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THE ROLE OF THE CEREBELLUM IN THE ZEBRA FINCH SONG CIRCUIT

by
Matthew DiGiusto

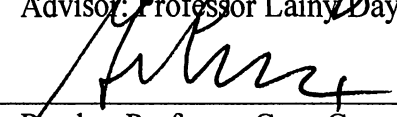
A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2011

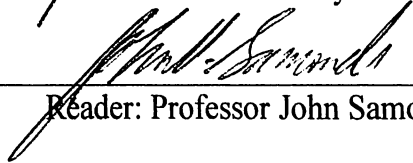
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ABSTRACT

The cerebellum was originally thought to be a brain area used just for motor coordination, but it is now known to play a role in complex cognition. Some of these complex roles include aspects of human speech such as rhyming, silent rehearsal, and timing of speech. There are many similarities between human speech and songbird song including a babbling stage, the necessity of a tutor to copy, and specialized neural structures for learning and production; however, it is not clear whether the cerebellum plays a similar role in the songbird song circuit. In this experiment I examined the role of the cerebellum in the zebra finch song circuit. The song of male zebra finches is directed towards receptive females, and was recorded over several consecutive days. Surgery was then performed on the birds to disrupt the connection between the cerebellum and the rest of the brain. The songs were then recorded post surgery to deduce any cerebellum control on the song circuit. The results of the data show that after cerebellum lesion, birds were able to retain and faithfully reproduce their learned song; however their song had increased variability in its spectral qualities and additionally their song was longer in duration. These results are very similar to what is seen in humans with cerebellar dysarthria, which includes various deficiencies in speech that are seen after damage to the cerebellum. These data suggests that the cerebellum plays similar roles in the songbird song circuit, just as in human speech.

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LIST OF ABBREVIATIONS

AFP	anterior forebrain pathway
AM	amplitude modulation
CbM	medial nucleus of the cerebellum
DLM	dorsolateral division of the medial thalamus
FM	frequency modulation
fMRI	functional magnetic resonance imaging
FoxP2	forkhead box P2 gene
lMAN	lateral part of the magnocellular nucleus of anterior neostriatum
MAD	measure of deviation
nXIIIts	tracheosyringeal part of the hypoglossal nucleus
RA	robust nucleus of the arcopallium
shRNA	short hairpin RNA
SAP	Sound Analysis Pro
USVs	ultrasonic vocalizations
.wav	Waveform Audio file
.wmv	Windows Movie Video file

INTRODUCTION

The purpose of the present study was to determine the role of the cerebellum in the zebra finch song circuit. The cerebellum is a part of the vertebrate brain known to be involved in motor coordination across species. While the number of brain regions recognized to be part of the avian song circuit have expanded since its initial discovery (Kao & Brainard, 2006), the cerebellum is not currently believed to be one of the brain regions involved (Figure 1).

Courtship displays performed by males to attract mates vary from elaborate physical displays to complex vocal songs in the Passeriformes order of birds. For example, manakins (suborder Suboscine, family Pipridae) have elaborate courtship displays that may include wing whirring, wing popping, and even cooperation among males (Fusani *et al.*, 2007) but their vocal output is limited to chirps and other simple unlearned calls. True songbirds (suborder Oscines) have courtship displays that consist of a learned vocal and physical component though the complexity of these can vary widely within this suborder. Bowerbirds (family Ptilonorhynchidae) are known for their multiple display traits that include the building of a specialized stick structure, called a bower, as well as intense postural displays and songs that may mimic predators, cars, and chain saws all driven by female preference (Coleman *et al.*, 2004). Compared to the bowerbirds, zebra finches (*Taeniopygia guttata*) have a much less elaborate courtship display and song. The zebra finch courtship display consists of a nasally repetitive song directed at a female and accompanying small hops while pivoting (Morris, 1954). Not exactly the peacock of the songbird world. However, it is the simplicity of the zebra

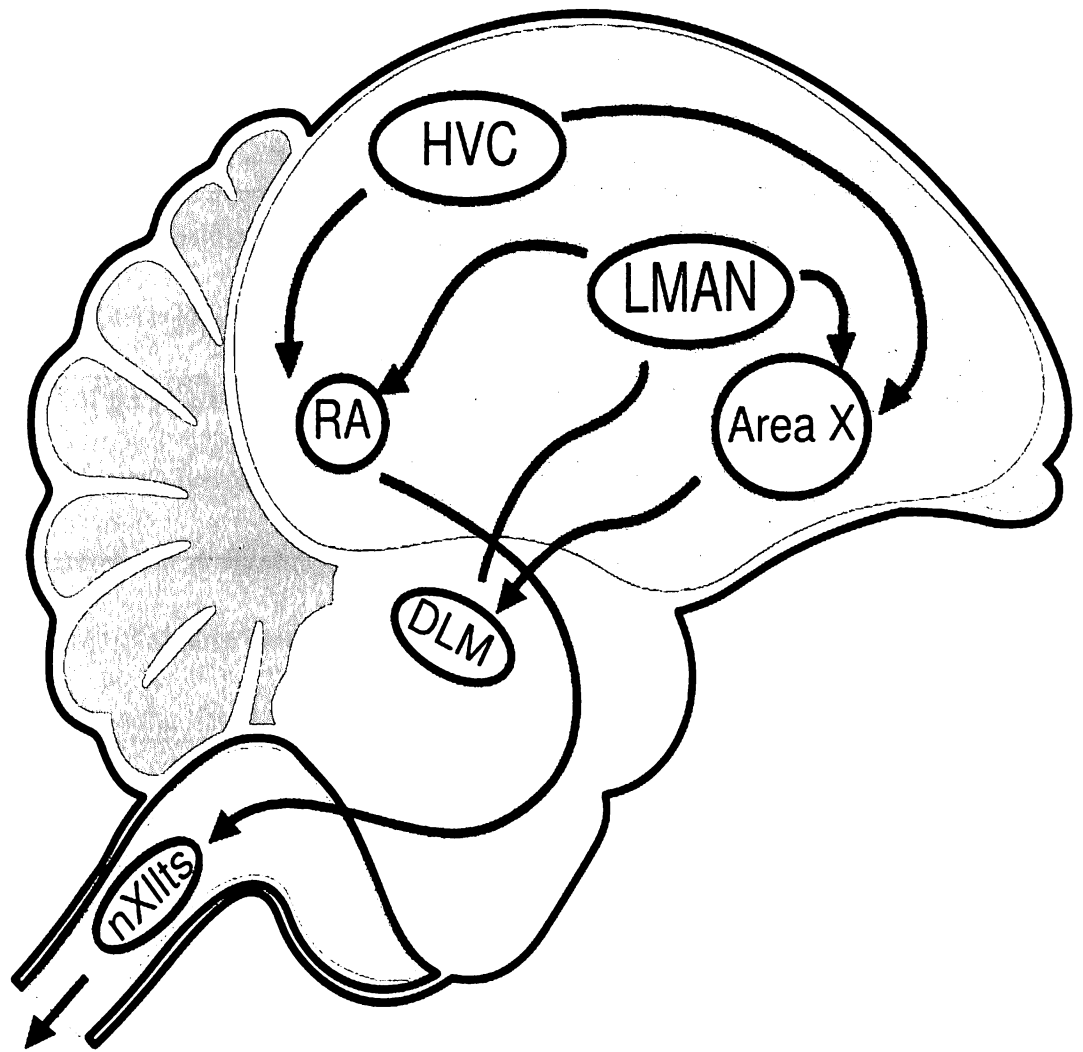


Figure 1 – Song Learning and Production Pathways in Songbirds (adapted from Nottebohm, 2005)

