying just one transcript of the intended target via a single peak in the primer melt curve and gene sequence verification (via GeneWiz) of PCR product isolated from a 1.5% electrophoresis gel using the Qiagen Gel Extraction Kit. Primers include: markers of apoptosis, Bax and Bcl-2 [21]; markers of microglia and microglial activation, Aif1 (or Iba1) [22], Csf1r [23], and Eda (or ED1) [24]; and markers of inflammation mediated through microglia activation, Il1-beta and Il6 [10, 11, 24, 25]. All samples for each primer set will be run in duplicate. All primers are designed to span at least one intronic region to avoid amplification of genomic DNA. To calculate the log-fold change all samples will be evaluated for levels of Gapdh mRNA and compared using the following equation: $C_T=2^{\Delta\Delta C_T} = 2^{(C_{T,X} - C_{T,R})_{control} - (C_{T,X} - C_{T,R})_{test}}$, where $C_{T,X}$ = test primer C_T, and $C_{T,R}$ = Gapdh C_T.

SI.1.2.8 Statistical analysis

For the LPS study, comparisons were made with two-way ANOVAs with breeding condition and treatment as the factor, and Tukey tests for post-hoc comparisons. For the anti-inflammatory experiment, comparisons between experimental groups were made with Student t-tests. For all statistical tests a p-value ≤0.05 was considered significant. All statistical analyses were made using JMP Version 8.0.1 (SAS, Cary NC). Data are presented as mean ± SEM.

SI.1.3 Preliminary results and conclusions

SI.1.3.1 The inflammatory agent LPS increases vVZ proliferation

To test whether local neuroinflammation and the activation of the innate immune response was mediating natural reactive neurogenesis, we tested the sufficiency of local inflammation within HVC to increase vVZ neural stem cell proliferation in both stable breeding and nonbreeding conditions. Micro-injection of LPS significantly increased the number of BrdU positive cells in the vVZ compared to controls in SD (SI Table 1 and SI Figure 1C; n=10; p=0.0128) and LD+T (n=6; p=0.0387). These results suggest that proliferation increases in response to a local inflammatory response within or near HVC. Moreover, the conditions in which the LPS-induced inflammatory response occurs (i.e. SD versus LD+T) does not alter the response in vVZ neural stem cell proliferation; vVZ proliferation in response to both sham and

LPS injections were the same when compared across breeding condition - a preiod in which levels of sex steroids and other factors such as BDNF [26][27, 28] are high in HVC - and nonbreeding condition (SI Figure 2C; sham, n=7; p=0.9878; LPS, n=9; p=0.9980). To confirm that the LPS is in fact increasing vVZ proliferation through an immune response, we will measure classic markers of inflammation and microglia activation using rt-QPCR from mRNA isolated from HVC tissue that received sham and LPS injections (SI Figure 2B). Histochemical analyses of microglia immunolabeled with an antibody to RCA-lectin revealed active microglia cells present in HVC in their ramified morphology [10, 29] that had engulfed Alexa Fluor conjugated LPS (SI Figure 2A). The presence of activated microglia in HVC suggests that microglia could be prompting local inflammation in response to LPS and that their activation could be quantified (SI Figure 2A&B). To ensure that the LPS did not mediate its effects through HVC cell death, we will measure cell death with immunohistochemistry (IHC) using an antibody to AC3, a marker of cell death, and QPCR for Bax and Bcl-2 mRNA. If we find that markers of inflammation and microglia activation are high while markers of cell death are low, we will be able to conclude that a local inflammatory response in the absence of cell death within HVC is sufficient to promote neural stem cell proliferation in the vVZ.

