Adult Song Stereotypy Linked To Neuronal Morphology in Two Song System Regions

by

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Abstract:

The pre-motor song control nucleus RA (robust nucleus of the arcopallium) receives projections from two distinct populations of projection neurons. Recent findings have shown that the ratio of dendritic arbor for input neurons from HVC (used here as proper name) to RA (HVC-RA) relative to arbor for input neurons from LMAN (lateral magnocellular nucleus of the nidopallium) (LMAN-RA) is positively correlated with post crystallization singing rate and adult age in zebra finches. HVC-RA projection neurons are known to promote motor program stereotypy, whereas LMAN-RA projection neurons have been linked to motor variability. When combined with earlier studies indicating that zebra finches continue to increase their song stereotypy well into adulthood, these findings suggest that changes in dendritic morphology may be functionally related to increases in song stereotypy. With increasing age, the bird’s cumulative singing experience may be focused towards a goal of improving song stereotypy. However, there have been no studies directly testing for a link between individual differences in dendritic arbor ratios and adult song stereotypy in the same birds. I proposed that higher singing rates and superior song stereotypy would correlate with greater dendritic arbor (as measured by average dendritic length, total dendritic length, and total number of dendritic segments) among HVC-RA cells and less dendritic arbor among LMAN-RA cells. Specifically, I predicted that there would be a positive correlation between both singing and song stereotypy with the ratio of HVC/LMAN dendritic arbor. To test this hypothesis, 10 male zebra finches between the ages of six to seven months were recorded for 24 hours for seven days. Immediately following these recordings, birds were injected
with rodamine dextran amines into RA to fluorescently label both HVC-RA cells and LMAN-RA cells for measuring dendritic attributes. I found that there were significant relationships between song stereotypy (but not singing rate) and the amount of dendritic arbor of HVC-RA cells, LMAN-RA cells, and the ratio of HVC-RA/LMAN-RA cells. These results suggest that the observed increase in this ratio is a cause or consequence of adult vocal motor refinement.
Introduction:

Using Song Birds as a Model for Vocal Learning:

Vocal learning is the ability to learn and produce vocalizations for the purpose of communication. Along with humans, songbirds are one of the few animal groups capable of vocal learning (Williams, 2004). More importantly, this vocal learning ability is remarkably similar to that of humans and this allows songbirds to act as a model organism for understanding vocal learning in humans (for review, Doupe & Kuhl, 1999). In both songbirds and humans, the ability to learn both song and speech, respectively, is limited by innate predispositions, is heavily dependent on social context, and diminishes with increasing age (Kirn, 2009). Additionally, learning both speech and song is an active process that relies on auditory input and feedback and is associated with specialized brain regions (Kirn, 2009, Doupe & Kuhl, 1999).

Another important characteristic of songbirds, which makes them an ideal model for research, is the remarkable plasticity of their brains during early development and throughout their lifetime (for review, Nottebohm, 2004). This important characteristic of the songbird will be discussed in greater depth later in this thesis. First, it will be necessary to have a basic understanding of the two major classifications of songbirds, and more importantly, the song learning system and the relevant brain regions involved.

Species Specific Song and Critical Periods:

Bird songs are known to be species-specific. In most species, males generally use calls for the purpose of communication and primarily employ songs as their
means of attracting mates. For this reason, only males sing in most species, and therefore most research on vocal learning is restricted to males. Although the song learning process is similar across species, each species has a unique way of learning their song. More importantly, while each individual bird produces a song that is species-specific (Immelmann, 1969), thereby keeping to a distinct set of rules, the song varies slightly from individual to individual, as each bird produces a unique adult song (for review, Williams, 1992).

There are two main classifications of songbirds that have been widely adopted over the past few decades. The first group is referred to as open-ended learners. Open-ended learners continue to learn and perfect their songs throughout their lifetime, and are capable of learning new songs from one breeding season to the next. The most common open-ended learner studied is the canary. The second group is known as close-ended learners. These birds do all their song learning during the first year of life. Close-ended learners have a fixed song by their first mating and the song does not change for the remainder of their lives (Immelmann, 1969; Brenowitz, 2004.)

While many aspects of song learning are different between these two classifications of songbirds, there is one major factor that is common among all species of oscine songbirds. This common feature is known as the critical period, and has been shown to exist not only in all species of song birds, but also among many other animals, including humans (Nottebohm, 2004). The critical period describes a relatively short and restricted window of time when the brain is particularly sensitive to certain environmental stimuli and it usually occurs quite early in life. If the animal
receives this external or self-generated input, the brain responds to this input and this causes specific behavioral and/or cellular transformations that result in acquisition of particular skills or abilities. However, when the necessary environmental inputs are not present, normal development of these abilities is stunted. In songbirds, this effect is demonstrated by isolation studies, where birds kept in isolation were found to develop abnormal songs (Eales, 1985). In humans, the idea of critical periods is often referred to when discussing the difficulty people often encounter when learning second languages past early childhood (for review, Bernhardt, 1984; Brainard & Doupe, 2000b). Unless children are exposed to certain phonemes or accents early in life, they lose the ability to fully incorporate them into language (Johnson & Newport, 1989).

While it was believed for many decades that the end of the critical period corresponded to the completion of song learning in the zebra finch and other close-ended learners, several studies have proven otherwise. Recent findings suggest that zebra finches continue to perfect their songs well into adulthood, and perhaps throughout their entire life (Pytte et al., 2007; McDonald & Kirn, in preparation). It has become clear in the past decade that adult song is closely monitored and dynamic, which may ultimately lead to redefining the characterization of close-ended learners, however there is still no evidence that close-ended learners can add song elements to their vocal repertoire in adulthood.
Figure 1. Spectrogram of a single motif from Big Boi, with time (seconds) on the x-axis and sound frequency on the y-axis. Sound intensity is depicted in pseudocolor, with red/yellow at the high end and blue/black at the low end.