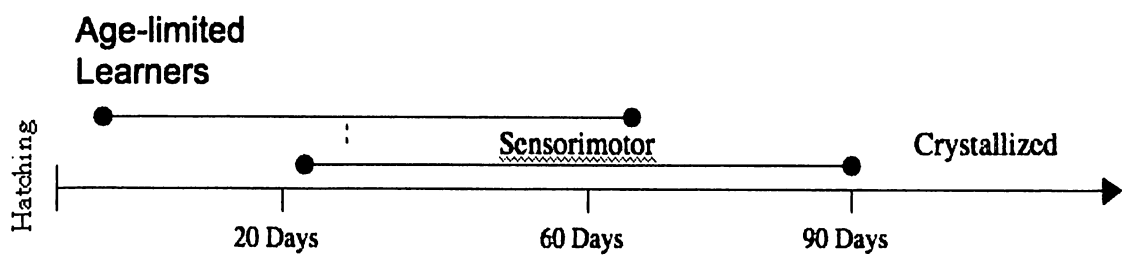


Figure 2 – Timeline of Song Learning

finches song as well as the extensively studied mechanisms for song learning that make the zebra finch exquisite for studies of how vocalizations are acquired.

The zebra finch song is learned from a tutor, usually the father, and there is a particular period after hatching, 25 to 65 days, when learning of the song can occur (Roper & Zann, 2006) (Figure 2). This pattern is similar to the way that humans learn speech and one of the prime reasons that the neural underpinnings of vocal learning, the “song circuit”, of zebra finches has garnered so much attention.

The reason for investigating the role of the cerebellum in the song circuit is that there are a number of studies that either explicitly state or imply a link between the cerebellum and the song circuit. The main points behind the belief that the cerebellum may be involved in the song circuit are as follows: (1) There is a necessity to coordinate song with the closely associated physical display, “dance”; (2) It is theorized that vocal learning evolved from motor systems; (3) There is a reported link between a part of the songbird song circuit necessary for vocal learning (Area X) and the cerebellum (Person *et al.*, 2008); (4) There is a high expression of FoxP2, a gene known to play a role in human speech, in the avian cerebellum; and (5) There is a known role of the cerebellum in human speech and many similarities between human speech and avian song learning. A better understanding of the zebra finch song and how it is learned is necessary before delving deeper into each of these topics individually.

Song learning in what are known as close-ended or age-limited passerine birds, such as the zebra finch, is a complex process that is divided into several distinct phases (Immelmann, 1969). First, young birds listen to both directed and undirected song from adults around them. Directed song is that sung by a male that is directed towards a female

as part of a pre-copulatory courtship display. Undirected song is that sung by a male where no female is present and/or no display is involved (Roper & Zann, 2006). Next, juveniles will begin to make their own vocalizations that, after practice, begin to sound more like songs of the adults around them. These initial vocalizations are called subsong and have been likened to the babbling of a human infant. This is one of the reasons that zebra-finch song learning has been compared to human speech learning. Finally, as the birds mature, their song becomes less variable until it is ultimately stereotyped (Franz & Goller, 2002). Zebra finch males follow this general pattern for vocal learning and develop their song during a time known as the sensitive period, between 30 and 90 days after hatching. Once the male zebra finch reaches sexual maturity its song becomes fully crystallized and does not change under normal circumstances (Immelmann, 1969).

Songbird songs are composed of several different, individual parts. Songs are usually portioned into smaller segments known as syllables. Tchernichovski *et al.* (2001) defines a syllable as “continuous sounds preceded and followed by silent intervals or by abrupt changes in frequency.” Despite the relative simplicity of the zebra finch song, it is more difficult to separate into syllables than most songbirds, because their song has many changes in frequency without intervening silent intervals. Juvenile zebra finches learn songs by copying syllables, either individually or as a series of consecutive syllables (known as a string), from adult male tutors. The reason for dividing a song into smaller units, such as syllables, is twofold: (1) the analysis of a song, such as the scoring of song similarity, can be made easier through the division of the song into syllables (Tchernichovski *et al.*, 2001), and (2) it has been shown that the syllable is the smallest unit of song that is transferred from tutor to juvenile (Franz & Goller, 2002). Juvenile

zebra finches imitate tutor songs by copying syllables or strings of syllables and it has been found that string boundaries are at the end, and not within, syllables (Franz & Goller, 2002). Only on rare occasions are syllables broken into smaller units of learning, such as notes, leading to the creation of new syllables. A juvenile's song may differ from its tutor's, typically because of insertion, deletion, or rearrangement of syllables rather than changes in notes (Williams, 2001). While a juvenile's song may differ from its tutor's due to these possible syllable rearrangements, the two songs should still score relatively high in similarity due to the juvenile's song containing only syllables learned from the tutor. When learning a song a juvenile may take syllables, or a string of syllables, from its father; however, it is also possible that a juvenile will hear the song of several different males and compile them to create a unique song. While it is possible that a juvenile will compile a unique song, it has been shown that there is strong bias towards the copying the father's song (Roper & Zann, 2006). The current hypothesis is that preference for the father is due to a filial bond formed because only the father provides parental care and roosts in the nest with its fledglings.

The male zebra finch courtship display consists of a song and accompanying body movements directed at a female. These movements, termed a dance, are characterized by specifically choreographed hops and changes in body orientation and head position. Williams (2001) failed to see a strong pattern in the relationship between song and dance in individual birds; however, a pattern emerged when dance movements were compared among birds with similar songs. Sons that copied their father's song initiated dance movements at the same points within the song. Additionally, it was shown that a systematic pattern of dance movements did not emerge reliably from song to song, but

rather infrequent dance movements appear to be initiated at a number of specific ‘hot spots’ within a song (Williams, 2001). The distribution of dance movements represent a stereotyped pattern even though the initiation of the dance movements do not appear to be strongly associated to a particular syllable within a song (Williams, 2001). The presence of these ‘hot spots’ within a song suggests that dance patterns are learned in correlation with the song; thus, dance pattern is an attribute of the song and may be compared to the tutor’s dance pattern, even if the juvenile’s song differs from the tutor’s song due to insertion, deletion, or rearrangement of syllables. Similar dance movements should occur at the same position in each copy of the song, as this is the identified ‘hot spot’. Additionally, it may be expected that if a syllable is a ‘hot spot’ for dance movements, then these syllables should not be deleted from the tutor’s original. The cerebellum is the part of the brain that is responsible for sensory integration and the coordination and timing of motor control. This means that if the cerebellum is responsible for the timing and motor control necessary to perform a stereotyped dance and there is a linking of dance movements with the song, then there should be a connection between the song circuit and the cerebellum. The close association between song and dance necessitates the coordination and timing of the neural circuitry controlling each aspect. In addition to this behavioral evidence there is also an evolutionary theory that implicates cerebellum involvement in the song circuit.