SI.1.3.1 The inflammatory inhibitor minocycline prevents vVZ proliferation

To test the necessity of microglia activation and the resulting inflammatory response, we inhibited microglia activation with oral minocycline during the previously reported natural regression of HVC [4]. Minocycline inhibited the increase in the number of neural stem cells proliferating and the number of their progeny in the vVZ compared to controls (SI Table 2 and SI Figure 3B; n=11; p=0.0021). These results suggest that activation of microglia and resulting inflammation mediates the increase in vVZ proliferation with rapid regression of HVC during LDW. To confirm that microglia are activated with HVC regression and inhibited by minocycline, we will measure microglia activation and production of IL1-beta and IL6 by rt-QPCR (SI Figure 2B). We will also assay whether prevention of a local inflammatory response alters the levels of HVC cell death with rt-QPCR and IHC. Finding an increase in markers for microglia activation and interleukins in HVC of birds that received vehicle compared to HVC of birds that remained in LD+T (tissue collected and stored from a previous project [4]) would suggest that microglia mediate the clean-up of apoptotic HVC neurons and signal the vVZ to increase proliferation. Finding a decrease in microglia activation and interleukin production in the HVC of birds that received minocycline would confirm that minocycline worked as expected and that microglia activation is necessary for the increase in vVZ proliferation. Finally, observing no change in cell

death between birds that received vehicle and minocycline would suggest that HVC cell death is primarily mediated by photoperiod and T. Conversely, finding a decrease in cell death with minocycline treatment would suggest that the initial wave of cell death in HVC may be mediated by the decrease in photoperiod and T, but that subsequent cell death is at least in part promoted by a local inflammatory response to the first wave of dying neurons.

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SI Table 1. vVZ proliferation in response to local inflammation

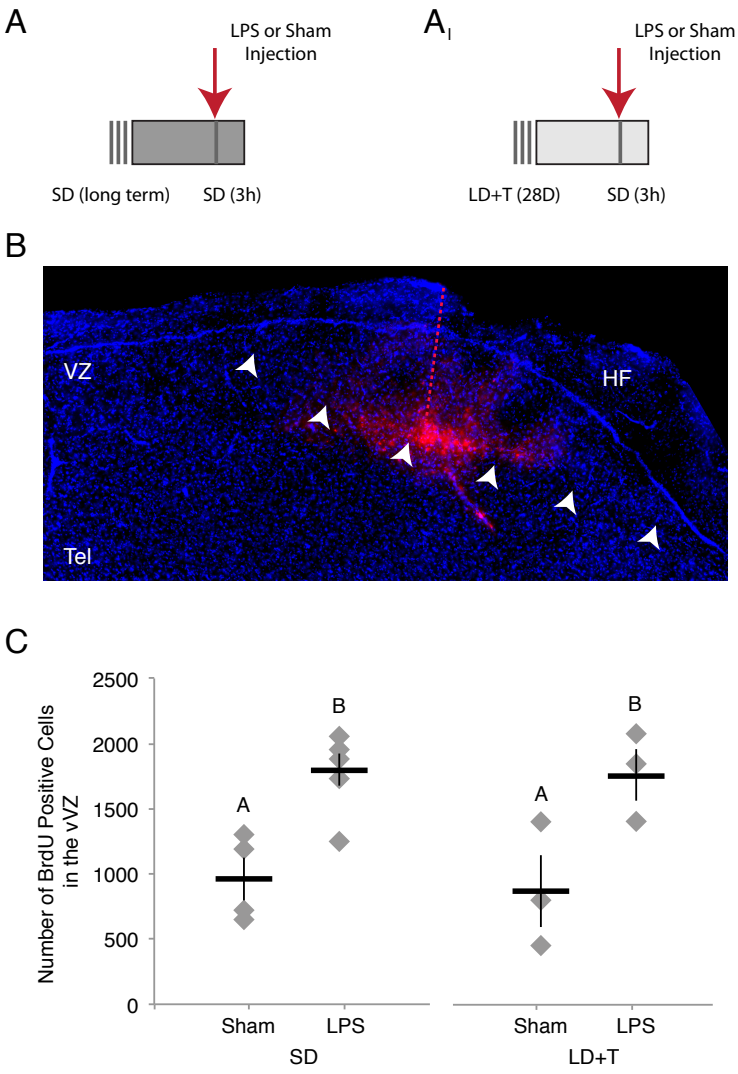
	Sample Size (Sham; LPS)	Sham	LPS	ANOVA	Post-Hoc Tukey
vVZ Proliferation					
SD	**4; 6**	971 ± 164	1819 ± 123	$F_{(2,12)}$=8.1536; p=0.0032	0.0128
LD+T	**3; 3**	884 ± 278	1776 ± 197		0.0387