*The Zebra Finch Song System and Song Learning Process:*

The bird chosen for this study is the zebra finch, an oscine species native to central Australia. The zebra finch is a close-ended song learner whose adult song is rather simple. The song is usually preceded by one-to-several short opening notes. Following these introductory notes, a song is repeated several times in rapid succession, each consisting of no more than four to seven individually distinct and relatively complex “syllables” (or note complexes) that in a typical adult are produced in the same sequence from rendition to rendition (Kirn et al., 2009). This fixed sequence of syllables is referred to as the bird’s motif, which is normally sung several times in rapid succession (See Figure 1).
The song learning process can be divided into four progressive phases. The first phase begins when juvenile birds listen to and memorize the songs of adult tutors (for review, Williams, 2004). This phase is known as sensory learning and begins roughly 25 days after hatching, before song-related vocalizations occur (Immelmann, 1969; Price, 1979). The second phase, which usually begins at approximately 35 days post hatching, is called the sensory-motor phase (Nottebohm, 2005). This phase is the process of using auditory memories as a guide to the development of the bird’s own song. This phase is characterized by rudimentary song vocalizations, known as sub-song, which have very little similarity to adult song, and are analogous to the jabber of newborns (Williams, 2004). The third phase, known as plastic song, is when recognizable notes emerge, but are highly inconsistent from one song rendition to the next (Aronov et al., 2008). This variability between song renditions gradually decreases throughout the sensory-motor phase and plastic song, until it reaches the final phase, song crystallization. This last stage typically occurs around 80-100 days of age and corresponds to the end of the sensitive period for song learning and the establishment of a stable adult song (Williams, 2004). Crystallized song, as it is often referred to, is made up of a highly complex, stereotyped motif (Aronov, 2008), and until recently, it was believed this stereotypy remained fixed through adulthood. As discussed earlier however, recent evidence suggests that song learning, specifically, song stereotypy, continues well beyond the end of the sensitive period for song learning (Pytte et al., 2007; McDonald & Kirn, in preparation).
The Song System of Song Birds:

The song system, also known as the song control system (SCS), which was first discovered by Fernando Nottebohm (1976), has since become an integral part of our current understanding of the learning, production, and maintenance of song in oscine birds (Nottebohm, 1976, 2004). The song system is a series of discrete brain nuclei and has been divided into two basic pathways (Figure 2). The first pathway, the direct motor pathway, is necessary for normal song production. This pathway begins with telencephalic nucleus HVC, which receives information from auditory areas in the brain. HVC neurons then send projections to the robust nucleus of arcopallium (RA), RA, in turn, sends projections to nXII in the hindbrain, which then projects to the tracheosyringeal muscles, where song is finally produced (Nottebohm, 2004).

The second pathway, the anterior forebrain pathway (AFP), involves a more complex set of nuclei, and while not necessary for song production, it is critical for song learning (Bottjer et al., 1984; Sharff & Nottebohm, 1991). This pathway also begins with HVC and eventually sends projections to RA, however, HVC neurons of the AFP take a less direct route. HVC neurons project to a region known as area X, area X projects to the dorsal lateral nucleus of the medial thalamus (DLM), and DLM then sends projections to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). The completion of the pathway occurs when LMAN sends projections to the same cells in RA that receive direct input from HVC within the motor pathway (for review, Brainard & Doupe, 2000a).

The motor pathway has been shown to be vital to the production of song.
Lesions of nuclei along this pathway have been shown to influence developing, as well as adult song. Lesions along this pathway have consistently been shown to cause the immediate break down of song, independent of the bird’s age (Nottebohm, 1976). Additionally, activity along this pathway has been shown to directly influence both the timing and sequencing of song production. For example, HVC-RA cells are extremely active while song is being produced, whereas this activity immediately drops to low spontaneous firing when singing ends (Rosen & Mooney, 2003). Furthermore, electrical stimulation to forebrain nuclei along this pathway has been shown to elicit complex vocal features specific to the bird’s own unique song (Vicario et al., 1995).

While the anterior forebrain pathway is not necessary for song production, it is essential to both the song learning process and the maintenance of song throughout adulthood. Research conducted during the past decade suggests that the AFP works by inducing or enabling variability within the motor program, which enables a form of trial and error song learning (Brainard, 2000a). During development, lesions along this pathway have been shown to result in monotonous repetitions of a single, simpler complex (Sharff & Nottebohm, 1991). Moreover, it has been shown that pharmacological inactivation of LMAN dramatically reduces acoustic and sequence variability in the songs of juvenile zebra finches (Olvecy et al., 2005).

Along with impacting song learning, this pathway is believed to be crucial to the maintenance of song in adulthood. Studies have shown that deafening of adult zebra finches results in the gradual breakdown of their song (Nordeen & Nordeen, 1992). However, with simultaneous lesions to LMAN, the gradual breakdown of
song that would otherwise occur is largely prevented (Brainard & Doupe, 2000a; 2001; Bottjer et al., 1984). It is believed the maintenance of stereotyped song is achieved by auditory error detection, which activates the anterior forebrain pathway, creating error signals that are intended to adjust commands in the motor pathway to match the bird’s desired song (Brainard & Doupe, 2000b). This model of song maintenance explains the deterioration that results from deafening as a result of an active process driven by error signals in the anterior forebrain pathway (Brainard & Doupe, 2000a). This deterioration is attenuated with LMAN lesions because these error signals can no longer be generated, therefore preventing changes in song.

Although the two pathways controlling song have been identified and separated by function, their efferent projections from HVC that converge on the dendrites of neurons in RA are essential to the ultimate production of zebra finch song (Hermann & Arnold, 1991). The AFP and motor pathways work together to produce a full circuit because each separate pathway ends by having their terminal dendrites forming synapses on the same neurons of RA (Hermann & Arnold, 1991). This point where the pathways converge creates an important potential site for learning. It has been theorized that increases in synaptic strength occur when simultaneous activation of two cells (sharing) with a functional connection occurs. More simply stated, “neurons that fire together, wire together”. This concept, known as Hebbian plasticity, was first proposed by Donald Hebb in 1949 and could be the underlying mechanism for the successful learning of song (for review, Cruikshank & Weinberger, 1996). It has been proposed that as a result of accidental and simultaneous firing of HVC and LMAN cells on RA, RA cells undergo neural
changes that ultimately lead to changes in song structure. Initial evidence for the possibility of Hebbian learning occurring at synapses in RA, came from Nordeen and Nordeen’s work, which showed the presence of both AMPA and NMDA receptors at these synapses (Nordeen & Nordeen, 2004). Additional support for this concept comes from an electrical stimulation study that looked at intracellular recordings from in vitro brain slices containing RA. This study found that LMAN and HVC axons provide pharmacologically distinct types of excitatory input to many of the same RA cells, which could modulate the output of RA (Mooney & Konishi, 1991).