The ability to memorize a template song, produce song, and modify vocal output to mirror the template indicates that there must be neural circuitry present for these complex tasks. Vocal learning is a rare trait found in three distantly related groups of birds (parrots, hummingbirds, and songbirds) (Nottebohm, 1972). It is likely that there

was anatomical similarity in ancestral avian brains that would allow vocal learning since it independently evolved in three separate groups of birds. The current theory for vocal learning origin is that it evolved out of specialization of pre-existing motor systems (Feenders *et al.*, 2008). This hypothesis proposes that previous motor nuclei have specialized to song learning. These vocal nuclei are now collectively known as the ‘song circuit.’ The song circuit is divided into two interconnected circuits: (1) the posterior vocal motor; and (2) the anterior forebrain pathway (AFP) (Nottebohm *et al.*, 1976). The posterior vocal motor pathway is composed of the HVC (formerly known as the *hyperstriatum ventrale, pars caudale*, or high vocal center), robust nucleus of the *arcopallium* (RA), and the *tracheosyringeal* part of the *hypoglossal* nucleus (nXIIIts). Lesioning the HVC, RA, or nXIIIts results in serious defects in song production, demonstrating that the posterior vocal motor pathway is responsible for the production of song (Nottenbohm *et al.*, 1976). The AFP is composed of the lateral part of the magnocellular nucleus of anterior neostriatum (IMAN), Area X, and the dorsolateral division of the medial thalamus (DLM). The AFP is innervated by the posterior vocal motor pathway via the HVC (which innervates Area X) (Nottenbohm *et al.*, 1976). Lesions of IMAN or Area X result in abnormal songs that do not crystallize, demonstrating that the AFP is responsible for song learning (Scharff & Nottebohm, 1991).

These vocal learning areas are embedded in motor regions. For example, Area X is part of the avian equivalent of the basal ganglia, the brain region damaged in Parkinson’s disease, and RA is part of an area that sends inputs to premotor neurons of the lower brain stem and back to areas around Area X. While those positing a motor

origin for vocal learning pathways recognized that various movements in birds activate the cerebellum, a role for the cerebellum in song has not been suggested (Feenders *et al.*, 2008).

The cerebellum is known to be connected with downstream motor circuits to influence timing and coordination but also has influence on upstream cognitive aspects like the sequencing of thoughts. In very basic terms, the vertebrate cerebellum receives information from the cerebral cortex and motor areas that code where the organism is, what sensory stimuli are being processed, and the overall emotional and cognitive context. Then the cerebellum processes the information to tell the organism exactly when it should make a particular motor output or sequential thought.

Given that the cerebellum is part of the motor system, if the motor-origins hypothesis for vocal nuclei evolution is correct, then parts of the cerebellum should contribute to the song circuit but this has not been demonstrated previously. One difficulty in demonstrating this connection lies in recognizing the motor pathways that might allow the cerebellum to directly influence the song circuit (Figure 3). Unfortunately, connections between the cerebellum and those motor regions involved in song, such as the basal ganglia (avian striatum, containing Area X), are not well understood. However, recent evidence suggests that there may be distinct loops between the cerebellum and basal ganglia supporting both motor control and cognitive function (Middleton & Strick, 2000). This data in mammals, suggest that a basal ganglia to cerebellum link is possible. While not widely cited, there is recent evidence that there is an indirect cerebellar input to Area X via the thalamus (Person *et al.*, 2008). This is similar to the basal ganglia and cerebellum inputs to the mammalian thalamus. While

other connections similar to mammals have not yet been mapped in birds, these results suggest continued study may find them. In addition, it is possible that the cerebellum functions somewhat independently of the song circuitry but influences the final timing and coordination of the motor outputs coded by the song circuit via influence on the thalamus and forebrain. In addition to this evolutionary motor origins theory, it appears that there is a molecular basis for cerebellum involvement in song.

The expression levels of certain genes have been under study as a molecular basis for the learning of speech. The forkhead box P2 (FoxP2) gene came under study after it was found that a family with a genetic speech-language disorder carried a FoxP2 mutation (Lai *et al.*, 2001). One way to study the effects of a gene is to use a short hairpin RNA (shRNA) technique. In this technique, a gene is virally inserted into the genome and this gene silences the expression of a target gene via mRNA interference. When FoxP2 mRNA and protein level in Area X were decreased, via a shRNA technique, during the sensitive period of learning, it was found that zebra finches could not accurately imitate their tutor's song; additionally, their crystallized adult song showed higher levels of variability (Haesler *et al.*, 2007). This demonstrates that in zebra finches, as in humans, the FoxP2 gene plays a very important role in vocalizations. In respect to the cerebellum, it has been shown that there is a strong expression of FoxP2 in the cerebellum (Lai *et al.*, 2003). Additionally, mice born with a FoxP2 mutation had incomplete cerebellum formation and impairment in ultrasonic vocalizations (USVs) (Fujita *et al.*, 2008). Their USVs, which play an important role in communication between mother and offspring (Branchi *et al.*, 2001), may or may not parallel human speech or bird vocalizations but

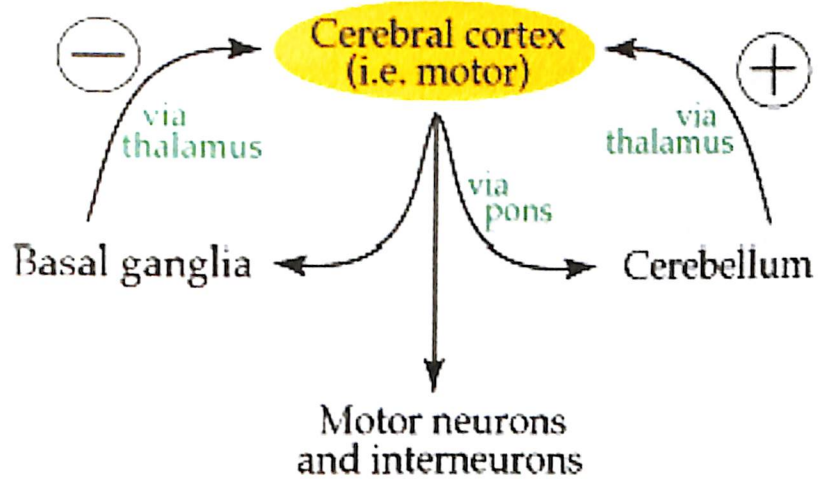


Figure 3 – Cerebellum and Basal Ganglia Connections with the Cerebral Cortex

(<http://thalamus.wustl.edu/course/cerebell.html>)

are vocalizations that mice pups make to ensure they get their necessary attention. The FoxP2 gene has been demonstrated to be important in human language and the zebra finch song circuit. Silencing it results in an underdeveloped cerebellum. This strongly points to a role for the cerebellum in the song circuit, while the deficit in USVs, an unlearned vocalization, suggests the cerebellum may be involved in learned and unlearned vocalizations. In addition to this genetic association between the cerebellum and song, the parallels between human speech learning and the zebra finch song circuit also point to a possible role for the cerebellum.

The cerebellum was once believed to be involved mainly in coordination of motor control, but now is known to participate in higher-level cognitive processes in humans (Middleton & Strick, 1994). Patients with cerebellar lesions showed impairment in executive function, working memory, and attention (Gottwald *et al.*, 2004). This clearly demonstrates the idea that the cerebellum is not only involved in motor coordination in humans. Initial studies in humans spurred others to look at possible roles for the cerebellum in cognition in other species. The zebra finch cerebellum has now been shown to play a role in spatial working memory (Spence *et al.* 2009) and in spatial learning (Stinson *et al.*, 2009). So, zebra finches share with humans both a role for the cerebellum in cognition and motor coordination. Additionally, the identification of speech-sound categories in humans appears to depend on the cerebellum. The right human cerebellum is thought to be involved in language through connections with the left cerebral cortex (Desmond & Fiez, 1998). A role for the cerebellum in human speech raises the question of whether or not there is a parallel link in the avian song circuit since there are numerous other known similarities between the two.