Data presented as Mean ± SEM

SI Table 2. vVZ proliferation during rapid regression with anti-inflammatory

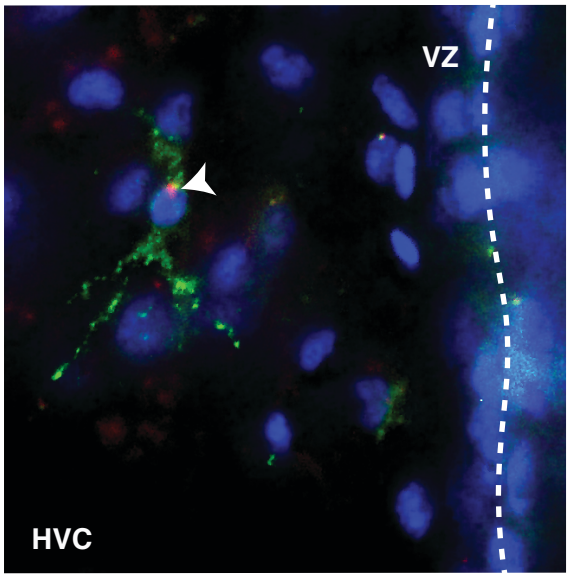
	Sample Size (Sham; LPS)	Vehicle	Minocycline	ANOVA
vVZ Proliferation				
LDW	**6; 5**	2228 ± 199	899 ± 245	$F_{(1,10)}$=18.1331; p=0.0021

Data presented as Mean ± SEM



SI Figure 1. The inflammatory agent LPS increases vVZ proliferation independently of breeding condition. *(A)* Experimental design increasing proliferation in nonbreeding (i.e. SD) conditions. *(A_l)* Experimental design increasing proliferation in breeding (i.e. LD+T) conditions. *(B)* An image showing the injection site of LPS within HVC. LPS was conjugated with Alexa Fluor 568 and is shown in red. Tissue was counterstained for 30 min with Hoechst 33342, a maker of cell nuclei, and is shown in blue. Arrow heads indicate the ventral border of HVC. HF = hippocampal formation, Tel = telencephalon. *(C)* The number of BrdU positive cells within the vVZ in breeding and nonbreeding condition birds sham injected or injected with LPS. LPS significantly increased vVZ BrdU positive cells in both breeding and nonbreeding conditions. Letters denote significant differences among groups obtained with a post-hoc Tukey test.

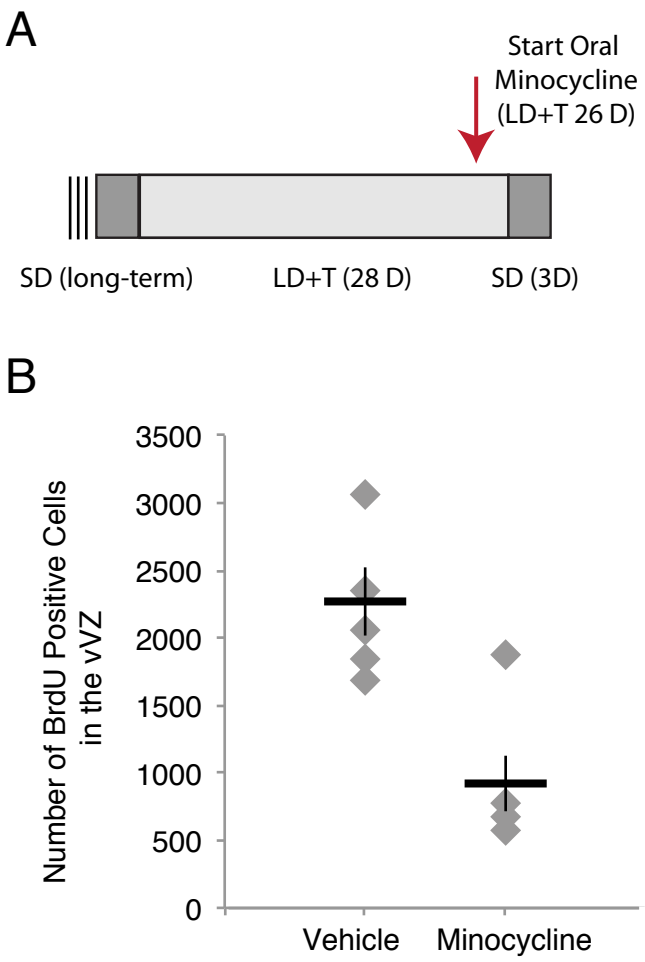
A



B

	Cell Death	Inflammation	Microglia Activation
IHC	AC3	–	–
rt-QPCR	Bax Bcl2	IL1b IL6	Csf1R Eda Aif