Comparison of HVC and LMAN:

Functional distinctions between the motor pathway and AFP are associated with differences in the cells converging on RA from HVC and LMAN. Certain cell types in the brain are continually replaced throughout the life of songbirds, while others are not. Cells projecting from HVC to RA, but not cells projecting from LMAN to RA are replaced throughout life. The process by which new neurons are generated is known as neurogenesis and it may have an important effect on song system function in birds (for review, Alvarez-Buylla & Kirn, 1997). New neurons are born in the ventricular zone of the two lateral ventricles and slowly migrate throughout the telencephalon until they reach their final destination (Alvarez-Buylla & Nottebohm, 1988). This migration can take anywhere from one to three weeks. While many neurons die before reaching their destination, the rest become fully incorporated into pathways in the song system (Paton & Nottebohm, 1984; Kirn et al., 1999). While the exact function of neurogenesis in the avian brain remains largely
unresolved, the replacement and incorporation of new neurons within the song circuitry populations has been correlated to singing experience, song structure, and auditory stimulation. The three cell populations that are replaced include HVC-RA neurons, HVC interneurons, and area X interneurons.

The cells that have received the most attention are HVC-RA neurons. The extensive changes that occur in the size of HVC in juvenile zebra finches have been found to be the result of increasing numbers of HVC-RA neurons caused by high rates of neurogenesis (Bottjer et al., 1985). HVC, along with other brain nuclei directly involved in song production, increase dramatically in size when birds first begin to sing, whereas areas not involved, exhibit much smaller size increases (Bottjer et al., 1985). It has been demonstrated that the first HVC-RA neurons make functional connections at age 35 days post hatch and that the production of song-related vocalizations begins when these HVC-RA connections are first made (Konishi & Akutagawa, 1985). From day 35 to 53, the number of HVC-RA connections continues to increase significantly. (Hermann & Arnold, 1991).

In contrast to the replaceable population of cells within the song system, there are several cell populations that do not undergo neurogenesis, and decrease in size during the course of song development. The most frequently studied of these brain regions is nucleus LMAN, which has been previously discussed as an integral part of the AFP because of its connections with RA. While there is great synaptic growth and addition in the HVC-RA pathway, there is corresponding large-scale loss of synapses in the LMAN-RA pathway. It has been demonstrated that LMAN losses about 70% of its synaptic connections to RA between day 25 and adulthood, most of
which occurs between days 25-53 when the birds are learning their song (Herrmann and Arnold, 1991). This is accompanied by a decrease in dendritic arborization and spine densities within LMAN (Nixdorf-Bergweiler at al., 1995). Furthermore, LMAN axon branches within RA are pruned during song learning, resulting in a total LMAN-RA synapse reduction of about 80% (Iyengar & Bottjer, 2002b, Hermann & Arnold, 1991). These morphological changes are significant because they underscore the hypothesis that the transition from song learning to song stereotypy is a result of shifts in the input to RA from these projection neuron populations. HVC continues to increase in size and HVC-RA neuron number and makes more synapses with RA; meanwhile LMAN decreases in size and makes fewer synapses with RA. Over the course of the song learning process as song stereotypy increases, these changes in input likely lead to a reversal in their influence over RA.

Role of Experience on Morphology during Song Learning:

The ratio of the size of HVC to LMAN is about 2:1 in the adult zebra finch, but in early development, this picture is reversed (Herrmann & Arnold, 1991). LMAN projections to RA undergo many morphological changes that coincide with the development of song in juvenile zebra finches. Several recent studies have shown that deafening and early isolation cause a delay or prevention of normal topographic organization and development of LMAN cells (Iyengar & Bottjer, 2002a). These findings support the belief that these changes are experience dependent, and therefore, that singing experience may influence these changes. Differences in both the amount of singing and bird age have been shown to influence many
morphological attributes. In adult male canaries, it has been shown that there is a positive relationship between the amount of singing and the number of new neurons in HVC (Alvarez-Borda & Nottebohm, 2002). More recently, in our lab, it was shown in post-crystallization male zebra finches that with increasing age, there is an increase in the amount of HVC-RA arbor relative to LMAN-RA arbor. Additionally, it was shown that individuals that sang more had more HVC-RA dendritic arbor relative to LMAN-RA dendritic arbor (McDonald & Kirn, in preparation). While cause and effect remains unknown, there is a clear positive correlation between both age and singing rate and the arbor of HVC-RA and LMAN-RA neurons.

There is substantial evidence that suggests that changes in the morphology of the HVC and LMAN could be associated with changes in song stereotypy. It has been shown in juveniles that early isolation from tutors delays the normal progression of spine and soma development of LMAN cells (Wallhauser-Frank et al., 1995). Additionally, the decrease in the frequencies of dendritic spines of LMAN neurons were shown to be related to the vocal learning process, since males deprived of the opportunity to hear a tutor song exhibited high spine frequencies, whereas those given a song model displayed reduced spine frequencies on LMAN neurons (Nixdorf-Bergweiller, 2001). The question remains whether these changes in morphology of the two song system populations continue into adulthood along with changes in song stereotypy.