The purpose of this experiment was to determine the role of the cerebellum in the zebra finch song circuit, because several different studies have implied a possible link. In summary, an indirect link, via the thalamus, has been found between the cerebellum and Area X, a known portion of the zebra finch song circuit (Person *et al.*, 2008). The FoxP2 gene has been linked with human speech and language (Lai *et al.*, 2001) and is expressed in motor areas of the brain, including in the cerebellum (Lai *et al.*, 2003). Mice born with a FoxP2 gene mutation had abnormal cerebellum formation (Fujita *et al.*, 2008) demonstrating the possible importance of the cerebellum and this speech-linked gene. The cerebellum has long been known to be involved in motor coordination. The hypothesis that avian song circuits arose from previous pre-existing motor systems (Feender *et al.*, 2008) and the link between the zebra finch song and dance (Williams, 2001) shows that there is a link between motor systems and song. Human speech and learned vocalizations in oscine birds show similarities in both behavioral forms of learning and neural learning pathways (Doupe & Kuhl, 1999). The cerebellum has been shown to play a role in human speech, such as rhyming (Fulbright *et al.*, 1999). This current experiment investigates this possible parallel between the neural circuitry in the zebra finch song circuit and the role of the cerebellum in human speech by lesioning the cerebellum in zebra finches, recording song pre and post lesion, and using sound analyses software to detect even minute changes in song due to the lesion.

PILOT STUDY

Pre-testing: The purpose of the initial pilot study was to identify an experimental condition where all necessary data could be collected in a reproducible matter at high

fidelity to allow automated data processing, which took place over the course of eight months. The first part of the pilot study was selection of test subjects. Experimental adult male zebra finches were taken from the general population and isolated in a small carrier cage (12" x 6" x 6"). Two adult females were then presented to the males, each also isolated in a carrier cage, one female cage on each side of the male cage. The male was then given approximately ten minutes to see if he would produce directed song. Roughly forty adult male zebra finches were tested in this manner until a total of six males were selected for producing varying degrees of directed song. Anecdotally, from the beginning it appeared that some males were better suited for this study than others. Some males began singing immediately when presented with a female, regardless of other external stimuli (observer visible, other birds audible, etc.). Other males would sing with varying regularity (i.e., only one bout immediately upon female presentation) or only under certain conditions (i.e., only when presented with restless females that continuously hopped around the cage and/or made audible vocalizations). Yet a vast majority of males tested would not sing directed song under any tested set of variables despite observational confirmation of indirect song practice. The six selected adult males were isolated in a separate cage from the general population, but housed in the same room. Four female adult zebra finches were also isolated from the general population in the same manner as these appeared to induce male song most often. Males were often presented with two females at a time, so a group of four was selected so that the females could be rotated and reduce a possibility of complacency on the part of the males.

Testing cage: A cage was constructed for proper data acquisition, which was defined as having a multidirectional microphone close enough to the singing male to obtain optimal audio collection and having at least one clear side of the cage so that the male could be video recorded. The cage construction process underwent two major iterations with the later undergoing several refinements until completion. The first cage was an all-plastic enclosure with a vented top and a plastic piece mounted to the top as a microphone attachment point. This first cage provided several lessons learned that carried over to the construction of the final test chamber. First, the all-plastic enclosure created a noticeable echo. No data was processed under these conditions. Sound dampening material was utilized, but its effect never fully resolved the echo. Additionally, the microphone was initially situated above the subject. This position allowed for recording but placing the microphone directly in front of the subject seemed to be ideal if feasible. This position is ideal because directed song is always sung with the male facing the female, so the microphone would be directed in between the singing male and receptive female. Another problem with this initial enclosure, was that the male zebra finch was often confused by the all-plastic enclosure. A common response to being placed in the enclosure was to spend the entire time exploring the perimeter and pecking at the plastic in an attempt to escape. This was initially corrected by covering one side and the back of the enclosure (leaving the side view available to the observer and a front view for female presentation) and placing a boarder around the two remaining clear sides in an attempt to define the boundaries of the enclosure. At first this helped but soon gave rise to another issue; the subject would often see its reflection in the plastic despite attempting to reduce this with alternate lighting techniques. The final lesson learned with the initial cage was

that the male zebra finches would often utilize all of the space provided, making videotaping rather difficult. With all this information at hand, a new cage was constructed by cannibalizing two metal carrier cages available in the lab (Figure 4). The new cage provided a smaller area for the male test subject and made easy the addition of a perch after it was realized that males in the breeding population were always on a perch when performing directed song. In the new cage, the presented females were much closer to the males than in the original cage and were separated by a cage divider rather than plastic, so drastically fewer sound waves were reflected back towards the male and, additionally, the male birds were able to better hear the females. Plastic plexiglass was more judiciously utilized to allow a clear viewing angle from the side and the top. These two angles were selected to allow the possible utilization of additional cameras in this and/or future studies. The use of sound dampening foam was carried over to the new cage with the added benefit of microphone placement directed in front of the test subject.

Subject selection: Of the initial six males selected for the study, only one male sang directed song every time presented with a female. For this reason several different conditions were tested to see if any would affect whether or not the birds would sing. Some of the conditions tested were as follows: a quiet room versus a room where the breeding colony could be heard, observer visible versus not visible, external stimuli visible versus not visible (via a black sheet encircling the cage), and one female presented versus multiple females. Observed differences in these conditions were noted and the most favorable conditions for directed song presentation were chosen. Conditions for testing were the following: a room where the breeding colony could be heard, having the



Figure 4 – Testing Cage Utilized for Data Acquisition

test cage elevated, and having the observer not visible. Additionally, during this testing the six males were isolated in a room by themselves in hopes that any duration without visual perception of a female would enhance excitability upon presentation.

When five of the six subjects selected would not sing on a regular basis, despite isolation from the rest of the breeding colony, a hypothesis was formulated that perhaps the five subjects had formed a mating pair in the colony prior to selection. Perhaps the males had formed a lifelong mating pair and therefore was less interested in any of the females presented. In order to isolate males that had not formed mating pairs, a strategy was implemented where new male fledglings were placed in a separate room as soon as they began to display sexual differentiation, approximately twenty to thirty days of age. These males were raised with similarly aged males and a tutor as needed for vocal development. The males were raised under these conditions until an age of 120 days where they were presented with a female for the first time upon reaching sexual maturity. Out of the twenty-five males raised in this fashion only one was found to be a reliable singer. This one male and one male from the pre-testing trials were the first two subjects of the study. The complete study was performed on these two males. All the males in the breeding colony were then retested under the finalized experimental conditions to find additional test subjects that would sing. Combining the general population resulted in three more males selected as subjects and tested for a total of five subjects. We have since learned that a crucial element may be implementation of a prolonged habituation period in the test chamber. During pilot testing we varied habituation period from 10 minutes to a couple of hours; we have now learned that others habituate subjects over night or a few days.