SI Figure 2. The presence of activated microglia in HVC following LPS injection. *(A)* An image of an activated microglial cell (shown in green) in HVC of a bird injected with LPS conjugated to Alexa Fluor 568 (red). Tissue was counterstained with Hoechst 33342, shown in blue. The close proximity of the LPS with the nucleus of the microglial cell suggests that this microglia engulfed LPS. Note the morphology of the microglial cell (i.e. the long projections), suggesting that this microglial cell is activate. Also note the close proximity of this microglial cell to the VZ, outlined with the white dashed line. *(B)* Planned analyses to determine levels of cell death, inflammation, and microglia activation within HVC.



SI Figure 3. The anti-inflammatory minocycline prevents the increase in proliferation that occurs with natural HVC regression. *(A)* Experimental design decreasing inflammation with LDW. *(B)* Minocycline decreases vVZ proliferation compared to controls. The asterisk indicates a p-value less than 0.0005.

SI.2 The Curriculum Vitae of Tracy Larson

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Education

2014 (exp)	Ph.D. Biology, University of Washington
2014 (exp)	Certificate of Law, International Bioethics, Social Justice and Health, UW School of Law
2006	B.S. Molecular, Cellular and Developmental Biology, University of Washington
2006	B.S. Neurobiology, University of Washington
2001-2003	Post-Secondary Options Program, Biology, Ohio University

Research Experience

2015-	Post-doctoral position, Fred Hutchinson Research Center Mentor: Katie Peichel Behavioral genomics of stickleback fish
2009-2014	Ph.D. Thesis, University of Washington, Department of Biology Mentor: Eliot Brenowitz Mechanisms of adult neurogenesis and neuroprotection in songbirds
2008	Research Engineer, University of Washington, Department of Biology PI: Horacio de la Iglesia The role of circadian rhythms in mood disorders
2007-2008	Research Engineer, University of Washington, Department of Biology PI: David Parichy Zebrafish neural-crest cell derivative (i.e. glia and craniofacial) development
2004-2006	Undergraduate Researcher, University of Washington, Department of Biology Mentor: Horacio de la Iglesia Cloning and expression of circadian clock gene homologs in crustaceans

Fellowships and Grants

2015-	NIH NRSA, Auditory Neuroscience Training Grant, University of Washington
2014	Washington Research Foundation Hall Fellowship, University of Washington ($10,600+tuition)
2010-2013	NIH NRSA (T32 GM007270), Cell and Molecular Biology Training Grant, University of Washington ($72,200+tuition)
2009-2010	NIH NRSA (T32 DC005361), Auditory Neuroscience Training Grant, University of Washington ($18,600+tuition)
2005	Arts and Sciences Research Fellowship, University of Washington ($1,500)
2004-2005	Mary Gates Research Endowment, Mary Gates Foundation (4,500)
2004	Howard Hughes Research Fellowship, Howard Hughes Medical Institute ($3,000)
2004	Mary Gates Research Endowment, Mary Gates Foundation ($2,500)

Publications

Larson TA, Thatra NM†, Lee B†, Brenowitz EA. (2014) Reactive neurogenesis in response to naturally occurring apoptosis in an adult brain. *The Journal of Neuroscience.* 34(39): 13066-76 doi: 10.1523/JNEUROSCI.3316-13.2014 PMID: 25253853

Larson TA, Wang TW, Gale SD, Miller KE, Thatra NM†, Caras ML, Perkel DJ, Brenowitz EA. (2013) Postsynaptic neural activity regulates neuronal addition in the adult avian song control system, *Proceedings of the National Academy of Sciences USA.* 110 (41): 16640-44 doi: 10.1073/pnas.1310237110, PMID: 24062453