Song stereotypy has been shown to increase significantly post-crystallization with increasing age (Pytte et al., 2007). More specifically, analysis of bird’s individual repertoires has shown that for at least one year after song crystallization,
song becomes more stereotyped. For this reason, it can be assumed that birds of age 6-7 months will have varying levels of song stereotypy. Based on the reasoning that song stereotypy increases with increasing age, perhaps as a result of cumulative singing experience, combined with evidence that HVC:LMAN dendritic ratio increases with increasing age and amount of singing, I hypothesized that there would be morphological differences in the dendritic arbor among zebra finches associated with the amount of singing and song stereotypy. There have been no direct demonstrations of the dendritic ratio relating to stereotypy in the same birds and controlling for age and thus, I proposed that higher levels of singing would correlate with song stereotypy and both would correlate with greater dendritic arbor (average dendritic length, total dendritic length, and total number of dendritic segments) among HVC-RA cells and less dendritic arbor among LMAN-RA cells within individual birds. I predicted that there would be positive correlations between singing, song stereotypy and the ratio of HVC/LMAN dendritic arbor.
Figure 2. This schematic diagram is a sagittal view of the songbird brain, specifically indicating the song system nuclei and pathways responsible for song production, acquisition, and maintenance. The motor pathway, illustrated by black arrows begins with nucleus HVC, which receives information from auditory areas in the brain. HVC neurons then send projections to RA (robust nucleus of the arcopallium), which projects to nXII in the hindbrain, and finally these cells project to the syrinx, the avian vocal organ. The second pathway, the anterior forebrain pathway (AFP), illustrated by green arrows, involves a more complex set of nuclei, but is critical for song learning. This pathway also begins with HVC and eventually sends projections to RA, however, HVC neurons of the AFP take a less direct route. HVC neurons project first to a region known as area X, then from area X to the DLM, which projects to LMAN. The final step occurs when LMAN sends projections to the same cells in RA that receive direct input from HVC within the motor pathway. The pipette shows the path of injection into RA I used for the purpose of retrogradely labeling the 2 cell populations under study (Figure modified from Nottebohm, 2005).
Materials and Methods:

Subjects:

The experiment was performed in accordance with the Wesleyan University Institutional Animal Care and Use Committee and NIH guidelines. Subjects were ten adult male zebra finches (*Taeniopygia guttata*) between six to seven months old (176-244 days post hatch). Subjects were all reared in the aviary of the Kirn laboratory at Wesleyan University. Birds were provided with food and water ad libitum and kept on a 14:10 hour light/dark cycle while maintaining a temperature between 19-22°C. Birds were housed with their parents and other males and females until approximately 90 days of age, at which time they were moved to single sex cages housing groups of 7-15. Birds were then housed individually in open sound attenuation chambers (Industrial Acoustics Company, Bronx, NY) during the 7-day song recordings. The two chambers were kept open to allow for auditory and visual accessibility to a cage of two females placed approximately two feet in front of the two cages.

Song Recording:

All birds were recorded for 24 consecutive hours for seven days using a microphone (Omnidirectional Model 3308, Radio Shack) placed behind the cage of each bird. All songs were recorded using Avisoft (Avisoft SASLab Pro Recorder; Avisoft Bioacoustics, Berlin, Germany). Each cage had its own microphone, allowing for two simultaneous recordings through separate channels.
The last 48 hours of recordings were used to analyze singing rate. This 48-hour recording was split into two 24hr periods, allowing for two separate counts of singing rate. The total number of motifs produced during each 24-hr interval was calculated by creating syllable templates and using the batch-processing tool of Avisoft SAS Lab Pro Version 4.15 to count the number of matches. These calculations were done blind to the identity of birds. Singing rate was relatively consistent across the two days (see Figure 3), and so song rates were averaged to yield total singing rate per 24 hrs.

*Surgery:*

Following song recordings, birds were injected with fluorescent dextran amines (dextra tetramethylrhodamine 3000MW; Invitrogen, Carlsbad, CA) into RA, which backfilled HVC and LMAN neurons. All surgeries were performed within four days of the completion of song recordings. Birds were first deprived of food for twenty minutes and were then anesthetized with 0.035 mL intramuscular injections each of ketamine (Ketalar, Parke-Davis, Fort Dodge, IA; concentration 10 mg/mL) and xylazine (Rompun, Haver, The Butler Co., Columbus, OH; concentration 20 mg/mL). Each individual was secured into a stereotaxic apparatus. Feathers were then plucked from the top of the head, skin was cleaned with 70% ethanol, and a small incision was made along the anterior-posterior midline. The skin was then pulled back to expose the dorsal surface of the skull. The skull above the bifurcation in the sinus between telencephalic hemispheres and cerebellum was removed to derive the injection sites (from zero point at the bifurcation: posterior -1.7 mm, lateral
+/− 2.35 mm, ventral (from the brain surface) −1.7 mm). Small holes were made at these sites and birds received bilateral pressure-injections of 0.04 µL of Rhodamine Dextran Amine (RDA, dextratetramethylrhodamine, 10% dilution in .1M phosphate buffered saline (PBS)) using a glass micropipette (tip diameter = 40 µm) at an angle 10° from vertical to avoid passing through HVC with the injection pipette on its path to RA. Following injections, the skin was moistened with PBS and the incision was closed with adhesive surgical tape. Birds were then given an intramuscular injection of .35 mL Yohimbe (.5mg/ml, Lloyd Laboratories, Shenandoah, IA), an agent intended to speed recovery from ketamine/xylazine anesthesia. Birds recovered in a small holding cage under a heat lamp for 24 hours and were provided with an additional egg mixture to aid recovery. Birds remained in these cages until they were perfused four days later.

*Perfusions and Tissue Processing:*

Birds were deeply anesthetized with an overdose of methoxyflurane (Metaphane; Mallinckrodt Inc., Mundelgn, IL). When unresponsive to a toe pinch, birds were perfused with 20 mL of 0.1M PB (pH 7.4) followed by 50 mL of 4% paraformaldehyde (in .1M PB, pH 7.4). A small incision was made below the rib cage and the skin was cut around the rib cage to lift the rib cage and expose the heart. The right atrium was then cut and a small needle was inserted into the heart. Perfusions were done through the left ventricle. Brains were then removed from the skulls and were post-fixed in 4% paraformaldehyde at 4°C for 48 hours.
Once removed from the fixative, brains were rinsed with deionized water and stored at 4° C in 1X PBS until cut. Brains were then cut into two separate hemispheres along the midsagittal plane. Using a vibratome, each hemisphere was cut into seventy-five µm thick sagittal sections and all those sections containing LMAN and/or HVC cells were mounted on superfrost slides and immediately cover-slipped with Aqua Mount (Polysciences, Inc., Warrington, PA.) Slides dried overnight in darkness and were then moved into slide boxes and stored at room temperature until analyzed.