METHODS

Animals. All experiments were conducted in accordance with the University of Mississippi ICUAC rules and regulations. All subjects were adult zebra finches greater than 120 days in age. Birds were housed in same-sex groups in separate rooms, each on a 14/10 light/dark cycle. Food and water were freely available unless otherwise noted.

Apparatus. A cage (12" x 6" x 6") was divided into two separate sides (Figure 4). The two sides were intentionally unequal in size. The female side measured 7.5" x 6" x 6", contained a perch, and the floor was covered with sound dampening material. The subject side measured 4.5" x 6" x 6" and, like the female side, contained a perch. The floor and one wall of the subject side were covered with sound dampening material. Two sides of the subject side of the cage were made of plexiglass to facilitate video recording, the roof and the wall opposite the wall were covered with sound dampening material. The additional caging that separated the two sides provided the optimal attachment point for a microphone.

Testing. Two females were always presented to an individual male. These females were rotated every recording session to ensure that the males did not become too accustomed to any one female. The females were never in the cage for longer than one hour per day. They were allowed free access to water but food was withheld to ensure engagement with the test subject. Males were tested in ten-minute intervals and both food and water were withheld during this period to garner the highest proportion of directed singing possible. Recording was done with a Sony Handycam camera (HDR-HC7) recording on Sony

MiniDV tapes (DVM80) using a directional microphone. Recording took place for ten days prior to administering lesions. Recording took place over ten days post-lesion as well. The first two test subjects were allowed two days to recover post-lesion before testing again. The final three test subjects began testing the day after lesions to make sure we had not missed in initial deficits

Lesions. Equithesian was administered to initiate a deep plane of anesthesia. Feathers from the caudal region of the skull were removed under brief isoflurane exposure. A stereotaxic frame was then prepared by coating the ear bars with lidocaine. The birds were placed in the stereotaxic frame where anesthesia was maintained with isoflurane gas/O₂ mix. Once in the stereotaxic frame, an incision was made and a craniotomy was performed over the cerebellum. Lesions were made with a 22 G needle at coordinates lateral (± 0.6 mm) and rostral (-2.5 mm) to the midline central sinus and ventral (-4.6 mm) to the surface of the brain. After the lesion, the skin was replaced over the skull and sealed with vetbond surgical glue and coated with Neosporin. Birds were monitored post-surgery until they were awake and alert.

Data acquisition. Two different recordings were made for each bird, one pre-lesion and one post-lesion. Each recording was downloaded and transferred by connecting the video camera to a laptop and using Windows Movie Maker, turning the entire tape into a single Windows Movie Video file (.wmv). The audio was then extracted from the movie file with a video converter (Video Converter for Mac), resulting in a Waveform Audio File Format (.wav) file. The program Audacity was then used to partition the .wav files from

bouts (Figure 5) into motifs (Figure 6) and individual syllables. Motifs and syllables were then analyzed using Sound Analysis Pro (SAP).

Lesion conformation. After testing was complete, birds were sacrificed by isoflurane overdose. Brains were removed and fast frozen in optimal cutting temperature on dry ice and stored at -80°C. Brains were sectioned on a cryostat at 50 µm, mounted, dehydrated through graded alcohols, and stained with crystal violet for lesion conformation.

Sound Analysis Pro

SAP is a program that allows for the automated analysis of song similarity and it was created specifically for zebra finches (Tchernichovski *et al.*, 2000). SAP uses a variety of features to characterize and compare songs, these features are as follows: Weiner entropy; pitch; goodness of pitch; amplitude modification (AM); and frequency modulation (FM). Each of these features has a different unit of measurement. In order to compare all the features SAP calculates a unitless number, the measure of deviation (MAD). The time window (FTT window) is the smallest unit of time over which all of these features are compared, for this study it was set at 10 ms. In order for the program to compare two songs it has been programmed with a threshold value for detecting similarities in the zebra finch song. Using the MAD for each feature created the program threshold, the Euclidean distance between all interval pairs are calculated, and a threshold value for each interval pair is calculated. The threshold value is estimated across a large sample (250,000) random pairs of frames obtained from comparisons across 25 random pairs of zebra finch songs (Sound Analysis Pro Manual). Thus, comparisons on similarity

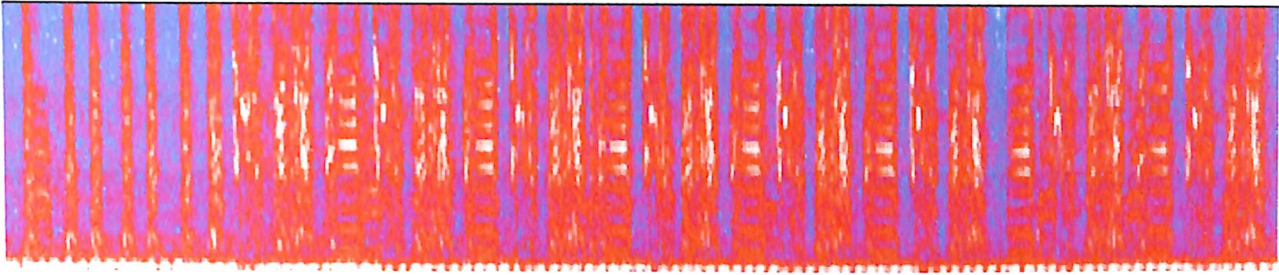


Figure 5 – Bout of Song from a Zebra Finch. A bout contains multiple renditions of a motif.

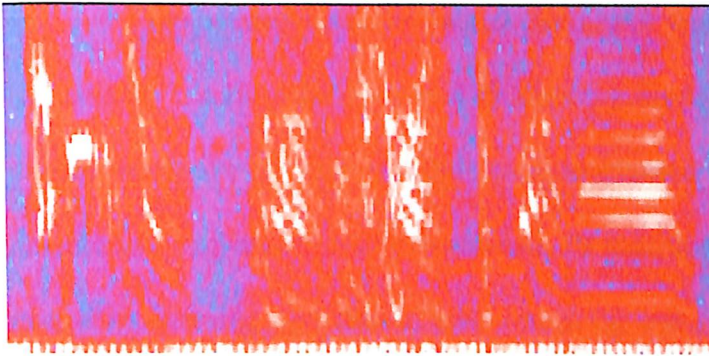


Figure 6 – Motif of a Zebra Finch Song. A motif contains a sequenced pattern of syllables (in this instance three different syllables).

in songs are compared to this background measure of similarity in random pairings of songs. They call this the p -value and it was set at 0.05 for this study. Note that this value is more a yardstick than an actual measure of significance. Larger similarity segments known as intervals (70ms) are formed as neighboring segments pass the p -value threshold. These large segments (intervals, 70 ms) are the basis for the similarity score while the smaller segments (FTT window, 10 ms) are the basis for the accuracy score.

Two scores were extracted from SAP when comparing songs: percent similarity (similarity) and mean accuracy (accuracy). Each of these scores has a different meaning when analyzing motifs or syllables as explained in subsequent sections. Under each condition, pre-lesion and post-lesion, 20 motifs were pseudorandomly selected for analysis making sure that at least one song was selected from each testing day. The syllables that constituted each of these motifs were extracted for the syllable analysis. Whether analyzing motifs or syllables, each sound file was compared to every sound file within that condition (i.e., all 20 pre-lesion motifs were compared to each other and all 20 post-lesion motifs were compared to each other) for a total of 380 comparisons (comparisons to two identical sounds were omitted). The reason for comparison within the condition was to get a measure of variability within, rather than across, a condition.