Larson TA, Gordon TN, Lau EM, Parichy DM. (2010) Defective adult oligodendrocyte and Schwann cell development, pigment pattern, and craniofacial morphology in puma mutant zebrafish having an alpha tubulin mutation, *Developmental Biology*, 346(2): 296-309 doi: 10.1016/j.ydbio.2010.07.035 PMID: 20692250

† Mentored undergraduate students

Manuscripts

Brenowitz EA & Larson TA. Neurogenesis in the Adult Avian Song Control System. "Adult Neurogenesis." 2nd ed. Gage, Kemperman, and Song. Cold Spring Harbor Press. To be published 2015

Larson TA, Hou D‡, Brenowitz EA. Daily rhythmic changes in singing behavior of Gambel's white-crowned sparrow. In prep for *PlosOne*

Larson TA, Lent KL, Bamler TK, MacDonald JW, Farin FM, Perkel DJ, Brenowitz EA. Network analysis of microRNA and mRNA dynamics in a highly neurogenic sensorimotor neural circuit. In prep for *BMC Genomics*

Larson TA, Thatra NT†, Hou D†, Brenowitz EA. The seasonal modulation of neuronal turnover in an adult sensorimotor circuit. In prep for *Journal of Comparative Neurology*

Larson TA, Lima M, Cole MM†, Brenowitz EA, Cohen R. The seasonal deconstruction and reconstruction of an adult sensorimotor neural circuit. In prep for *Hormones and Behavior*

† Mentored undergraduate students
‡ Mentored high school students

Press Coverage

Local and National

S Hines, *University of Washington News and Information*. (2014) Dying brain cells cue new brain cells to grow in songbird

T Dubnicoff, *California Institute of Regenerative Medicine*. (2014) The sparrow's dying song: a possible path toward natural, stem cell-based repair of human brain diseases.

C Griffith, *Science World Report*. (2014) New brain cells grown in songbirds each year: Neuron growth investigated

S Hines, *Futurity*. (2014) Do dying neurons tell bird brains to regenerate?

K Nayan, *Counsel & Heal*. (2014) Dying brain cells cue new brain cells to grown in songbird, study finds

O Nunez, *Design & Trend*. (2014) Regenerating brain neurons help birds sing better during breeding season

Three Sentence Science. (2014) Dying brain cells cue up new ones in songbirds

International

Ians, *ZeeNews,* India. (2014) Dying cells send signal to stimulate new cells in birds.

Honors and Awards

2014	Undergraduate Research Mentor Award, University of Washington (5-6 awarded per year among all UW faculty, staff, and students)
2013	Travel Award, Department of Biology, University of Washington ($350.00)
2012	Travel Award, Department of Biology, University of Washington ($400.00)
2009	Honorable Mention, NSF Graduate Student Fellowship Program
2006	1st Place Poster Winner, Woodland Park Zoo Research Meeting
2006	Wildlife Care Aid of the Month, Progressive Animal Welfare Society
2001-2006	Annual and Quarterly Dean's List, Ohio University and University of Washington

Meetings and Symposia

Oral Presentations

2014	Annual University of Washington - South Lake Union Research Symposium, Seattle WA, "Natural reactive neurogenesis in the avian brain"
2014	Graduate Student Symposium, University of Washington, Seattle WA, "Natural reactive neurogenesis in the avian brain: a powerful model for regenerative neurogenesis"
2011	Graduate Student Symposium, University of Washington, Seattle WA, "Efferent neural activity regulates adult neuronal recruitment"
2008	Graduate Student Symposium, University of Washington, Seattle WA, "Desynchronized rat as a model for mood disorders"
2005	Mary Gates Undergraduate Research Symposium, Seattle WA, "Crustacean circadian rhythmicity"
2004	Friday Harbor Research Apprentice Symposium, Friday Harbor WA, "In search of clock neurons in decapod crustaceans"

Poster Presentations

2013	Society for Neuroscience Annual Meeting, San Diego, CA, "Rapid changes in microRNA expression during seasonal growth and regression of the adult avian song control system"
2013	Society for Neuroscience Annual Meeting, San Diego, CA, "Turnover of adult born neurons in the avian song control system"
2012	Society for Developmental Biology Northwest Meeting, Friday Harbor, WA, "Changes in neuronal recruitment, apoptosis, and behavior during periods of seasonal growth and regression"
2012	Society for Neuroscience Annual Meeting, New Orleans, LA, "Changes in neuronal recruitment, apoptosis, and behavior during periods of seasonal growth and regression"
2011	Society for Neuroscience Annual Meeting, Washington, D.C., "Rapid changes in neuronal recruitment and apoptosis during periods of seasonal growth and regression"
2008	Zebrafish Development and Genetics, Madison WI, "puma Encodes an alpha-tubulin required for craniofacial morphogenesis, myelination and adult pigment pattern formation"

2006 Society for Neuroscience Annual Meeting, Atlanta GA, “Cloning and expression of clock gene homologs”

2006 Mary Gates Undergraduate Research Symposium, Seattle WA, “Cloning and expression of clock gene homolog in crustacean”

2006 Woodland Park Zoo Research Meeting, Seattle WA, “The Nocturnal Exhibit: A visibility study and proposal for future studies”; *awarded - Best Poster*

Attended

2011 Clinical Neuroscience and Society Conference, Philadelphia, PA

2011 Annual Meeting of the International Neuroethics Society, Washington, D.C.

2010 Annual Meeting of the Neuroethics Society, San Diego, CA

2010 Society for Neuroscience Annual Meeting, San Diego, CA

2009 Annual Society for Bioethics and Humanities Meeting, Washington, D.C.

Teaching Experience

Courses

Pending Primary Instructor, Biology 497, Practical Ethics in Neuroscience, University of Washington

2009 Teaching Assistant, Biology 418, Biological Rhythms, University of Washington

2009 Teaching Assistant, Biology 350, Physiology, University of Washington

2008 Teaching Assistant, Biology 200, Introductory Biology, University of Washington

Mentoring

Graduate Students

2014 Kali Esancy, Rotation Student, University of Washington
Histological and behavioral techniques and analyses

2013 Lisa Voelker, Rotation Student, University of Washington
Histological, surgical, and behavioral techniques and analyses

2012 Eric Thomas, Rotation Student, University of Washington
Histological and behavioral techniques and analyses

2008 Fabio Everton Maciel, Visiting Student, Universaidade Federal de Rio Grande
Molecular cloning techniques and animal husbandry

Undergraduate Students

2014-2015 Marriane Cole, University of Washington
Designing and conducting an independent research project
Publications: 2 (in prep)
Presentations: Mary Gates Research Symposium, UW (1)

2013-2014 Darren Hou, University of Washington
Conducting independent research projects
Publications : 2 (in prep)
Presentations: Mary Gates Research Symposium, UW (1)

2011-2012 Brian Lee, Johns Hopkins University
Histological techniques and analysis
Publications: 1

2010-2014 Nivretta Thatra, University of Washington
Designing and conducting independent research projects
Awards: Mary Gates Research Fellowship (2), Sargent Award (1)
Posters: Biology Undergraduate Research Symposium, UW (1); Mary Gates Research Symposium, UW (1)
Presentations: Mary Gates Research Symposium, UW (2)
Publications: 3 (1 in prep)

2010-2011 Matt Shapiro, University of Washington
Designing and conducting an independent research project
Awards: Mary Gates Research Fellowship (1)
Posters: Mary Gates Research Symposium, UW (1)

High School Students

2012-2013 Darren Hou, Interlake High School, WA
Conducting research in neurobiology and behavior

2010-2011 Hamdi Malin, Cleveland High School, WA
College applications and financial aid

2009 Nivretta Thatra, Bellevue High School, WA
Designing and conducting research in neurobiology and behavior

2008-2010 Tracy Wanjiku, Des Moines High School, WA
College applications and financial aid

Membership and Service

Professional Membership

2010- Society for Neuroscience, Student Member
2009-2012 International Neuroethics Society, Student Member

University Service

2013-2014 Representative, Graduate Student Body Coordinating Committee, elected position, Department of Biology, UW
2012-2013 Graduate Student Representative, Faculty Appointments Committee, Department of Biology, UW
2012-2013 Organizer, Cell and Molecular Biology Training Grant Monthly Meeting, UW
2011-2014 Founder and organizer, Department of Biology Monthly Social, UW
2011-2012 Representative, Graduate Student Body Coordinating Committee, elected position, Department of Biology, UW
2009-2011 Graduate Student Representative, Graduate Program Committee, Department of Biology, UW
2009 Co-organizer, Graduate Student Symposium, UW Department of Biology

2008-2009 Graduate Student Representative, Undergraduate Curriculum Committee, Department of Biology, UW
2006 Associate President, Alpha Epsilon Delta (Honor Society)
2005-2006 Secretary, Alpha Epsilon Delta (Honor Society)

Community Service and Outreach

2011, 2013, 2014 Brain Awareness Exhibitor, Brain Awareness Week, Seattle, WA (18 hrs)
2011 Interview with Taka Olds, middle school student; discussed legal, ethical and social issues of human cloning (1 hr)
2008-2011 High School Student Mentor, Making Connections Program, Women's Center, UW (100+ hrs)
2005-2007 Wildlife Care Aid, Progressive Animal Welfare Society, Lynnwood, WA (500+ hrs)
2005 Counselor, Stanley Stamm Summer Camp for disabled children, Children's Hospital, Seattle, WA (1 wk)
2004-2005 English, Math and Science Tutor, Meany Middle School, Seattle, WA (50+ hrs)

Relevant Skills

Molecular

Cloning from model (zebrafish, *Arabidopsis*) and non-model (crustacean, songbird) organisms; quantitative RT-PCR; transgenics (construct design to animal screening)

Histological

Cryo-, vibrotome and freezing microtome sectioning; immunohistochemistry, single to triple labeling, sections and whole-mount; cell proliferation (BrdU, EDU; single and double cohort label) and death assays (acridine orange, TUNEL, ApopTag); *in situ* hybridization, single and double labeling, sections and whole-mount

Biochemical

Yeast-two-hybrid; immunoblotting (Western Blot); ELISA

Behavioral Analyses

Forced swim test, marble burying assay, open field test, and locomotor activity (rat); locomotor activity (crustacean); song behaviour (bird)

Surgical

Brain electrode implantation (rat); iButton implantation surgeries (rat); single cell injections of oligomorpholinos and expression plasmids (zebrafish); brain cannula implantation (bird); targeted brain retrograde/anterograde tracer injections (bird)

Microscopy

Confocal, fluorescent, bright field, and 2-photon imaging using Zeiss, Leica, and Image J Software platforms

Colony Maintenance

Genetic stock and colony maintenance (rat, zebrafish, *Arabidopsis*, bird); transgenic stock screening and maintenance (zebrafish)

Software/Databases

JMP, Ingenuity IPA, FileMaker Pro, Omni Plan (Gantt Charting), Microsoft Office Products, iWork Products, Adobe Creative Suite Design Products, Endnote, Raven Pro, Pubmed, Web of Science, Lexus Nexus, Bloomberg Law

Graduate Course Work

2014	Practicum in Health Law, International Bioethics, Social Justice and Health Seminar
2013	Biology Seminar, Topics in Biology - Manuscript Writing, Genetics and the Law
2011	Molecular Medicine, International Bioethics, Social Justice and Health Seminar, Seminar in Behavioral Neuroscience, Health and Human Rights, Introduction to Systems Biology and Quantitative Approaches to Biomedical Sciences
2010	Biology Seminar, Seminar in Behavioral Neuroscience, Seminar in Neurodevelopment, Signal Transduction, Cell Signaling, Law Ethics in Public Health Genetics
2009	Biomedical Research Ethics, Neuroendocrinology, Topics in MCD Biology, Developmental Neurobiology, Advanced Animal Behavior, Biology Seminar, Pathology of Aging, International Research Ethics, Law and Policy, Seminar in Behavioral Neuroscience, International Bioethics, Social Justice and Health Seminar, Seminar in Neurodevelopment
2008	Topics in Biology - NSF Grant Writing, Topics in Physiology, Biology Seminar