Song Analysis:

Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu/sound_analysis_pro) is a computer software program that calculates a similarity score based on point-to-point comparisons of two sound patterns across 50-ms time frames centered at successive 7-ms intervals (Pytte, 2007). Similarity scores are based on measurements of three features; Wiener entropy, pitch, and mean frequency modulation, which are first converted into a common unit that can be compared across features. The average similarity that is calculated is output as “accuracy”, but will be referred to as stereotypy score for the purposes of this thesis.

The stereotypy of acoustic structure was analyzed using motifs produced over a 5-hour time window during the last day of each recording session between the hours of 7 AM and 2PM, a period which began 2 hours after lights on, and ended 7 hours before lights off. For each individual and each recording session, 14-35 motifs were compared using a matrix design such that each motif was compared with each of the
others (excluding duplicate comparisons). For example, if 25 motifs were compared, there would be a total of \(25 \times 24/2\) comparisons (300 comparisons, \(((n \times (n-1)/2))\)). Once all the similarity score comparisons were run, a mean was then calculated for each individual bird. In one recording session, one bird produced only 12 motifs with identical syllable sequences that could be used to calculate stereotypy. However, when using the same time window from the previous day of recording (comparing 25 motifs), the average stereotypy score was very similar to the stereotypy score from the 12 motifs, and therefore the scores presented are from the appropriate day and time window (day 2 between 7AM-2PM).

Motifs were chosen based on their location within the song bout and by their syllable structure. All first and last motifs were ignored, as these motifs are commonly found to have the most variability and thus do not accurately represent the bird’s peak stereotypy (Pytte et al., 2007). Additionally, all motifs were chosen where the same syllables were produced in the same order. Any motifs with deleted or additional syllables were not included. As a result, the stereotypy score for the motif provided an average measure of mean similarity in acoustic features based on a syllable-by-syllable comparison of like syllables. More simply stated, the stereotypy score provided is a measure of similarity in acoustic structure of multiple renditions of the same sequence of syllables.

*Spectral Features:*

Once stereotypy scores were found for each bird, the specific parameters that, when combined, generate the stereotypy score were analyzed independently. These
included mean Wiener entropy (expresses randomness in the frequency domain), mean pitch (measure of sound periodicity), and mean frequency modulation (slope of changing frequencies). In order to identify the particular acoustic features that contributed to the change in stereotypy scores, each syllable was measured for these three features, a standard deviation was calculated for each syllable and then an average standard deviation across the motif repetitions was calculated. The standard deviation presented is an average across all song files used to calculate stereotypy score. In addition to these three variables, the tonality of syllables (termed ‘‘pitch goodness’’ in Sound Analysis Pro) was also measured.

Temporal Features:

In order to ensure the stereotypy score produced was a result of differences in acoustic structure, and not the result of differences in song delivery speed, temporal features were measured for each bird. Mean motif duration, mean syllable duration, and mean inter-syllable duration were all measured. Motif duration was measured from the start of the first sound element to the end of the last element, including all syllables and inter-syllable intervals. Syllable duration was determined by measuring the length of each syllable individually. Mean inter-syllable duration was determined by subtracting the sum of the syllable durations from the motif duration and dividing by the number of inter-syllable intervals. Once all measures were calculated, an average standard deviation was used to determine variation of tempo.
**Microscope Analysis and Data Collection:**

All microscope analysis was done blind to the individual. Using dark-field optics on a fluorescent microscope (Olympus BX50), and computer software (Neurolucida software, MicroBrightfield Inc., Williston, VT) the boundaries of HVC and LMAN were outlined using a 10X objective. Once contours were traced at 10X magnification, tracings of LMAN and HVC labeled cells were done using a 40X objective. For each bird, fifteen to thirty cells per region were analyzed using the computer-yoked microscope system, and Neurolucida software. Cells were chosen based on three criteria; they were brightly labeled with fluorescence, it was determined through scrolling in the z-plane that their branches did not suddenly terminate in the same focal plane, and their branches did not overlap with other cells, making them easily identifiable. Once all cells were traced, NeuroExplorer (MicroBrightfield Inc., Williston, VT) was used to determine the average area and perimeter of each soma, the total and average dendritic length, the dendritic segment length, and the number of segments by branch order. These numbers were then copied into Excel spreadsheets and averages were calculated for each bird in order to determine the amount of dendritic arbor for each cell type and to calculate the ratio of HVC/LMAN dendritic arbor.

**Statistics:**

To determine whether there was a correlation between song stereotypy and singing rate, and between these behavioral measures and dendritic arbor attributes, multiple sets of regressions were run using Microsoft Excel. Linear regressions were
performed between LMAN-RA dendritic arbor, HVC-RA dendritic arbor, and the ratio of HVC-RA: LMAN-RA dendritic arbor against both singing rate and song stereotypy.

**Results:**

**Behavior:**

The singing rate used for comparisons was based on the average singing rate across the last two days of recording. The range was 45 songs to 1869 songs per day, providing a large variation in singing rate. The average song stereotypy score also spanned a relatively large range from 81.83 to 89.33 (on a scale of 1-100).

**Temporal Features:**

As previously discussed in the Methods section, it was important to determine the main contributors to the stereotypy score produced by Sound Analysis Pro. The best way to determine whether the stereotypy score was a result of changes within the acoustic structure of the song, temporal features within the song had to be individually assessed. Several regressions were run comparing stereotypy to a variety of temporal features. These features included the standard deviation of motif duration, standard deviation of syllable duration, and the standard deviation of inter-syllable interval duration. No significant relationships were found between any of these measures and the song stereotypy score, which is strong evidence that the similarity score cannot be accounted for by temporal features. The closest
relationship found was between stereotypy and syllable duration, but this was still far from a significant correlation (p = .18).