Motif analysis. This analysis allowed me to look at the whole motif, or the repeated sequence of syllables that constitutes the zebra finch song. When discussing motifs it is common to designate each different syllable a different letter (i.e., AABCD is a motif where there are four different syllables and the first syllable is sung twice). Analysis at the motif level was run under asymmetric conditions (Figure 7). This means that the most

similar sound elements within each motif are matched and then compared regardless of their position. So whether the bird sings ABBC or ACBB, as long as each syllable was identical, no differences would be found. The similarity score represents the amount of the first sound from before the lesion that was included in this same sound after lesion. This score is measured across an interval time frame (70ms) and so it provides a gross analysis – comparing composites of 70 ms sampling windows of the first song to other 70ms samples of the other song. The accuracy score represents how well the two sounds matched each other across the similarity segments. The score is calculated as the product of all the average accuracy values across the intervals (70ms) used in the similarity score, measured at the FTT window scale (10ms). So, this measurement is finer, asking if within each 70 ms window how well each 10ms window matches the comparison 10ms window.

Syllable analysis. This analysis looked at each individual syllable that composed the motifs. It was run under symmetric conditions meaning the FTT windows (10ms) are compared sequentially from the beginning to the end of the syllable. The interval time frame (70ms) is ignored in this analysis. So, each 10 ms window (10ms, 20ms, 30 ms etc.) from beginning to end is compared to these same windows in the compared syllable (10ms to 10ms, 20ms to 20ms, 30ms to 30 ms) (Figure 8). The similarity score represents the percentage of sound intervals above the p -value – how often the syllables were more similar to each other than to a random syllable. The accuracy score represents the average of similarity scores ($1 - p$ -value). In the event that a syllable was repeated several times

in a motif, only the last rendition of that syllable was used in the analysis (i.e., only one of each syllable from each motif was used in the analysis).

Song length. Song length was manually measured using tools available in SAP. Each syllable and each silent interval between syllables was measured to the nearest millisecond (ms). These measurements were added together to arrive at the length of the song. These measurements were then used for comparison of whole song length, syllable length, and pause length.

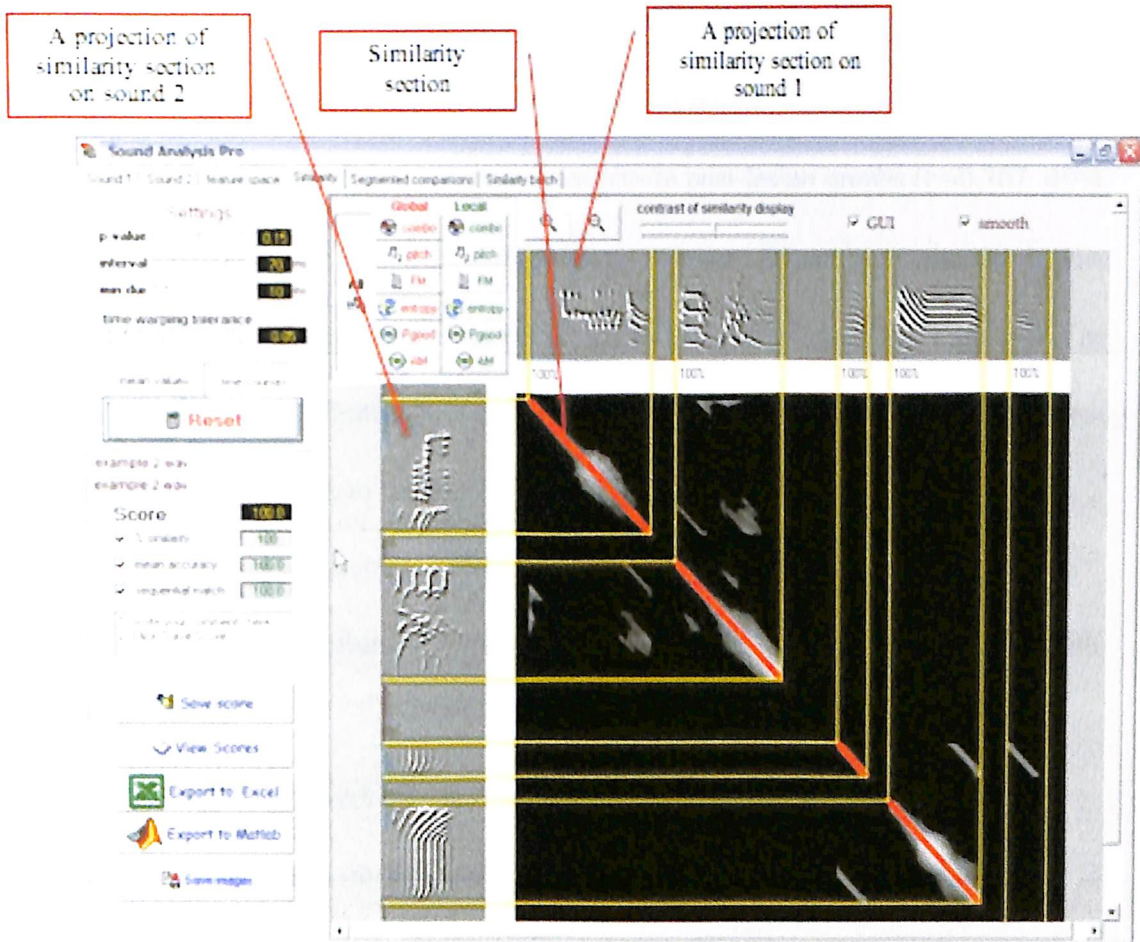


Figure 7 – Sound Analysis Pro Motif Analysis (asymmetric analysis)

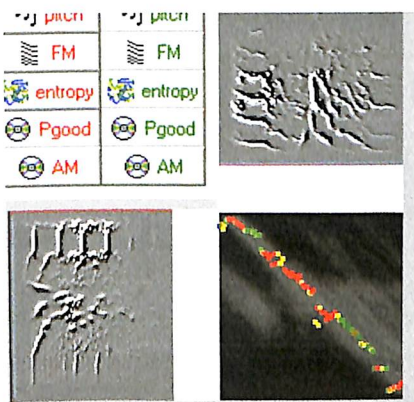


Figure 8 – Sound Analysis Pro Syllable Analysis (symmetric analysis)

RESULTS

Increased song element variability after cerebellum lesion

Motif similarity did not change from pre- to post-lesion groups ($t=-0.307$, $df=4$, $p=0.774$). The ability to sing a stereotyped song was not affected by cerebellum lesions. Motif accuracy showed a significant decrease ($t=4.226$, $df=4$, $p<0.05$) (Figure 9). The variability of the song elements (amplitude, frequency, etc.) within the post-lesion group increased in comparison to the pre-lesion group. Neither syllable similarity ($t=0.823$, $df=4$, $p=0.457$) nor accuracy ($t=.993$, $df=4$, $p=0.377$) showed a significant difference between groups. Cerebellum lesions did not preferentially affect any particular syllable within the motif.

Increased song length with cerebellum lesion

One of the birds (referred to by its birdband, White11 or W11) had an oddity in his song that was not seen in the four other subjects. The other four subjects sang a stereotyped song that never changed from rendition to rendition. On the other hand, W11 sings one of his syllables (the 'B' syllable) a variable number of times. Uncertain of how to handle this oddity, the total length of the zebra finch motif was calculated without data from W11 and found to significantly increase ($t=-3.510$, $df=3$, $p<0.05$) from pre- to post-lesion groups. The increase in length is seen in the song overall. Neither syllables nor the silent intervals between them were lengthened post-lesion but rather the whole song was, suggesting different renditions of the song had different elements – silence or syllables drawn out but that this was not consistent across songs.

The data from W11 was addressed after this analysis was complete. The method devised was to match pre- and post- lesion songs that had the same number of variable

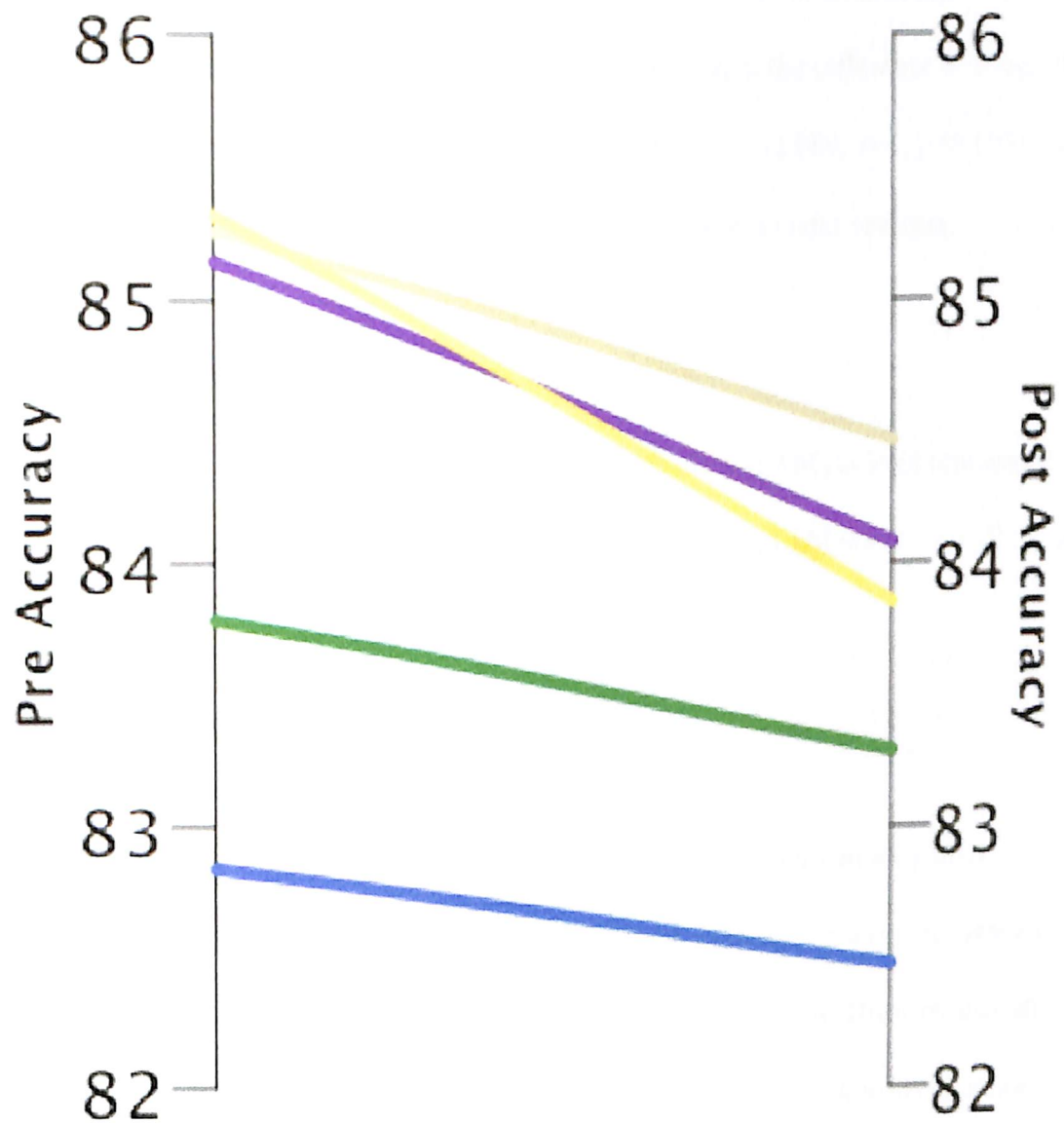


Figure 9 – Motif Accuracy Pre- and Post-Lesion

and hence total, syllables. So, only 11 of the original 20 motifs were included when analyzing the data from W11. With the inclusion of this data, the difference between the length of pre- and post-lesion motifs became insignificant ($t=-2.089$, $z=4$, $p=0.105$). A method to evaluate the data from W11 more accurately is still under revision.

Lesions damaged deep cerebellum nuclei

The lesions hit the medial nucleus of the cerebellum (CbM) at least unilaterally on 3 of the 5 subjects. White matter superficial to or surrounding CbM was hit on all other lesions (Figure 10).

DISCUSSION

Birds were able to retain and faithfully reproduce their learned song after cerebellum lesion at the same similarity level as prior to lesions; however, accuracy of the song decreased and the length of the song increased after lesion. These results are similar to those observed in humans with cerebellar dysarthria (also known as ataxic dysarthria). Affected humans are usually classified into one of three categories and may show imprecise consonants, distorted vowels, disruption of intonation or stress, and pitch problems (Stocks *et al.*, 2009). Any one of these afflictions would provide an explanation as to why lesioned zebra finches are able to reproduce a known song, but with slight differences in the spectral qualities (pitch, FM, AM, etc.) from rendition to rendition. Another symptom of cerebellar dysarthria is a slowed tempo of spoken language (Ackermann *et al.*, 2007). This may be similar to the increase in song length after cerebellum lesion. These results point to the possibility that the cerebellum

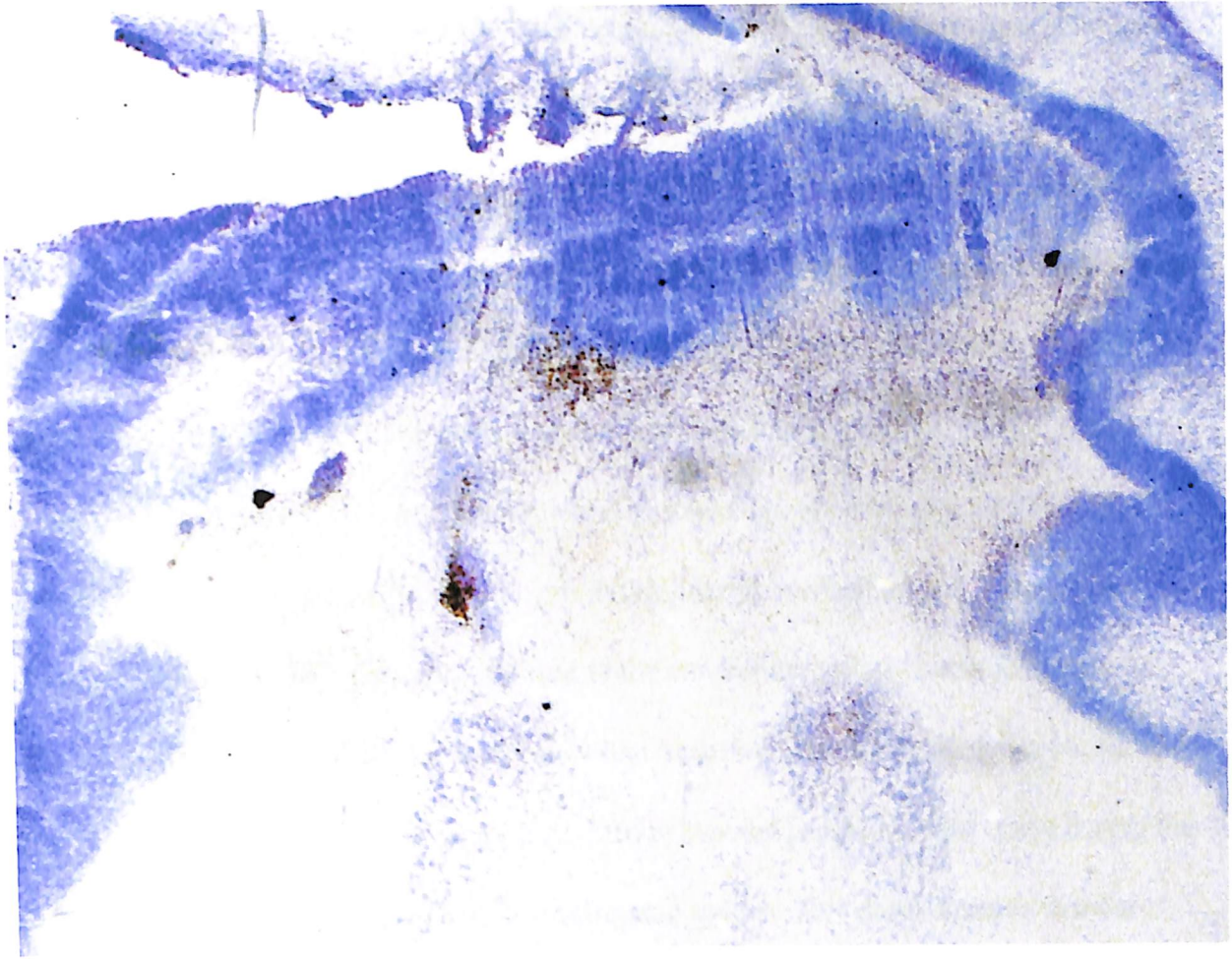


Figure 10 – Lesion Confirmation on W64

plays a role in the zebra finch song circuit that is similar to the role it plays in human speech production.

The cerebellum, originally believed to play a role mainly in motor coordination and control, is now thought to play a role in higher-level cognitive processes (Middleton & Strick, 1994). Patients with cerebellar lesions have been shown to have impairment in executive function, working memory, and attention (Gottwald *et al.*, 2004). These discoveries influenced many investigators to reevaluate their perception of the role of the cerebellum. Soon investigations began looking at the cerebellum and its role in human speech and song. Most studies looking at the cerebellum utilize functional magnetic resonance imaging (fMRI) to indicate when a particular region of the brain becomes activated (or inactivated). Callan *et al.* (2007) showed cerebellum activation during the perception and production of both singing and speech. This demonstration that the cerebellum is activated in human speech and song gives more credence to the hypothesis that developmental dyslexia may arise from cerebellar deficits (Nicolson *et al.*, 2001). Additional direct evidence that the cerebellum is involved in human speech is seen with the cerebellum abnormalities of the KE family.

The KE family has a severe genetic disorder of speech and language. Half the members of the family have a deficit in the sequencing of articulation patterns, often making their speech unintelligible. They exhibit a developmental form of verbal dyspraxia. The KE family genetic disorder has been linked to a mutation in the FOXP2 gene (Lai *et al.*, 2001). Affected members of the KE family have been shown to have cerebellum abnormalities (Watkins *et al.*, 2002). Thus it is not too surprising when it was found that there is a strong expression pattern for FOXP2 in the cerebellar cortex (Lai *et*

al., 2003). It has also been found that FoxP2 is regulated in areas of the songbird song circuit (Scharff & Haesler, 2005) and additionally, the cerebellum of songbirds' exhibits strong FoxP2 activity (Haesler *et al.*, 2004). Despite the evidence for similarities in genes related to song and speech in the cerebellum, the cerebellum was not believed to play a role in the songbird song circuit and the songbird has not been used to model the role of the cerebellum in human speech.

Human speech and birdsong have numerous parallels (Doupe & Kuhl, 1999). Both humans and songbirds learn their vocalizations early in life. They usually require hearing speech or song from a proficient individual so that they may learn by imitation. Juveniles will begin to make their own vocalizations that, after practice, begin to sound more like the song of the adults around them. Initial vocalizations are called subsong in songbirds and have been likened to the babbling of a human infant. Studies in human infants have shown that syllables are the basic units of speech motor control (MacNeilage, 1998). Likewise, it has been shown that the syllable is the smallest unit of song that is transferred from tutor to juvenile in the zebra finch (Franz & Goller, 2002). The parallels between the songbird song and human speech are why the songbird is often used as an animal model for human speech. The evidence that the cerebellum is involved in human speech and birdsong points to further similarities between the two.

While this study was limited in number of subjects, the evidence for a change in accuracy was found for all birds in the study and indicated that even a limited group of subjects can reveal that cerebellum damage in the zebra finch has effects similar to what would be expected in a human that suffered cerebellum damage. These initial results

indicate that the role of the cerebellum in the songbird song circuit should be more extensively evaluated.

Initially, I additionally hypothesized that the timing of the song and dance would be disrupted with cerebellum lesions. It has been demonstrated that zebra finch cerebellum lesions initially cause gross motor deficits (Spence *et al.*, 2009). The close association between song and dance necessitates the coordination and timing of the neural circuitry controlling each aspect. The zebra finch dance consists of infrequent movements initiated at a number of specific 'hot spots' within a song (Williams, 2001). It would be an interesting study to see if lesions of the cerebellum disrupt the close association of the song and dance.

It was shown that the cerebellum is involved in both the perception and production of human speech (Callan *et al.*, 2007). Damages to the cerebellum have been shown to produce deficits in both motor functions and cognitive processes (Levisohn *et al.*, 2000). Deficits in cognitive processing indicate that there may be dramatic effects of lesions on song learning. The next logical progression would be to investigate the role of the cerebellum in song learning in the zebra finch. The cerebellum is involved in the perception of human speech (Callan *et al.*, 2007) and most likely plays some role in a young zebra finches' learning of its tutor's song since it has been shown to play a role in working memory (Spence *et al.*, 2009). There is a reported possible link between a known area of the songbird brain necessary for vocal learning (Area X) and the cerebellum (Person *et al.*, 2008). Lesions of Area X result in abnormal songs that do not crystallize (Scharff & Nottebohm, 1991) indicating that it plays an important role in song learning. This possible link between Area X and the cerebellum could be transmitting

information about the perception of a tutor's song (processed in the cerebellum) to Area X so that the juvenile could learn the song. Lesions to the songbird cerebellum prior to song learning could distort the perception of tutor song or disrupt a possible connection between the cerebellum and Area X. This would most likely result in a song less like the tutor's than would normally be expected.

In conclusion this study has demonstrated that the cerebellum plays a role in the zebra finch song circuit that is analogous to human speech. This opens the door to the possibility of using the zebra finch as a model organism for investigation of the role of the cerebellum in human speech and learning.

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