*Acoustic Structure:*

In order to determine whether there was a relationship between average singing rate, song stereotypy and multiple song attributes, pair-wise correlations were run with stereotypy, standard deviation of entropy, standard deviation of motif duration, average singing rate, standard deviation of pitch, and standard deviation of FM. Among all these comparisons, there were only two correlations that approached significance, stereotypy versus entropy (p=.09), and entropy versus FM (p=.055). Two important conclusions can be drawn from these results. The first is that there was no significant relationship found between singing rate just prior to surgery and overall song stereotypy. The second conclusion was that there was no single acoustic attribute that could predict stereotypy score. However, it is possible that variation in entropy and FM were greater contributors than the other features.
<table>
<thead>
<tr>
<th></th>
<th>Stereotypy score</th>
<th>St dev entropy</th>
<th>St dev duration</th>
<th>Singing rate</th>
<th>St dev pitch</th>
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Table 1. This table displays a song attribute pair-wise matrix. The values displayed are the $R^2$ values for each attribute comparison. The values highlighted in red and blue display the $R^2$ values with their corresponding $p$-value below for the correlations that were close to significance.
Brain Morphology

The morphology of the brain regions can be visualized by looking at Figure 4 found below. These images are photomicrographs taken with a fluorescence confocal microscope. (A) Low power magnification fluorescence confocal image of a seventy-five \( \mu \text{m} \) parasagittal section of RDA backfilled HVC. (B) Low power magnification fluorescence confocal image of a seventy-five \( \mu \text{m} \) parasagittal section of RDA backfilled LMAN. (C) High power magnification fluorescence confocal image of a seventy-five \( \mu \text{m} \) parasagittal section of an HVC backfilled neuron. (D) High power magnification fluorescence confocal image of a seventy-five \( \mu \text{m} \) parasagittal section of an LMAN backfilled neuron.
Figure 4. These images are photomicrographs taken with a fluorescence confocal microscope. (A) Low power magnification fluorescence confocal image of a seventy-five µm parasagittal section of RDA backfilled HVC. (B) Low power magnification fluorescence confocal image of a seventy-five µm parasagittal section of RDA backfilled LMAN. (C) High power magnification fluorescence confocal image of a seventy-five µm parasagittal section of an HVC backfilled neuron. (D) High power magnification fluorescence confocal image of a seventy-five µm parasagittal section of an LMAN backfilled neuron. Scale bars for all images are 50µm. Images courtesy of Kate McDonald.

Soma Size:

Average soma size was not found to be correlated to either song stereotypy or singing rate (SR) for either LMAN (SR: p=. 170, Stereotypy: p = .209), HVC (SR: p=. 808, Stereotypy: p = .140), or the HVC/LMAN ratio (SR: p=. 749, Stereotypy: p = .193). Additionally, a comparison was run between average soma size and total dendritic length for both HVC and LMAN and no relationship was found. It can be
concluded that within 6-7 month old birds, there was no relationship between soma size and any other measured feature.

*Total Dendritic Length vs. Stereotypy:*

Figure 5a shows the relationship between total dendritic length of LMAN neurons plotted against song stereotypy, Figure 5b shows the relationship between total dendritic length of HVC cells and song stereotypy, and 5c shows the relationship between the ratio of total dendritic length for HVC/LMAN and stereotypy. There was a significant negative correlation between song stereotypy and the total dendritic arbor of LMAN-RA cells ($R^2 = .598$, $p=.008$), and a significant positive correlation between song stereotypy and HVC-RA cell total arbor ($R^2 = .516$, $p=.019$), and the ratio of HVC-RA: LMAN-RA cell arbor ($R^2 = .587$, $p= .0098$). There was also one low outlier, which was removed to ensure it was not artificially inflating the correlations. When this outlier was removed, the significant relationships found actually increased, confirming the significant relationship found. The results of one of these correlations can be found in Figure 5d.
Figure 5. A) shows the relationship between total dendritic length of LMAN and song stereotypy, B) shows the relationship between total dendritic length of HVC and accuracy, C) shows the relationship between total dendritic length of the ratio of HVC/LMAN and song stereotypy. D) shows the same relationship as C) omitting the outlier, and the already significant relationship increases ($R^2 = .7605$, $p = .002$), confirming the significant relationship found.
Total Dendritic Length versus Singing Rate:

Figure 6a shows the relationship between total dendritic length of LMAN neurons plotted against singing rate, Figure 6b shows the relationship between total dendritic length of HVC cells and singing rate, and 6c shows the relationship between the ratio of total dendritic length for HVC/LMAN and singing rate. There was a just significant correlation between singing rate and total dendritic arbor of HVC cells ($R^2 = .409$, $p = .046$) and the ratio of HVC-RA:LMAN-RA cells ($R^2 = .417$, $p = .0436$). There was no relationship between singing rate and total dendritic arbor of LMAN cells. However, there was one outlier in the analysis (see Figure 6d) and when removed from the regression, there were no significant correlations between singing rate and any dendritic arbor measurements (HVC: $R^2 = .026$, $p = .728$; HVC/LMAN: $R^2 = .018$, $p = .728$). As a result of the small sample size used in this study, it is important to examine and remove significant outliers, as they can easily cause false correlations to appear in the data.
Figure 6. These three graphs show the relationship between singing rate (total songs per 24hr) and total dendritic length of LMAN-RA cells, HVC-RA cells, and the ratio of HVC/LMAN cells. There was no relationship found between LMAN–RA cells and singing rate (A), however there was a just significant relationship between their HVC-RA cells (B, \( R^2 = .409, p = .046 \)) and their ratios and singing rate (C, \( R^2 = .417, p = .044 \)). However when the outlier was removed, these relationships disappeared. Graph D shows the non-significant relationship once the outlier has been removed.

**Dendritic Segment Order Versus Song Stereotypy:**

Individual regressions were run comparing the number and length of dendritic branches by segment order (see Table 2). Significant correlations were found
between dendritic length at some (but not all) branch orders and song stereotypy. When comparing HVC morphology to stereotypy, significant relationships were found for 2\textsuperscript{nd} order length (R$^2$ = .495, p = .023) and 3\textsuperscript{rd} order length (R$^2$ = .479, p = .026) and number (R$^2$ = .61, p = .008). When comparing LMAN neuron morphology to stereotypy, significant relationships were found for 1\textsuperscript{st} order length (R$^2$ = .61, p = .008) and 2\textsuperscript{nd} order length (R$^2$ = .61, p = .008). The largest number of statistically significant relationships was found between the HVC/LMAN ratio and stereotypy, suggesting the ratio of dendritic attributes contributes most to overall song stereotypy. Significant relationships were found for the length ratio of 2\textsuperscript{nd} (R$^2$ = .505, p = .021), 3\textsuperscript{rd} (R$^2$ = .790, p = .0001), and 4\textsuperscript{th} (R$^2$ = .624, p = .011) branches.

*Dendritic Segment Order Versus Singing Rate:*

Individual regressions were also run comparing the number and length of dendritic branches by segment order to singing rate (see Table 2). When comparing HVC morphology to singing rate, only one significant positive relationship was found, which involved the number of 4\textsuperscript{th} order branches (R$^2$ = .76, p = .001). When comparing LMAN morphology to singing rate, there were no significant relationships found. As with the total dendritic length findings discussed above, the most significant relationships were once again found between the ratio of HVC/LMAN and singing rate. Significant correlations were found between both the number of 3\textsuperscript{rd} (R$^2$ = .473, p = .028) and 4\textsuperscript{th} (R$^2$ = .764, p = .002) order branches and singing rate.
### HVC vs. Stereotypy

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### LMAN vs. Stereotypy

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### Ratio vs. Stereotypy

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Table 2. This table displays the p-values and R-squared values comparing dendritic attribute to song attributes. Comparisons were made by branch order for both length of branches and number of branches compared to song stereotypy and singing rate. The numbers in bold represent the insignificant correlations.
Discussion:

Overview:

Previous research showed that with increasing bird age, as well as with increased singing rate, there is a significant increase in the amount of HVC-RA dendritic arbor relative to LMAN-RA dendritic arbor (McDonald & Kirn, in preparation). In this work it was inferred that singing rate is an indirect measure of song stereotypy. However, no direct measures comparing the relationship between neuron morphology and song stereotypy were made. The data presented here are the first to directly link dendritic arborization and song stereotypy.

Soma Size:

Previous work has shown that there is a significant decrease in soma size of both LMAN and HVC cells with increasing age post crystallization (McDonald & Kirn, in preparation). The lack of any significant correlations between soma size and stereotypy or singing rate in the present work suggests that while overall soma size is influenced by age, soma size likely has little relationship to the song attributes measured. This is consistent with previous work from our lab, which found differences when comparing groups across a large age range (3-4, 6-7, and 11-12 months), but no significant correlations between dendritic arbor and soma size (McDonald & Kirn, in preparation). This study looked at only one age group, and found little variability in soma sizes across the birds.
Singing Rate:

While some analyses of dendrite segments yielded significant correlations with singing rate, the lack of any significant relationships between total dendritic arbor and singing rate is inconsistent with my hypothesis and earlier work performed in this laboratory. This previous research found that there was a correlation between singing rate and the ratio of dendritic arbor of HVC/LMAN in post crystallization zebra finches (McDonald & Kirn, in preparation), however, the measurement used to assess singing rate in these earlier studies was taken over a longer interval. These data suggest that cumulative singing rate (or more than two days worth of sampling) may be a better predictor of neuron morphology, and that a sampling of the singing rate over a short two-day period is simply insufficient to demonstrate this relationship.

One possible explanation for the lack of significance found between acute measures of singing rate and neuronal morphology could be a result of variable rates of development. For example, birds who sang more earlier in life and thus have higher stereotypy scores could be decreasing their singing rate later in life, whereas other birds who sang less when younger, and have less cumulative singing experience have to catch up, and therefore are now singing at much higher rates.

Measuring total singing experience would likely give a better correlation. In a follow-up study, it would be crucial to measure singing rate over the birds lifetime, instead of restricting the analysis to the two days prior to surgery. For example, by recording singing rate for one day each week over a period of many weeks or months, the measurements would much more accurately represent the cumulative singing
experience over the bird’s lifetime. Such a measurement would be a reasonably reliable indicator of the bird’s total practice time and thus would likely provide a better test of the relationship between singing rate and song stereotypy.

*Stereotypy:*

The significant relationships found between the dendritic morphology of HVC-RA and LMAN-RA cells and song stereotypy are consistent with my hypothesis and previous work conducted in this lab. In independent studies, previous work showed age related increases in song stereotypy (Pytte et al., 2007) and in the ratio of HVC:LMAN arbor (McDonald & Kirn, in preparation). This study compliments and extends these results, by establishing a connection between song stereotypy and dendritic arbor in the same birds. This further enhances our understanding of how these two input pathways to RA might work together to improve motor output.

As discussed earlier, HVC is known to contribute to motor program stereotypy, whereas LMAN plays a crucial role in motor variability. Therefore by increasing dendritic arbor of HVC-RA cells, and decreasing dendritic arbor of LMAN-RA cells, the overall ratio change in dendritic length provides the potential for differential weighting of synapses in RA due to the greater input to HVC-RA cells than to LMAN-RA cells. As a result of the greater input to HVC-RA cells, one could speculate that this might lead to less variability in the signals HVC sends to RA, which could lead HVC to exert a stronger influence over RA. The strong correlation
between the ratio of total dendritic arbor and song stereotypy found in this study provides one potential neuronal mechanism for adult differences in song stereotypy.

Taken together with previous work that song-related morphological changes continue well beyond crystallization (McDonald & Kirn, in preparation), this work further challenges the notion that learning and neuronal change end with crystallization in close-ended song learners. While there may still be developmental distinctions between close-ended and open-ended learners with respect to the critical period, this thesis, when combined with earlier work from our lab, suggests the differences between these two classes of song learners are much smaller than previously thought.

Methodological Issues:

The primary reason for selecting RDA as the tracer for analyzing the morphological attributes of HVC-RA and LMAN-RA neurons over other neuron tracers is that RDA allows for visualization of particular target populations of cells and there are currently no immunohistochemical markers that selectively label these two cell populations in their entirety. Both HVC and LMAN cells have projections to RA, and by injecting RA and backfilling these cell populations with RDA, HVC-RA and LMAN-RA cell populations are targeted. Additionally, by using a backfill process, both target populations could be labeled with a single injection. Although there were several cases when one region received stronger labeling than the other region, only birds with traceable backfill in both HVC and LMAN were used for analysis.
Although there are many advantages to using RDA compared to other visualization techniques, there are also a number of potential limitations that accompany its use. The most important of these limitations is that the use of RDA provides no direct information about the changes in synapses with RA, thus restricting the analysis to measurements of the soma and dendrites of HVC and LMAN cells. Although no measurements of HVC or LMAN synapses with RA could be observed in this study, the results still provide important information, as it is well understood that the greater the amount of dendritic arbor, the greater the potential for synapses on these dendrites.

Another important concern associated with the use of RDA involves the efficacy of the backfill produced. For example, if the tracer does not adequately reach the ends of the dendritic branches, the results could be due to the efficiency of the retrograde transport, rather than a true measure of the dendritic morphology. If the relationship found was caused by the efficacy of the tracer, then we might expect to see relationships between song attributes and dendrites only with the highest order branches, as those branches would be most impacted by variation in transport efficiency. To address this issue, we looked at the relationship of the lower order branches, to song stereotypy, limiting the analyses to those cells that also had higher order branch labeling. In this way, we could assess whether tracer efficiency might account for our results. In other words, was the length of lower order branches still related to song stereotypy when analyses were restricted to cells that also had higher order branches labeled? When this hypothesis was tested with HVC cells by branch order, there remained a significant relationship between second order branch length
and song stereotypy (p = .013), and the relationship between song stereotypy and third order branch length approached statistical significance (p = .08). The lower p value in this latter case may be due to a sample size issue, as there were fewer cells with both third and fourth order labeling. Thus, it is unlikely that the significant brain-behavior relationships found were the result of variation in the extent of backfilling. Additionally, further support that the relationships found were not caused by the efficacy of the tracer is that many cells, within LMAN in particular, had comparably filled 5th order branches. Although no fifth order segments were actually used for analysis, as there were too few birds with enough 5th order branches make good comparisons, the mere fact that fifth order branches appeared to be as thoroughly filled as lower order branches in those cells with little arbor, supports the claim that the tracer was able to sufficiently fill even 5th order branches when such branches were present.

The most significant shortcoming of this study is the small sample size. Provided with more time, it would have been beneficial to have a larger sample size. However, due to various difficulties encountered with certain procedures, and the limited time available, only ten subjects could be analyzed for this study. Moreover, many of the trends observed were very strong, suggesting that the sample size of 10 was sufficient to address the main questions of this thesis.

Additionally, although all males were placed in individual cages, with both visual and auditory accessibility to a cage of female birds placed two feet in front, it is possible that song recordings could be a mix of directed and undirected song. A male facing and singing in the direction of a female defines directed song, which is
more stereotyped than undirected song. Though it is assumed that the presence of two females would result in predominantly directed song, there were no visual recordings of the birds during singing, and so it is possible that undirected song was included in the analysis of song stereotypy. While this could play into the overall stereotypy score of individual birds, it is also likely that the affect of this would be insignificant, as the likelihood of including undirected song would be similar across all individuals. It is also likely that undirected song was relatively rare, based on previous work where birds were monitored visually and it was found that 85-100% of song was directed toward females (Teramitsu & White, 2006; Jarvis et al., 1998).

Future Directions:

This study found a significant relationship between dendritic arbor and stereotypy, however, there were no data collected from which to determine whether song stereotypy is driving morphology or whether morphology is driving song stereotypy. To examine more directly the relationship between cause and effect, a new study could be done to disrupt song stereotypy, either by deafening or ts-nerve cuts, in post-crystallization zebra finches. The study could compare morphology of the song system populations of deafened birds against controls with a large sample size. By including multiple groups, this proposed study could allow for an analysis of neuronal morphology at various (post-deafening) time intervals to observe how deterioration of song would correlate with changes to dendritic arbor. Using variable survival times, one might be able to determine which changes first, song structure or song system neuronal morphology.
One theory that would be interesting to test would involve deafening birds and observing the affect on the dendritic arbor ratio of HVC/LMAN. Changes in song after deafening are believed to be driven by LMAN, as LMAN is influencing song variability. Therefore, by deafening birds, one would expect the ratio of HVC/LMAN to be flipped, as LMAN’s influence over RA would become stronger.

Another direction for future study on this subject would be to investigate alternative effects, such as circulating blood testosterone levels. By collecting blood samples and analyzing the amount of circulating testosterone, such a study could address the influence of a possible confounding variable that could be driving, or contributing to the relationships observed between stereotypy and neuron morphology.

In addition to looking at the dendritic arbor of HVC and LMAN cells, it would also be helpful to conduct a follow-up study looking at the changes in synapse number in RA to see if the changes seen in the dendrites relate to RA synapses. Following Hermann and Arnold’s protocol (Hermann & Arnold, 1991) it would be possible to identify and count the number of synapses from either HVC or LMAN. This study would provide even more convincing evidence that the changes in song stereotypy are influenced by changes in the weighting of input from HVC and LMAN to RA.

**Conclusion:**

This is the first study to show a relationship between dendritic arborization and song stereotypy in post-crystallization zebra finches. The results of this study will
significantly improve our understanding of the link between brain morphology and song learning. The strong correlations found between HVC, LMAN, and HVC/LMAN arbor and song stereotypy strongly support the hypothesis that the two primary inputs to RA are influencing the motor output of RA, and subsequently, overall song stereotypy. In summary, the results reported in this thesis provide strong additional support for the idea that changes in song stereotypy are directly linked to morphological changes in the underlying neural populations of the song system.

**Acknowledgements:**

I would like to thank Professor John Kirn for all his guidance and support throughout this research project. I would also like to thank the rest of the Kirn lab, especially Kate McDonald for her help and patience throughout this project. Additionally, I would like to thank my father, for his encouragement and emotional support through all the ups and downs.
Literature Cited:


