The Neural and Behavioral Mechanisms of Adult Song Plasticity in a Critical Period Song Learner

by

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Abstract

Studying vocal learning in zebra finches can elucidate how complex motor skills are learned during critical periods of development and then maintained during adulthood. Previous studies have shown that during both juvenile song learning and adulthood, the HVC-RA pathway (a pre-motor pathway) promotes song stereotypy, whereas the LMAN-RA pathway (output of a pathway analogous to basal ganglia-thalamo-cortical loops) is critical for song plasticity and variability. Additionally, studies have suggested that the ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor is associated with the degree of song stereotypy. Deafening in adulthood causes degradation of song stereotypy, and therefore deafening can be used as a manipulation to elucidate if there is a functional relationship between dendritic arbor and the degree of song stereotypy. Cell morphology of both projection neuron types can be assessed by retrograde labeling from RA. The length of the dendritic arbor of deafened birds was compared to the arbor of hearing-intact controls given a sham surgery, and dendritic arbor length was plotted against the extent of song degradation. Deafening-induced song changes are due to an active process, and the widely held model is that a complete lack of auditory feedback triggers maladaptive error-signals from auditory feedback-driven error-correction mechanisms. Therefore, dendritic arbor changes due to deafening could reflect the cellular basis of adult song plasticity. Possibly due to a small sample size, no significant differences in dendritic arbor characteristics were found between deaf and sham birds. However, a marginally significant trend was found indicating that greater LMAN-RA dendritic arbor relative to HVC-RA arbor was associated with greater post-operative reduction of song
stereotypy in deaf birds and was associated with a lesser increase in song stereotypy in hearing-intact birds. This result supports a model in which greater LMAN dendritic arbor relative to HVC arbor promotes greater loss of song stereotypy whereas greater HVC dendritic arbor relative to LMAN arbor promotes higher song stereotypy in both deaf and hearing-intact birds.

A widely held hypothesis is that the auditory feedback (or lack thereof) associated with the act of singing provides the information for error-correction during song learning and maintenance. Therefore, the singing rate for each bird was plotted against song stereotypy, and we predicted that in deaf birds, more singing would lead to more maladaptive error signals and greater song degradation. We found that deafened birds that sang at higher rates in the first few days after deafening had more pronounced song degradation later on, but conversely, birds that had a higher average singing rate throughout the post-deafening period were more likely to have less song degradation. The latter result indicates that there could be additional proprioceptive feedback—perhaps from the vocal muscles—that promotes increased song stereotypy through increased singing independent of auditory feedback.
Introduction

Songbirds as a model for vocal learning

Vocal learning is the ability to learn and then produce vocalizations for communication. In addition to humans, songbirds are one of the few animal groups capable of vocal learning (Doupe and Kuhl, 1999, Williams, 2004). Songbirds, specifically male zebra finches, are useful animal models for studying vocal learning, as there are numerous parallels between the vocal learning of humans and songbirds. Both humans and male zebra finches have a critical period of development for vocal learning early in life that relies on hearing language or song from an adult (Doupe and Kuhl, 1999). Songbirds have specialized regions of the forebrain devoted to producing song (the song system), which is parallel to the existence of language regions in the human forebrain (Doupe and Kuhl, 1999). In addition, most songbirds, including zebra finches, maintain their song through an active process that relies on auditory feedback, which is also true of speech maintenance (Doupe and Kuhl, 1999). In general, studying vocal learning can give insights into the neural basis of how complex motor skills are learned during critical periods of development and then maintained during adulthood.

Zebra finch song behavior, learning, and critical periods

Each songbird species produces a species-specific song. Normal adult zebra finch song includes a repertoire of 3-15 notes, which are defined as continuous sounds separated from one another by brief periods of silence. Zebra finches sing a “motif” that most often consists of the entire repertoire of notes sung in a fixed
sequence, though sometimes these motifs are truncated. This motif is also often called
the bird’s canonical song. Zebra finches usually sing multiple repetitions of these
motifs, which is called a song “bout,” and these bouts are often preceded by
repetitions of identical short notes called introductory notes (Williams, 2004) (Figure
1). Each individual zebra finch produces a unique adult song, but the songs follow the
species-specific rules outlined above.

![Spectrogram of a zebra finch song bout.](image)

Figure 1. Spectrogram of a zebra finch song bout. Time(seconds) on the x-axis and
sound frequency on the y-axis. Recording is from bird 50. a-e represent notes, i1 and
i2 represent introductory notes. Bracket indicates one complete motif. Sound intensity
is depicted in color, with red/yellow representing high intensity, and blue/black
representing low intensity.

Only male zebra finches sing, and they use song mainly to attract mates.

When a male is singing towards a female and displaying courtship behavior, the song
is “directed”, and when a male is not singing to an obvious target, the song is
considered “undirected.” The song structure is somewhat different in these different
contexts. The note sequence and acoustic structure of undirected song is more
variable, whereas directed song is more stereotyped (Williams, 2004).

In order to develop normal species-specific song, all species of songbirds must
listen to a conspecific adult tutor during development, and they must have auditory
feedback of their vocal practice (Doupe and Kuhl, 1999). Song learning consists of a
sensory phase and a sensorimotor phase. During the sensory phase, a young male
listens to and memorizes the song of an adult male, who is most often the juvenile’s father (Williams, 2004). In zebra finches, the sensory phase begins approximately 20-25 days after hatching (Immelmann, 1969, Zann, 1997). The bird is thought to memorize an adult male’s song during this stage, because if a young bird is exposed to the song of an adult male for a short period of time prior to the onset of song-like vocalizations, their adult song will resemble this tutor’s song (Nelson, 1997, Zann, 1997).

During the sensorimotor phase, birds vocalize and then rely on auditory feedback for proper learning to occur; the widely held model is that through trial and error learning, birds are attempting to match their vocal output to a memorized acoustic model of the tutor’s song that was acquired during the sensory phase (Nelson, 1997, Nottebohm, 2004) (Figure 2). The beginning of the sensorimotor phase is marked by the first production of song-like sounds, and this phase overlaps substantially with the sensory phase in zebra finches (Immelmann, 1969). The sensorimotor phase has three distinct stages: subsong, plastic song, and crystalized song (Hultsch and Todt, 2008). Subsong occurs around 30 to 45 days post hatch, during which the bird produces very rudimentary and variable sounds; this stage is analogous to the babblings of an infant and is thought to allow the adjustment of the vocal apparatus to improve sensorimotor control (Williams, 2004, Aronov et al., 2008, Hultsch and Todt, 2008). The next stage is plastic song, during which the bird begins to produce notes that resemble the tutor song (Hultsch and Todt, 2008). Vocalizations are variable throughout this stage, though the song structure gradually becomes more and more stereotyped through vocal practice. A highly stereotyped
song is one in which the acoustic structure and note sequence of the motif is very consistent from rendition to rendition. In zebra finches, 90 days marks song crystallization, when song becomes very stereotyped and is fully developed in its adult form (Williams, 2004, Hultsch and Todt, 2008). After crystallization, zebra finch song is fixed and is not affected by exposure to new acoustic models (Immelmann, 1969). The adult song includes many notes that are near or exact matches of the tutor’s song notes, but during song development young birds may alter a learned note, change the note sequence, or introduce what seem to be original notes into their songs, and thus each individual bird develops a unique song (Williams, 2004).

![Figure 2. Model of sensorimotor song learning. Proper song learning is reliant on auditory feedback (Doupe and Kuhl, 1999). The hypothesis is that through trial and error learning, birds attempt to match their vocal output to a memorized stored template (acoustic model) of the tutor’s song acquired during the sensory phase. Figure adapted from (Brainard and Doupe, 2000a).](image)

The song learning process described above occurs during a critical period of development, the close of which is marked by song crystallization at 90 days. A critical period is a limited phase during development when the effect of specific environmental inputs on the brain and behavior is particularly strong. The
brain/behavior is altered permanently due to the experience received during a critical period, and if proper inputs are not received, development is not normal (Knudsen, 2004). Song learning in zebra finches fits this definition of a critical period: young zebra finches must be exposed to conspecific tutor song early in life for species-typical song to develop, as isolated birds develop very abnormal songs (Doupe and Kuhl, 1999). The timing of the close of the critical period in zebra finches is somewhat plastic however. Raising zebra finches in the absence of an adult male tutor delays the close of the critical period past 90 days, such that if a tutor is introduced to an isolated bird after day 100, the bird will modify its isolate song and copy from this tutor (Zann, 1997).

Because sensorimotor learning in zebra finches is restricted to the first year of life, zebra finches are classified as “closed-ended” learners (Brenowitz, 2008). The song that becomes crystalized at 90 days is most often the song that a zebra finch will sing for the rest of its life. Other species of songbirds, such as canaries, are classified as “open-ended” learners because song learning continues throughout adulthood. During the breeding season, the song is stereotyped, but then song becomes variable when the breeding season ends. Song becomes stereotyped again by the next breeding season, and it is often altered somewhat from the song produced in the previous season (Williams, 2004, Brenowitz, 2008).

It was previously thought that there were no song changes in closed-ended learners such as zebra finches after the critical period (Brenowitz, 2008). However, recently it has been shown that the adult songs of closed-ended learners can change (Brenowitz, 2008, Kirn, 2010). For example, note stereotypy increases in adult zebra
finches after crystallization (Pytte et al., 2007). In addition, disruption of auditory feedback causes song modifications, indicating that closed-ended learners actively monitor production of song (Lombardino and Nottebohm, 2000, Tschida and Mooney, 2012a). Therefore, Brenowitz argues that closed-ended learners should not be defined as having an absence of plasticity in adulthood, but rather they should be considered as having a lower degree of adult song plasticity than open-ended learners (Brenowitz, 2008). Important questions are what causes song crystallization and the close of the critical period, and what cellular and behavioral processes involved in juvenile song learning are retained in the adult brain (Brainard and Doupe, 2000a).

**Song system anatomy**

The song system is a set of interconnected brain nuclei that underlie song production, learning, and maintenance in oscine songbirds (Nottebohm, 2004). The song system consists of two pathways: the motor pathway and the anterior forebrain pathway (Figure 3). The motor pathway controls the stereotyped production of learned song (Nottebohm, 2004). This pathway begins with the forebrain nucleus HVC, which receives input from auditory areas. A cell population within HVC projects down to the robust nucleus of the archopallium (RA). From RA, the pathway continues to a nucleus in the hindbrain (nXII), which contains motor neurons that innervate the muscles of the syrinx (the vocal organ).

The anterior forebrain pathway (AFP) is a circuit critically involved in song learning and maintenance (Bottjer et al., 1984, Brainard and Doupe 2000b). The AFP begins with a population of cells within HVC that project to Area X. Area X sends
projections to the medial nucleus of the dorsolateral thalamus (DLM), which projects to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). LMAN then projects to RA, which is the spot of convergence of the two pathways. The AFP is analogous to the cortico-basal ganglia-thalamo-cortical loops found in mammals.

Figure 3. The Song System. The motor pathway (black arrows) and the anterior forebrain pathway (white arrows) both start with HVC, which receives auditory input from UVA and NIF. The population within HVC that projects to RA begins the motor pathway, which controls the stereotyped production of song. The population in HVC that projects to Area X begins the anterior forebrain pathway, which is critical for juvenile song learning and modification of song in adulthood. The projection from Area X to DLM to LMAN is homologous to basal ganglia-thalamo-cortical loops in mammals. LMAN then projects to RA, the point at which the two pathways converge. Figure adapted from (Nottebohm, 2004).

Motor pathway

There is much evidence that demonstrates that the motor pathway is important for the stereotyped production of learned song. Lesions of HVC and RA immediately result in severe song deficits (Nottebohm et al., 1976). In addition, individual HVC neurons that project to RA (HVC-RA neurons) reliably produce sparse bursts at precise times during each rendition of a song note, and different HVC neurons tend to
burst at different time points in the song motif (Hahnloser et al., 2002). RA neurons also generate patterns of activity that correlate closely with the produced song across multiple song renditions (Yu and Margoliash, 1996). These results suggest that the motor pathway controls the production and timing of stereotyped song.

*Anterior forebrain pathway*

There is much evidence demonstrating that the AFP is critical for song learning and plasticity. Lesions of LMAN and Area X in juvenile birds prevent normal song development, indicating the importance of the AFP in song learning (Bottjer et al., 1984, Scharff and Nottebohm, 1991). Inactivation or lesion of LMAN in juveniles causes song to become prematurely crystalized and stereotyped, which supports a role for the AFP in allowing for song plasticity (Scharff and Nottebohm, 1991, Olveczky et al., 2005, Aronov et al., 2008). During the sensorimotor phase of song learning, it is widely believed that birds attempt to match their vocal output to an auditory memory template of the tutor’s song acquired during the sensory phase (Figure 2). It is possible that LMAN provides random variability that allows for an evaluation mechanism to select and reinforce correct motor actions while weakening incorrect ones (Kao and Brainard, 2006). Alternatively, LMAN could be providing an instructive input that directly guides adaptive song modifications (Andalman and Fee, 2009).

Originally, studies indicated that lesions of the AFP in adulthood had no effect on song, and therefore it was concluded the AFP is not required for normal production of adult song (Bottjer et al., 1984, Scharff and Nottebohm, 1991). However, more recent evidence using sensitive sound analysis software
(Tchernichovski et al., 2000) shows that LMAN lesions in adulthood cause a decrease in the natural variability of undirected song (Kao and Brainard, 2006), which suggests that LMAN could provide song variability needed for song plasticity. Additionally, the AFP is critical for adult song plasticity due to experimental manipulations of auditory feedback. For example, LMAN lesions prevent the song destabilization that normally occurs after deafening (Brainard and Doupe, 2000b). The above evidence suggests that the AFP is important for song plasticity during both song learning and adulthood.

Convergence of the pathways onto RA

The motor pathway and the AFP converge on RA, therefore implicating RA as a potential site of integration important for song learning (Kirn, 2010). LMAN neurons that project to RA (LMAN-RA neurons) and HVC-RA neurons form synapses on the same dendrites of RA cells (Herrmann and Arnold, 1991). HVC-RA synapses contain both AMPA and NMDA receptors, and LMAN-RA synapses use mainly NMDA receptors (Mooney, 1992). This mix of AMPA and NMDA receptors allows for Hebbian plasticity in RA (Nordeen and Nordeen, 2004). Hebbian plasticity is a theoretical model in which the coincident activation of cells causes synaptic strengthening. One mechanism of Hebbian plasticity is long-term potentiation (LTP), which is a molecular process that strengthens synapses and is caused by the opening of NMDA receptors. NMDA receptors can only open if bound to glutamate while the cell is depolarized, and therefore the receptors are only open during coincident activation of pre-and post-synaptic cells (Shors and Matzel, 1997). It is possible that coincident activation of HVC-RA and LMAN-RA neurons on the same RA cell could
cause LTP that changes the activity of RA cells and consequently modifies song behavior, and this LTP could be an important mechanism underlying song development (Nordeen and Nordeen, 2004).

**Changes in the song system during song development**

During song learning, song behavior is initially variable and then becomes increasingly stereotyped (Williams, 2004). Over the same developmental time period, there are also many cellular changes that occur, which may be influencing or underlying the increase in stereotypy during song learning (Kirn, 2010).

In zebra finches, HVC-RA axons do not innervate RA until 30-35 days after hatching, which is after the beginning of the sensory and sensorimotor phases of song development (Kirn, 2010). Many of the connections in the anterior forebrain pathway develop much earlier (by day 20), which further supports that the AFP has a critical role in the early stages of song development (Kirn, 2010). During song learning there is a shift in which cell type makes relatively more synapses on RA: LMAN inputs dominate during early development when song is variable, and then HVC inputs dominate as song becomes more stereotyped (Herrmann and Arnold, 1991, Kirn, 2010). HVC volume triples between post-hatch day 12 and 53 in the zebra finch, mostly due to neurogenesis, and after the first HVC-RA axons innervate at around 30 days, there is a significant increase in the number of synapses HVC makes with RA during this time period (Herrmann and Arnold, 1991, Kirn, 2010). Conversely, LMAN volume decreases over the same time period (during which song is becoming more stereotyped), and LMAN-derived synapses in RA are reduced by roughly 80%
In conjunction with the decrease in LMAN-RA synapses, there is also a decrease in dendritic arborization and spine densities over the same time period, which is partially responsible for the shrinkage of LMAN volume (Nixdorf-Bergweiler et al., 1995). It is possible that the weighting of synapses on RA from HVC and LMAN is functionally related to the degree of song stereotypy, such that a relatively large number of synapses from HVC create a more stereotyped song, and a relatively large number of synapses from LMAN cause a less stereotyped song.

Singing rates are generally highest during song learning when there is a rapid increase in stereotypy (Johnson et al., 2002) and therefore singing rate could be affecting song stereotypy and/or the morphological changes described above.

Changes in song behavior and song system in adulthood

As mentioned earlier, the close of the critical period in “closed-ended” learners such as zebra finches does not signify the end of all changes in song behavior. In addition, recent evidence has shown that there is plasticity during adulthood at the cellular level as well. It is possible that this cellular plasticity during adulthood is due to mechanisms that are similar to the ones that occur during development.

Adult changes in behavior

It was recently shown that the stereotypy of zebra finch song increases in adulthood between three months and twelve months (Pytte et al., 2007). In addition,
disrupting auditory feedback in adulthood can create song modifications in closed-ended learner species. For example, deafening causes degradation of song stereotypy in zebra finches, such that song structure is no longer consistent with the song produced before deafening, and song continually degrades such that the renditions of song produced after deafening are also inconsistent with one another (Lombardino and Nottebohm, 2000). Additionally, song is modified when distorted auditory feedback (such as white noise) is played to zebra finches during singing, and then song recovers when the distorted feedback is turned off (Tschida and Mooney, 2012a). Also, when singing-related auditory feedback was artificially pitch-shifted when played back through headphones, Bengalese finches attempted to adaptively compensate by shifting the pitch of their song output in the opposite direction of the artificially pitch-shifted feedback (Sober and Brainard, 2009).

Collectively, these findings show that adult birds rely on auditory feedback to maintain their stereotyped songs, and they implicate that stable adult song is actively monitored by an auditory feedback mechanism similar to the one present during song learning (Figure 4A). The adaptive pitch shifts found by Sober and Brainard strongly support a model in which there is a stable internal representation of song during adulthood against which auditory feedback is compared, and then mismatches lead to error-correction (2009). This auditory feedback error mechanism would be helpful in maintaining a stable song in cases where there is perturbation of the motor program or changes in the physical properties of the vocal periphery (Sober and Brainard, 2009). This model is opposed to a previously held model, which proposed that song was
maintained by a simple and unchanging pattern generator after song crystallization (Konishi, 1965).

Interestingly, the extent of song deterioration after deafening is related to the age of the bird; the songs of young birds (about 3-6 months old) degrade after a few weeks, whereas birds who are two years or older often do not show any severe song degradation for many months after deafening (Lombardino and Nottebohm, 2000). These results indicate that as adult birds age, song stability becomes decreasingly reliant on auditory feedback.

**AFP is essential for song behavior changes in adulthood**

In addition to its importance during song learning, the anterior forebrain pathway is also critical for song plasticity during adulthood. For example, LMAN lesions prevent the song degradation that normally occurs after deafening (Brainard and Doupe, 2000b). This result suggests that deafening-induced song destabilization is due to an active process, and the widely held model is that this process is similar to what occurs during song learning, in which the mismatch between the auditory feedback (removed due to deafening) and the stored auditory template causes aberrant error-correction signals that cause a maladaptive modification of the motor program (Brainard and Doupe, 2000a) (Figure 4B). Therefore, although somewhat counterintuitive, greater song degradation after deafening could indicate more song plasticity. Additionally, pharmacological inactivation of LMAN prevents acoustic changes that normally occur due to the playback of white noise during singing (Warren et al., 2011). Furthermore, LMAN is necessary for the song recovery that
occurs after prolonged exposure to distorted auditory feedback is terminated, indicating that LMAN is also important for adaptive plasticity in adulthood (Warren et al., 2011). The combined evidence above implicates the AFP as critical for song plasticity that attempts to create a match between auditory feedback and the stored auditory template, whether it is adaptive error-correction (during song learning or song recovery) or maladaptive error-correction (due to deafening).

Figure 4. A) Model of adult song stability as reliant on auditory feedback. It is hypothesized that stereotyped song is maintained throughout adulthood by an error-correction mechanism in which subtle differences between the auditory feedback and the stored auditory template lead to small adaptive changes in the song program that keep song stable. This mechanism would be adaptive in cases where there is a small perturbation of the motor program. B) Model of deafening-induced song degradation. It is hypothesized that the complete lack of auditory feedback causes a huge
mismatch between this interrupted feedback and the stored auditory template, therefore causing large, but aberrant, error-correction signals and a maladaptive modification of the motor program leading to song degradation. Therefore, greater song degradation could indicate greater song plasticity. Both of the above models are analogous to what occurs during juvenile song learning (figure 2). Figures adapted from (Brainard and Doupe, 2000a).

Evidence suggests that LMAN is actively and directly driving song changes during auditory feedback perturbations in adulthood. Lesions to LMAN after deafening cause a reversal of deafening-induced song changes (Nordeen and Nordeen, 2010), and inactivation of LMAN causes reversal of song changes induced by playbacks of white noise (Andalman and Fee, 2009). It is possible that LMAN provides random variation that allows for an evaluation mechanism to select and reinforce correct motor actions while weakening incorrect ones (Kao and Brainard, 2006). In support of this model, lesions to LMAN during adulthood cause a decrease in the natural variability of song (Kao and Brainard, 2006), and pharmacological augmentation of LMAN activity leads to increased variability of both note sequence and note acoustic features in adults (Hamaguchi and Mooney, 2012). It is also possible that in addition to song variability, LMAN and the entire AFP is providing the instructive input to guide song plasticity. Note pitch has natural variability, and if white noise is played conditionally when the pitch of a bird’s note is above a certain threshold, then the bird will adaptively shift the targeted note’s pitch downward to avoid white-noise playback. LMAN inactivations will reverse this adaptive pitch shift to baseline, which indicates that LMAN is partially responsible for the implementation of adaptive behavioral changes, and supports a model in which LMAN provides instructive signals for song modification (Andalman and Fee, 2009).
As mentioned above, the increase in the weighting of HVC synapses on RA relative to LMAN synapses that occurs during song learning could be functionally related to the corresponding increase in song stereotypy (Kirn, 2010). Because the AFP has similar functions during song learning and adulthood, it is possible that the ratio of HVC-RA:LMAN-RA synapses is related to the degree of song stereotypy during adulthood as well. Because LMAN is critical for song plasticity and HVC is essential for song stereotypy, it follows that the anatomy could change to favor LMAN-RA synapses due to deafening, which causes song modifications leading to loss of song stereotypy in adulthood.

*Age-related changes in cell morphology in adulthood*

In addition to behavioral changes, there are also changes in the dendritic morphology of the song system during adulthood (McDonald and Kirn, 2012). Interestingly, it was found that there is an increase in HVC-RA dendritic arbor relative to LMAN-RA dendritic arbor between the ages of three months and one year (McDonald and Kirn, 2012). These changes are reminiscent of what occurs during song development: as song becomes increasingly stereotyped, there is a decrease in dendritic arborization in LMAN (Nixdorf-Bergweiler et al., 1995). In addition, this adulthood increase in the HVC-RA:LMAN-RA dendritic arbor ratio occurs over the same time period (3-12 months) as the stereotypy increases that occur in adulthood (Pytte et al., 2007). Therefore, it is possible that the ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor could be functionally related to song stereotypy during development and adulthood. Pilot data in the Kirn laboratory show a trend for a
positive correlation between the ratio of HVC-RA:LMAN-RA dendritic arbor and degree of song stereotypy, which further indicates that there may be a functional relationship (Smith, 2011).

An increase in the dendritic arbor of HVC-RA cells relative to LMAN-RA cells could be related to a relative increase in the number of HVC-RA synapses. Previous work has shown that during development, a decrease in the amount of LMAN arbor happens alongside a decrease in LMAN-RA synapse number (Herrmann and Arnold, 1991, Nixdorf-Bergweiler et al., 1995, McDonald and Kirn, 2012). Therefore it is possible that the ratio of HVC-RA:LMAN-RA dendritic arbor is an indirect measure of the number of HVC synapses on RA relative to LMAN synapses.

It is also possible that increased connectivity between HVC-RA cells due to an increase in dendritic arbor could increase song stereotypy (McDonald and Kirn, 2012). There are abundant connections between cells in HVC (Mooney and Prather, 2005), and HVC-RA neurons reliably produce sparse bursts at precise times during each rendition of a song note, but with some small random temporal error (Hahnloser et al., 2002). Therefore, it is possible that increased connectivity in HVC could decrease the temporal variability of pre-motor commands, and therefore increase song stereotypy (McDonald and Kirn, 2012).

The increase in the ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor also happens over the same time period as the age-related decrease in degree of song degradation after deafening: young birds between three and six months of age show severe song degradation a few weeks after deafening, whereas birds older than
one year maintained a stable song for much longer (Lombardino and Nottebohm, 2000). Therefore, decreased reliance on auditory feedback is another possible function of an increased HVC-RA:LMAN-RA dendritic arbor ratio (McDonald and Kirn, 2012). LMAN is responsible for deafening-induced song destabilization (Brainard and Doupe, 2000b, Nordeen and Nordeen, 2010). Therefore, if arbor correlates with synaptic strength, the increase in HVC-RA arbor relative to LMAN-RA arbor would reflect a greater influence of HVC on RA relative to LMAN, which could cause song to be more resistant to deafening-induced song destabilization.

*Experience-dependent cellular changes in the song system during adulthood*

There is evidence that experience in adulthood can influence the song system at the cellular level. Singing rate has been shown to affect neurogenesis and song system morphology. Canaries that were allowed to sing for 8 days had significantly more new neurons in HVC than those that were prevented from singing in the same time period, and there is a significant positive relationship between singing rate and new HVC neurons in castrated male canaries (Li et al., 2000, Alvarez-Borda and Nottebohm, 2002). In addition, there is a significant positive correlation such that a higher singing rate at the time of crystallization relates to a larger amount of HVC-RA arbor relative to LMAN-RA dendritic arbor in individually housed males (McDonald, 2009). Singing rates are generally highest when there is a rapid increase in stereotypy during song learning (Johnson et al., 2002), and therefore singing rate could be affecting song stereotypy and/or the morphological changes that occur
during development. Therefore, it is also possible that singing rate affects stereotypy or morphology during adulthood.

In addition, Tschida and Mooney recently showed that deafening affects spine morphology in the song system (2012b). Specifically, deafening reduces the size and stability of spines on the cells in HVC that project to Area X (the beginning of the AFP), and the magnitude of change in spine size predicted the subsequent degree of song degradation (Tschida and Mooney, 2012b). Alongside the morphological changes, there were changes in intrinsic excitability and spontaneous action potential output that could possibly affect the singing-related action potentials of these cells and consequently affect song behavior. This result shows that the AFP is plastic at the cellular level in response to auditory feedback perturbations.

**Experimental design**

In general, studying vocal learning in songbirds can give insights into the neural basis of how stereotyped motor skills are learned during critical periods of development and then maintained during adulthood. Evidence suggests that the balance of HVC-RA dendritic arbor or synaptic strength relative to LMAN-RA could be related to the degree of song stereotypy during both song learning and adulthood (Kirn, 2010, Smith, 2011, McDonald and Kirn, 2012). Because deafening causes degradation of song stereotypy, deafening can be used as a manipulation to elucidate if there is a functional relationship between the ratio of HVC-RA:LMAN-RA dendritic arbor and degree of song stereotypy. My thesis examined the dendritic morphology of HVC-RA and LMAN-RA cells in birds that are deafened in adulthood.
versus control hearing-intact birds, and compared this morphological data to the degree of song degradation. Because previous work has indicated that singing rate can have an effect on the morphology of the song system (Li et al., 2000, Alvarez-Borda and Nottebohm, 2002, McDonald, 2009), singing rate was also calculated for each bird.

Singing rate was also plotted against song stereotypy, because relationships between the two could elucidate information about the error-correction model. The error-correction model posits that the act of singing and the associated auditory feedback (or lack thereof) provide the information for error-signals. Therefore, the model predicts that in deaf birds, more singing would create more aberrant error-correction signals, and consequently a more degraded song (Figure 4B), whereas in hearing-intact birds, more singing would create more adaptive auditory feedback signals and therefore improve song stereotypy (Figure 4A).

Young adult birds (3 to 4.5 months old) were deafened, and 21 days afterwards the retrograde tracer RDA was injected into area RA in order to visualize the dendritic arbor in HVC and LMAN. Birds were perfused 4-days after RDA surgery in order to allow for complete backfill of cells. Young adult birds from 3 to 4.5 months old were chosen, because younger adult birds show deafening-induced song changes after only a few weeks, whereas older adult birds (6 months old and older) often do not show significant deafening-induced song changes for months, which indicates that young birds are more reliant on auditory feedback to maintain their songs (Lombardino and Nottebohm, 2000). A 25-day\(^1\) survival time was chosen,

\(^1\) RDA surgery was 21 days after deafening. Birds were perfused 4 days after RDA surgery, and therefore total survival time was 25 days.
because it was found that LMAN is most important for driving song degradation in the first few weeks after deafening (Nordeen and Nordeen, 2010), and in the present study we were interested in song changes produced by the AFP.

I predicted that deafening would promote dendritic growth in LMAN relative to HVC. Some deafened birds have a more degraded song than others (Lombardino and Nottebohm, 2000, Pytte et al., 2012), and I predicted that birds with the greatest song deficits after deafening would have the lowest ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor. If the hypothesis is true—that deafening promotes dendritic growth in LMAN relative to HVC—the data would support a model in which the ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor is causally related to the degree of song stereotypy during adulthood and during development. In this model, the loss of song stereotypy that occurs after deafening could be caused partially by an increase in LMAN dendritic arbor relative to HVC arbor, and the previously observed age-related increase in song stereotypy after the critical period (Pytte et al., 2007) could be partially explained by the observed increase in the HVC arbor relative to LMAN (McDonald and Kirn, 2012). Because song degradation after deafening is due to an active process, if LMAN-RA arbor causes loss of song stereotypy after deafening, this also implies that LMAN-RA arbor could be underlying song plasticity more generally. Additionally, because a relatively large amount of LMAN arbor is also seen when song is has low stereotypy during early song development (Nixdorf-Bergweiler et al., 1995), the HVC-RA:LMAN-RA ratio of dendritic arbor could also partially explain the low song stereotypy and high plasticity during song learning in juveniles. Alternatively, if deafening does not
promote dendritic growth in LMAN relative to HVC, it is possible that natural variation in dendritic morphology could affect the degree of song destabilization due to deafening, and therefore the age-related increase in HVC arbor relative to LMAN in young adulthood could be underlying the age-related decrease in reliance on auditory feedback to maintain stable song (Lombardino and Nottebohm, 2000).
Methods

Subjects

The experiment was done in accordance with the Wesleyan University Institutional Animal Care and Use Committee and NIH guidelines. Birds were housed with their families until about 90 days of age, at which time they were put into single-sex cages with 10-15 other birds. Twenty-two male zebra finches (Taeniopygia guttata) were used, and the birds were between 3 and 4.5 months of age (90 to 138 days post-hatch) at the time of deafening/sham surgery. Birds were singly housed in sound attenuating recording chambers for six to eight days before deafening and sham surgeries, and remained in the recording chambers until perfusion, which was 25 days after surgery. Because the birds were between 90 and 138 days old, some birds were transferred directly from their family cages to the recording chambers, and some were transferred from single sex cages. The soundproof recording chambers were set up as described in Tchernichovski, 2012: The chambers were made out of 100 quart coolers turned on their side, which were lined with soundproof foam. The coolers had a hole cut in the side for insertion of a microphone, and had metal fasteners screwed on for secure closure. Air was provided to the chambers by plastic tubes that were inserted into a hole on the side of the cooler and attached to an aquarium air pump on the other end. The air can escape, because the chamber is not air tight.

Deafening and Sham Surgeries

3- to 4.5-month old adult male zebra finches were deafened by bilateral removal of the cochlea or received a sham surgery during which birds were treated identically with the exception that the bony cochlea casing was not opened, as
described in Pytte et al. (2010). Birds were anesthetized with intramuscular injections of xylazine (.04 ml, concentration 20 mg/mL and ketamine (.04 ml, concentration 10 mg/mL), and were given additional injections of .01 ml of ketamine if the first injection was not sufficient to begin surgery. The birds were given .04 ml of yohimbine after the surgery in order to facilitate the recovery from the xylazine and ketamine anesthesia.

Song Recordings

One week before deafening, birds were individually housed in the soundproof recording chambers described above. Each chamber contained a microphone (Samson CL2 condenser microphone with the cardioid element, Samson Technologies, Hauppauge, NY), and the birds’ songs were recorded using a sound-activated computer program (Avisoft SASLab Pro Recorder; Avisoft Bioacoustics, Berlin, Germany). The threshold of the sound activation program was set in a conservative manner, such that all songs were recorded. Due to the conservative threshold, there were also many sound files that included noises that were not songs, so recording files were screened to delete those files that did not include song. The recording continued continuously until RDA surgery (21 days after deafening).

Rhodamine Dextran Amine Surgery

Twenty-one days after deafening, Rhodamine Dextran Amine (RDA; 3000MW; Invitrogen, Carlsbad, CA.), a retrograde fluorescent tracer, was injected into brain area RA using a stereotaxic surgery apparatus. The birds were perfused four days after surgery in order to allow for backfill of RDA into brain areas HVC and LMAN. Birds were anesthetized with intramuscular injections of xylazine (.04
ml, concentration 20 mg/ml) and ketamine (.04 ml, concentration 10 mg/mL). The birds were then placed in a stereotaxic apparatus and feathers were plucked from the head. The scalp was cleaned with 70% ethanol, and an incision was made along the anterior-posterior midline, and then the skin was pulled apart to reveal the skull. A small window was cut in the skull to reveal the bifurcation of the sinus between telencephalic hemispheres and cerebellum, and the stereotaxic instrument was adjusted such that the injection pipette zero-point was at this bifurcation. After the skull above the injection sites was removed, .04 µl of RDA (10% dilution in 0.1 M phosphate-buffered saline) was pressure injected bilaterally into RA (posterior −1.60 mm, lateral +/-2.30 mm, and ventral −1.7mm) at 10° from the vertical using a glass micropipette (tip diameter approximately 30-50 µm). The incision was closed with surgical tape, and then the birds were given .04 mL of yohimbine in order to facilitate the recovery from the xylazine and ketamine. The bird was allowed to recover in an individual cage with a heat lamp for 24 hours. The birds were then returned to the soundproof recording chambers, where they remained until perfusion four days later.

Perfusions and Tissue processing

Four days after RDA surgery, birds were strongly anesthetized with methoxyflurane (Metofane; Mallinckrodt, Mundelgn, IL) until unresponsive to a toe pinch. After the right atrium was cut, 20 ml of .1 M phosphate buffer and 50 mL of 4% paraformaldehyde (in 0.1 M PB; pH 7.4) were perfused through the left ventricle, and then the brain was extracted. Brains were stored in 4% paraformaldehyde at 4°C overnight and then stored in 0.1 M Phosphate-buffered saline until sectioned. Seventy-five µm sagittal sections were cut with a vibratome and all sections
containing HVC and LMAN were mounted on Unifrost microscope slides (Azer Scientific, Germany) and coverslipped with Aquamount (Polyscience, Warrington, PA.).

**Song Analysis**

Normal adult zebra finch song consists of three to ten notes produced in a consistent sequence. This note sequence is considered a motif, and most often motifs are repeated in rapid succession, which is classified as a song bout. Bouts are typically separated from one another by at least 0.5 seconds. Song stereotypy can be measured by looking at the consistency of note acoustic structure and note sequence from rendition to rendition. High stereotypy indicates high consistency in song structure across renditions.

In some analyses, song stereotypy before deafening and song changes after deafening were quantified using Sound Analysis Pro (SAP) (Tchernichovski et al., 2000). SAP compares sets of song files and analyzes differences in various acoustic features: pitch, frequency modulation, amplitude modulation, Weiner entropy, and goodness of pitch. The program calculates two scores based on these acoustic features: an “accuracy” score and a “% similarity” score. The accuracy score measures the degree of song likeness, whereas the % similarity score measures the proportion of song one that is similar to song two according to a similarity threshold. Figure 5A shows a pictorial representation of the song comparisons using SAP. The red lines indicate the portions of the songs that have passed a similarity threshold, and the % similarity score represents the portion of song 1 (vertical) that has a similar version in song two (horizontal). The accuracy score calculates degree of song
likeness frame-by-frame at the level of milliseconds, and then computes an average score. Therefore, % similarity is a more global representation of song stereotypy, whereas accuracy is a score that represents stereotypy at the local level. Figure 5B shows examples of spectrograms from two different song comparisons, and the accuracy and similarity scores calculated for each.

Figure 5. Song analysis using SAP. A) Pictorial representation of the song analyses performed using SAP. Song one (vertical spectrogram) is compared to song two (horizontal spectrogram). The red lines indicate the portions of the songs that have passed a similarity threshold. The % similarity score represents the portion of song one that has a similar version in song two. Alternatively, the accuracy score is measured by calculating the degree of song likeness frame-by-frame at the level of milliseconds, and then computing an average score. Figure adapted from Tchernichovski, et al., 2000. B) Song spectrograms of single motifs from two deaf birds, before and after deafening. The accuracy and % similarity scores shown were calculated from a comparison of the before and after deafening motifs using SAP. Time (seconds) is on the x-axis and sound frequency is on the y-axis. Sound intensity is depicted with red/yellow for high intensity and blue/black is low intensity. Figure created using Avisoft SAS lab Pro computer program (version 4.15, Raimund Specht, Berlin, Germany)
In order to quantify pre-surgery song stereotypy, the song recordings from the two days just prior to deafening and sham surgeries were visualized using spectrograms created with the Avisoft SAS lab Pro computer program (version 4.15, Raimund Specht, Berlin, Germany) or with the Audacity program (Free Software Foundation Inc., Boston, MA), and 30 song recordings were chosen from each day. The songs from day one were compared to the songs from day two using the “MxN” matrix analysis feature in SAP, in which each song from day one was individually compared to each song from day two. A mean accuracy score and mean % similarity score were then calculated from the multiple comparisons.

In order to quantify the structural changes in song due to deaf and sham surgeries, songs were analyzed in a manner similar to Pytte et al., (2012). 30 song recordings from 21 days after the surgery were compared to 30 of the pre-surgery recordings, and mean accuracy and % similarity scores were calculated. Scores calculated in this manner are referred to as post-operative stereotypy scores throughout the paper. For deaf birds, recordings from four days and twelve days after deafening were also compared to pre-deaf recordings to quantify the amount of song change at various time points. Four days after deafening was most often the first day that a bird sang post-surgery.

Two different kinds of analysis were done, in which different methods were used to choose the songs to be analyzed by SAP. In one type of analysis, the first 30 usable song recordings that contained the most common note sequence were chosen, and therefore all of the songs chosen contained the same notes in the same sequence. Therefore, the mean accuracy and mean % similarity scores from this analysis solely
provided a measure of the stereotypy of acoustic structure based on a syllable-by-syllable comparison of numerous renditions of the same syllables, and did not reflect note sequence variability.

In another type of analysis, the first 30 usable song recordings of the day were chosen regardless of what notes were contained in the song and what the sequence of notes was. The % similarity scores that were computed for this analysis reflected both the consistency of acoustic features and the consistency of note sequence. As mentioned above, a % similarity score measures the proportion of song one that is similar to song two according to a similarity threshold. Therefore, if we compare a motif with notes ABCDE to a motif with notes ABC using SAP, the % similarity score would be lower than when comparing the motif ABCDE to another rendition of ABCDE. The scores from this analysis are referred to as the % similarity (1st 30) scores throughout this paper.

In order to calculate a measure of the post-surgery change in song structure that is normalized to natural pre-surgery stereotypy, the mean accuracy and % similarity scores from the pre-surgery versus post-surgery analysis were subtracted from the pre-surgery scores. This score is referred to as the “normalized change” in accuracy and similarity throughout the paper. The percent change in accuracy and % similarity was calculated by dividing these normalized scores by the pre-surgery scores. A more negative normalized change or % change value indicates a larger reduction in post-operative song stereotypy relative to natural pre-operative stereotypy. A more positive normalized change or % change in values indicates a post-operative song that is more stereotyped relative to pre-operative song stereotypy.
The scores from the SAP analyses give two measures of the stereotypy of acoustic features independent of note sequence (accuracy and % similarity scores), and a measure that reflects both acoustic feature stereotypy and note sequence consistency (% similarity 1st 30). Another analysis was needed to calculate a value that represented note sequence consistency independent of note acoustic feature stereotypy. In order to quantify the consistency of note sequence pre-surgery, the proportion of typical note transitions was calculated (typical note transitions/total note transitions) (Scharff and Nottebohm 1991). The first usable 25-35 songs were analyzed from each of the days of interest: the two days just prior to deafening/sham surgery.

A “typical” note transition was operationally defined as the note transition most commonly sung as well as some very common note transitions defined here: in addition to the note transition most commonly sung, the transition between the most common last note in a motif and any intro note was considered a typical transition, as well as the transition between the most common last note and the most common first note; intro notes are present at the beginning of a song bout, but not before each rendition of the motif within a bout, and we did not want this variability to be considered in the analysis, as it is normal behavior. Transitions between repeating introductory notes were ignored, because introductory notes were also ignored in the SAP analyses.

The analysis also considered transitions between the ending note of a motif and silence. The transition between the most common ending note in a motif and
silence was considered typical, so that song truncations (when motifs do not end with the most common ending note) were considered atypical transitions.

In order to quantify the changes in note sequence that occurred post-surgery, the typical note-transition was defined as the most common note transition pre-surgery, and the proportion of typical note transitions at 21 days after surgery was calculated as described above from the first 25-35 usable songs of the day (Figure 6). This value reflects the proportion of the post-surgery song that is consistent with the typical sequence of the pre-surgery song. For deaf birds, sequence consistency was calculated at two additional time points: four days and twelve days after deafening.

A) Low Sequence Consistency
Before

![Song spectrogram showing low sequence consistency before surgery.]

After

![Song spectrogram showing low sequence consistency after surgery.]

B) High Sequence Consistency
Before

![Song spectrogram showing high sequence consistency before surgery.]

After

![Song spectrogram showing high sequence consistency after surgery.]

Figure 6. Song spectrograms representing high and low post-operative sequence consistency scores. A) Low sequence consistency. B) High sequence consistency
In addition, the percent change in sequence consistency was calculated by subtracting the post-surgery sequence consistency score from the pre-surgery score, and then dividing by the pre-surgery score. Because the pre-surgery song reflects the crystalized song, this % change score reflects the percent change in the proportion of songs that has the typical sequence of the original crystalized motif.

The two days just prior to deafening and sham surgeries were analyzed for pre-surgery singing rate. The total number of song motifs sung from lights on to lights off was quantified using the Avisoft SAS Lab Pro version 41.5 computer program. Templates of one or two syllables were created using the “Save spectrogram (ASCII/Binary)” tool, and then the “batch processing” and “scan for template spectrogram patterns” tools were used to automatically count the number of motifs in a set of recordings. Additionally, song counts were performed at 21 days after surgery for the sham birds, and at four, 12, and 21 days after surgery for the deaf birds. Post-deafening, the songs were too variable to use the “scan for template spectrogram patterns” feature, so the motifs were counted manually.

Morphological Analysis:
The slides were looked at with a fluorescence microscope (Olympus BX50) in order to identify the fluorescently labeled cells in HVC and LMAN and to measure their total dendritic length. The perimeter of HVC and LMAN and the dendritic arbor were traced manually using the Neurolucida program (MicroBrightField, Colchester, VT). The perimeter of HVC and LMAN was traced using a 10x objective and brightfield optics, and the dendrites were traced using a 40x air objective and fluorescence optics. The cells with the brightest fluorescence were traced so that the entire
dendritic tree would be included. Three to 14 cells were traced in each HVC and three to 13 cells in each LMAN per bird. The Neurolucida program created 3-dimensional reconstructions of the traced cells and calculated the length of each dendritic segment. Then, the total dendritic length was calculated for each cell by summing all of the segment lengths, regardless of branch order. Mean total dendritic length values were then calculated for each brain region of each bird. There were no significant differences in dendritic length found between left and right hemispheres. Therefore, when data from both hemispheres was available, the mean values for a brain region were calculated from a data set that was pooled from both hemispheres. Medial to lateral differences were analyzed, as there is some evidence that there are functional distinctions between medial and lateral HVC (Jarvis et al., 1998). The morphological data from each brain region was divided into a medial and a lateral half, and medial and lateral averages were calculated for the above attributes.

Statistics

All analyses were performed using JMP (SAS Statistical Software). To determine whether deafening had an effect on dendritic arbor length, one-way ANOVA analyses were performed to compare mean values in the deaf and sham groups. One-way ANOVAs were also run to investigate the effect of deafening on the four different post-operative song stereotypy scores, as well as the normalized change in scores. A Two-way ANOVA of dendritic arbor length was run, with experimental group (deaf/sham) and brain subregion (medial/lateral) as independent factors, to look for medial versus lateral differences in total dendritic length. Simple regressions were run
to determine whether there were significant correlations between song stereotypy and
dendritic arbor, and between song stereotypy and singing rate.

*Extra control birds*

Many birds died due to RDA surgery, and therefore an attempt was made to
add more control birds. These extra control birds were not given sham surgeries, but
instead they were housed in the recording chambers for 8 days, and then an RDA
surgery was performed. One bird from this procedure survived RDA surgery and had
proper RDA backfill that allowed for dendritic arbor tracing (unfortunately of LMAN
only). The dendritic arbor data from this bird were included in the “sham” group for
the ANOVA analysis that compared mean total dendritic arbor between the two
groups, and it was included in the medial versus lateral analysis, but it was not used
for any other analyses.

*Sample sizes*

Initially, twenty-two birds were used (9 deafened, 9 sham surgery, 4 extra
control birds) but many birds died during deafening or RDA surgery. It is possible
that it was too stressful for many birds to survive two surgeries. Additionally, a few
birds did not have proper RDA backfill. Therefore, there were six birds with dendritic
arbor data (HVC: n=3 deaf, n=2 sham, LMAN: n=3 deaf, n=3 sham), and twelve
birds with singing rate data (n=6 deaf, n=6 sham). For the medial versus lateral
analysis, some birds only had backfilled cells in one of the subregions, so the sample
sizes were a bit smaller. There were 4 birds with lateral HVC data (n=2 deaf, n=2
sham), 4 with medial HVC data (n=2 deaf, n=2 sham), 5 with lateral LMAN data
(n=2 sham, n=3 deaf), and 6 with medial LMAN data (n=3 deaf, n=3 sham).
Results

*Dendritic arbor morphology*

The retrograde tracer Rhodamine Dextran Amine (RDA) was injected into area RA 21 after deafening/sham surgeries. The birds were perfused four days after RDA surgery, thus allowing for backfill of the tracer into HVC and LMAN. Examples of cells backfilled with RDA can be seen in figure 7 below. Figure 7A shows the site where RDA was injected into area RA. Figure 7B shows a low power magnification of RDA backfilled cells in LMAN, and figure 7C shows a high power magnification merged z-stack image of a backfilled neuron in LMAN and its dendritic arbor. HVC-RA neurons were backfilled in a similar manner (not shown).

A strongly significant positive correlation was found between LMAN mean total dendritic length and HVC mean total dendritic length (Figure 8, \( R^2 = .984, \ p < .001, \ n=3 \) deaf, \( n=2 \) sham). This correlation could reflect a characteristic of dendritic arbor in zebra finches, but it could also reflect variation in the quality of RDA backfill between birds. Therefore, many analyses included the ratio of HVC total dendritic length relative to LMAN total dendritic length, because this ratio should not reflect variations in backfill quality. Both HVC-RA and LMAN-RA axons ramify throughout all of RA, and therefore backfill quality should not differ between the two brain areas (Kirn and Nottebohm 1993).
All images are of 75 µm parasagittal sections and were taken with a confocal fluorescence microscope. A) Low power magnification image of RDA injection site into RA. RA outlined with white arrows. B) Low power magnification image of RDA backfilled LMAN. C) High power magnification z-stack merged image of a RDA backfilled LMAN neuron and its dendritic arbor. Scale bar=100 µm.

Figure 8. Mean HVC total dendritic length plotted against mean LMAN total dendritic length for each bird. Correlation was significant (R²=.984, p<.001).
One-way ANOVAs were used to investigate the effect of deafening on HVC and LMAN mean total dendritic length, and the HVC/LMAN total dendritic length ratio. Contrary to our expectations, no significant differences or notable trends were found between deaf and sham groups (For LMAN, n=3 for each group. For HVC and HVC/LMAN ratio, n=3 for deaf, n=2 for sham. All p > .30).

There is some evidence for a functional distinction between the medial and lateral portions of HVC and LMAN (Jarvis et al., 1998). For LMAN and HVC, two-way ANOVAs of dendritic arbor characteristics were run, with experimental group (deaf/sham) and brain subdivision (medial/lateral) as independent factors. No significant results or notable trends were found, and there were no significant interactions (all p > .30).

**Song stereotypy**

For each bird, songs were recorded for seven to nine days prior to deafening/sham surgeries and for 21 days afterwards, at which point the RDA surgery was performed. Figure 9 shows changes in the four measures of song stereotypy—sequence consistency, accuracy, % similarity, and % similarity (1\textsuperscript{st} 30)—due to deafening and sham surgery. Measures of song stereotypy calculate the consistency of a bird’s song structure from rendition to rendition of song; higher scores indicate greater consistency. Deafening could alter song structure in a number of ways. It could cause degradation of acoustic features, changes in the note sequence, or a combination of the two. Four different measures of song stereotypy were used to evaluate these possible song changes. The % similarity score is a global measure of
the stereotypy of acoustic features that calculates the proportion of one song that is similar to another song according to a similarity threshold, whereas the accuracy score is a local measure of acoustic feature stereotypy that calculates the degree of song likeness at the level of milliseconds. These scores were calculated from a set of songs with the same note sequence, thus giving measures of acoustic stereotypy that exclude songs with sequence variability. In contrast, sequence consistency scores reflect the consistency of note sequence, and are calculated by measuring the proportion of song motifs produced with the most typical note sequence; this score is not at all related to acoustic stereotypy. The % similarity (1st 30) measure calculates a % similarity score from the first 30 songs produced regardless of note sequence, thus giving a combined measure of both acoustic and note sequence stereotypy 

([Tchernichovski et al., 2000) see methods for more details on song stereotypy analysis].

All stereotypy scores reflect the mean score of multiple pairwise comparisons of 30 song motifs sampled from two separate days. The song stereotypy scores from before deafening/sham surgery reflect a comparison of the songs from the two days just prior to surgery. The post-operative stereotypy scores were calculated by comparing songs from 21 days after surgery to songs from the day just prior to surgery, thus giving a measure of song consistency between pre- and post-operative songs. In the deaf group, stereotypy scores were also calculated in this manner at day 4 and day 12 after deafening.

In order to calculate a measure of the post-operative change in song structure that is normalized to natural pre-surgery stereotypy/variability, the post-operative
stereotypy scores were subtracted from the pre-operative scores (this measure is referred to as the “normalized change” throughout). The percent change in accuracy and % similarity scores was calculated by dividing these normalized scores by the pre-operative scores. A more negative normalized change or % change in stereotypy indicates more post-operative song degradation relative to natural pre-operative stereotypy. A more positive normalized change or % change indicates a post-operative song that was more stereotyped relative to pre-operative stereotypy.

In the sham group, some of the birds showed a small decline in song stereotypy after surgery, but many showed no change (Figure 9). In the deaf group, there was a gradual decline in all measures of song stereotypy across the three time points, although there was considerable variation in the degree of song degradation across birds; some birds had a fairly minor decline in stereotypy, while others had greater degradation (Figure 9). The similarity measure was the least sensitive to deafening; except for one bird (bird 67), most birds had very small declines in similarity score due to deafening (Figure 9B).
Figure 9: Song stereotypy scores for birds before and after deafening/sham surgeries. Figure legends reflect bird ID numbers. The “before deafening” and “before sham” data reflect stereotypy scores calculated from a comparison of songs from the two days just prior to deafening/sham surgeries. The stereotypy scores at “day 4,” “day 12,” and “day 21” reflect a comparison of songs from 4, 12, or 21 days after deafening/sham surgery, to songs from the day just prior to surgery. Stereotypy scores for sham birds were only sampled at 21 days after sham surgery, whereas scores for deaf birds were calculated at all sampling points.

A) Accuracy score, a measure of local acoustic stereotypy. B) % Similarity score, a global measure of acoustic stereotypy. C) % Similarity score (1st 30), a global measure of both acoustic and note sequence stereotypy. D) Sequence consistency, a measure of note sequence stereotypy. See methods section for more detail.
One-way ANOVAs were used to investigate the effect of deafening on all post-operative song stereotypy measures at day 21. The normalized change and % change in stereotypy scores were also analyzed. While there were only six birds with dendritic arbor data (due to deaths during RDA surgery), there were 12 birds with behavioral data (6 deaf, 6 sham) that were used for this analysis. Deaf birds had a significantly larger normalized decrease in accuracy scores by day 21 (normalized change: F(1,11) = 11.246, p = .007; % change: F(1,11) = 11.356, p = .007). None of the other comparisons were significant, although there were some notable trends. Deaf birds tended to have a lower post-operative similarity score (F(1,11) = 3.256, p = .101), and they also tended to have a greater normalized decrease in the similarity (1st 30) score (normalized change: F(1,11) = 3.406, p = .095; % change: F(1,11) = 3.32, p = .098).

There were no trends for sequence consistency, but at post-operative day 21 there was more variation in the deaf group than in the sham group (figure 9D).

**Song stereotypy versus dendritic arbor**

All song stereotypy measures at 21 days after deafening/sham surgery (the day before RDA surgery) were plotted against HVC mean total dendritic length and LMAN mean total dendritic length, and simple regressions were run. Because the correlation between LMAN and HVC dendritic arbor could possibly be due to variations in backfill quality, all song stereotypy measures were also compared to the ratio of HVC:LMAN total dendritic length. These simple regressions were run within the deaf group, within the sham group, and when the deaf and sham groups were pooled. The data were pooled because there were no significant differences in dendritic arbor measures or stereotypy measures between the two groups (except for
accuracy, and the data were not pooled in this case), and because it is possible that song motor stereotypy relates to dendritic arbor irrespective of auditory feedback.

No notable trends were found for accuracy, similarity, or similarity (1st 30) scores. When the deaf and sham data were pooled, a marginally significant positive correlation was found between the % change in sequence consistency at day 21 and HVC mean total dendritic length, such that a lower total dendritic length was associated with a more negative % change in sequence consistency (Figure 10A, R²=.729, p=.066). LMAN mean total dendritic length also showed a positive correlation with % change in sequence consistency that was marginally significant (Figure 10B, R²=.686, p=.083). It is likely that a more negative % change in sequence consistency was associated with both a lower HVC total dendritic length and a lower LMAN total dendritic length due to the positive correlation between HVC and LMAN total dendritic length (Figure 8). Interestingly, there was also a marginally significant positive correlation between the HVC:LMAN total dendritic length ratio and the % change in sequence consistency (Figure 10C, R²=.693, p=.080), indicating that a higher LMAN total dendritic length relative to HVC was associated with a more negative % change in sequence consistency. In other words, birds with more LMAN dendritic arbor relative to HVC also tended to sing post-operative note sequences that were more inconsistent with the pre-operative note sequences (normalized to natural pre-operative sequence consistency). Although the limited sample size makes definitive interpretations difficult, because these results were from an analysis that pooled the deaf and sham groups, it may be that this relationship holds in birds with and without auditory feedback.
Figure 10. Deaf and sham groups were pooled, and mean HVC total dendritic length, mean LMAN total dendritic length, and the HVC:LMAN dendritic length ratio were each plotted against the % change in sequence consistency at day 21. A) Mean HVC total dendritic length positively correlated with % change in sequence consistency ($R^2 = .729$, $p = .066$). B) Mean LMAN total dendritic length positively correlated with % change in sequence consistency ($R^2 = .686$, $p = .083$). C) HVC:LMAN dendritic length ratio positively correlated with % change in sequence consistency, indicating that higher LMAN total dendritic length relative to HVC (low ratio) was associated with a more negative % change in sequence consistency ($R^2 = .693$, $p = .080$)
Song stereotypy versus singing rate

Figure 11 shows the number of songs produced per day (from lights on to lights off, 14 hours) before and after deafening/sham surgeries. Singing rate on the day just prior to deafening positively correlated with the singing rate on the previous day ($R^2=265.368$, $p<.001$). Therefore, the singing rates from before deafening and sham surgery in figure 11 reflect an average of the singing rates from the two days before surgery. In the deaf group, singing rates were calculated at day four, day 12, and day 21 after deafening, and in the sham group singing rates were calculated at day 21 only. As shown in figure 11A, singing rate after deafening was highly variable. Some birds had a low singing rate in the first few days after deafening, and then an increase at day 12 and day 21, while one bird had a high singing rate at day four after deafening, which then decreased by day 12 and 21. Relatively high singing rates before deafening did not predict higher rates after deafening. Half of the deaf birds had a higher singing rate at day 21 than before surgery, and in contrast, all sham birds had a higher singing rate 21 days after sham surgery than before sham surgery. Additionally, higher singers before sham surgery tended to be higher singers after sham surgery (Figure 11B). However, it is unknown whether singing rate in hearing birds was consistent after surgery, because it was only sampled at day 21. The increased singing rates were possibly due to increased familiarity and comfort with the recording chambers as housing over time.
Figure 11. Singing rates for birds before and after deafening/sham surgery. Figure legend reflects bird ID number. The “before deafening” and “before sham” singing rates are averages of the rates from the two days prior to surgery. A) Deaf birds. Day 4, 12, and 21 reflect singing rates 4, 12, and 21 days after deafening surgery. B) Sham Birds. Day 21 reflects singing rates 21 days after deafening surgery.

Simple regression analyses were done to look for relationships between singing rate and all measures of song stereotypy (including the normalized change and % change in the stereotypy measures). Correlations between the two measures could elucidate information about the error-correction model, which posits that in hearing-intact birds, auditory feedback-driven error-correction signals cause adaptive modification of song (Figure 4A), while in deaf birds, the large mismatch between the auditory feedback (removed due to deafening) and the auditory template causes aberrant error-correction signals which cause maladaptive modification of song (Figure 4B) (Brainard and Doupe, 2000a). In this model, the act of singing and the associated auditory feedback (or lack thereof) provides the information for error.
correction, and therefore one might predict that in deafened birds, higher singing would be associated with greater song deterioration due to more error signals.

For deaf birds, the average singing rate after deafening (sampled at day 4, day 12, and day 21, and averaged across the three time points), was plotted against all measures of song stereotypy at post-operative day 21, and simple regressions were run. A marginally significant trend was found in which average post-deafening singing rate positively correlated with the normalized change in the similarity (1st 30) score (Figure 12, normalized change: $R^2=.609$, $p=.067$; % change: $R^2=.597$, $p=.0716$). In other words, the birds that sang fewer songs after deafening were more likely to have a more negative normalized change in similarity (1st 30), which indicates greater degradation of both acoustic features and note sequence relative to natural pre-op similarity (1st 30). No trends were found for the other song stereotypy measures (all $p > .30$).

![Figure 12](image)

Figure 12. The average singing rate after deafening (sampled at day 4, day 12, and day 21) plotted against the normalized change in similarity (1st 30) score, showing a marginally significant positive correlation ($R^2=.609$, $p=.067$). The birds that sang fewer songs after deafening were more likely to have a more negative normalized change in similarity (1st 30), indicating greater degradation of both acoustic features and note sequence.
If singing rate is related to song stereotypy, it is possible that the singing rates from the entire period of time post-surgery are relevant, but it is also possible that the most relevant singing rates are the measures taken from the same day as the stereotypy measures. Therefore, singing rate at day 21 was plotted against each of the measures of song stereotypy at day 21 and simple regressions were run. With this analysis, trends were seen for additional measures of stereotypy. Regressions were run for the deaf and sham groups separately, and then additional regressions were done in which the deaf and sham data were pooled for similarity, similarity (1\textsuperscript{st} 30), and sequence consistency scores. The data were pooled because there were no significant differences in singing rate or the above-mentioned stereotypy measures between deaf and sham groups, and it is possible that singing rate is related to stereotypy independent of auditory feedback. For both the deaf group and the sham group, a lower singing rate at day 21 tended to be associated with a more negative or a less positive normalized change in similarity score at day 21 (Figure 13 A and B). Lower similarity scores indicate lower stereotypy of acoustic features. This trend was also seen when the deaf and sham groups were pooled (Figure 13 C). After running simple regressions, significant correlations were found within the deaf group (normalized change: $R^2=.727, p=.0309$; % change: $R^2=.7225, p=.0313$) and in the pooled analysis (normalized change: $R^2=.485, p=.0119$; % change $R^2=.483, p=.0121$), whereas the p-value was not significant for the sham group analysis (normalized change: $R^2=.417, p=.166$; % change: $R^2=.419, p=.165$). The significant results within the deaf group and for the pooled group analysis seem largely due to one bird (bird
67) with relatively large stereotypy changes after deafening (data point marked with arrow), and the p-values were no longer significant when bird 67 was excluded.

Figure 13. Normalized change in similarity score at day 21 plotted against singing rate at day 21. Arrow points to a possible outlier (bird 67). A) Significant positive correlation was found within deaf birds ($R^2=.727$, $p=.0309$). A lower singing rate tended to be associated with a more negative normalized change in similarity, indicating that deaf birds that sang fewer songs after deafening had more degradation of acoustic features relative to natural pre-operative similarity. The correlation was non-significant when bird 67 was excluded ($p>.30$).

B) Within sham birds, a non-significant trend for a positive correlation was found ($R^2=.417$, $p=.166$). A higher singing rate tended to be associated with a more positive normalized change in similarity score, which indicates that hearing birds that sang more after sham surgery had a greater increase in the stereotypy of acoustic features relative to pre-operative stereotypy. C) A positive correlation was found also when the deaf and sham groups were combined ($R^2=.485$, $p=.0119$). The correlation was non-significant when bird 67 was excluded ($p>.30$).
Additionally, trends were also seen for similarity (1st 30) in the same direction: lower singing rates tended to be associated with a more negative or a less positive normalized change in similarity (1st 30). This trend was found in the deaf group and when the groups were pooled, and it was less pronounced in the sham group (Figure 14). In this case, the p-values were not significant with bird 67 included (deaf group: normalized change: $R^2=.336, p=.227$; % change: $R^2=.321, p=.241$. pooled groups: normalized change: $R^2=.150, p=.213$; % change: $R^2=.141, p=.230$). However, within the deaf group, the p-value became significant when excluding bird 67 (data point marked with arrow; normalized change: $R^2=.858, p=.024$; % change: $R^2=.857, p=.024$).
Figure 14. Normalized change in similarity (1st 30) scores at day 21 versus singing rate values from day 21. A) Deaf birds only. There were trends for singing rate to be positively correlated with the normalized change in similarity (1st 30), indicating that birds that sang less after surgery tended to have more degradation of acoustic structure and note sequence relative to pre-op similarity (1st 30). This correlation was non-significant (day 21: $R^2=.336, p=.227$), but became significant when bird 67 (arrow) was excluded (day 21: $R^2=.858, p=.024$). B) Sham birds only. Somewhat of a trend was found for singing rate to positively correlate with the normalized change in similarity (1st 30) at day 21. However, it was not at all significant ($p>.30$) and was not as strong as it was for deaf birds. 4 points are fairly linear, whereas two are outliers. C) Pooled deaf and sham groups. Somewhat of a trend was found for singing rate to positively correlate with the normalized change in similarity (1st 30) ($R^2=.150, p=.213$). This outcome was not much affected when bird 67 was excluded (arrow, $R^2=.122, p=.292$).
Similar trends were found when singing rate at day 12 was plotted against each of the measures of song stereotypy at day 12 in deaf birds (stereotypy and singing rate analysis at day 4 and day 12 after surgery was not done for sham birds). Like the results at day 21, lower singing rate tended to be associated with a more negative normalized change in song stereotypy scores. This relationship was found to be marginally significant when comparing singing rate at day 12 and the normalized change in similarity (1st 30) score on day 12 (normalized change: $R^2=.598$, $p=.071$; % change: $R^2=.577$, $p=.080$). Additionally, a marginally significant positive correlation was found between the singing rate at day 12 and the % change in sequence consistency at day 12 ($R^2=.638$, $p=.057$), which was not found for day 21, indicating that birds that sang fewer songs after surgery also tended to have more degradation of note sequence stereotypy irrespective of acoustic feature changes.

In sum, significant positive correlations were found between singing rates and stereotypy measures from the same day (day 12 or day 21) for deaf birds, and sham birds showed trends for the same relationship: the birds that produced fewer songs after surgery also had greater song degradation (deaf birds) or a lesser increase in song stereotypy (sham birds). Trends in this direction were found for all four stereotypy measures. These trends within deaf birds do not support the error-correction model, which predicts that less singing would create fewer aberrant error-correction signals, and therefore a less degraded song. The trends within sham birds fit the model, which predicts that in hearing-intact birds, more singing would create adaptive auditory feedback signals, and therefore improve song stereotypy. No
similar trends were found when comparing singing rate at day four versus stereotypy at day four for deaf birds. It is unknown whether there is a functional relationship between singing rate and song stereotypy, but it is plausible that song stereotypy on one day could be related to or affected by the singing rates a few days earlier. It is possible that there is a specific and limited early critical period after deafening during which singing rate impacts song stereotypy. Many studies have shown that changes in song stereotypy due to experience happen on the time scale of days (Lombardino and Nottebohm, 2000, Sober and Brainard, 2009). Therefore, singing rate at day four was compared to stereotypy scores at day 12 and day 21 (for deaf birds only). Within deaf birds, significantly or marginally significant relationships were found, such that a lower singing rate at day four correlated with a higher post-operative accuracy score (day 12: $R^2=.732$, $p=.030$, day 21: $R^2=.667$, $p=.047$) and a less negative normalized change in accuracy at day 12 (Figure 15B, normalized change: $R^2=.915$, $p=.003$; % change: $R^2=.924$, $p=.002$) and day 21 (Figure 15A, normalized change: $R^2=.626$, $p=.061$; % change: $R^2=.651$, $p=.052$). These correlations remain significant or marginally significant after excluding the possible outlier (bird 67) mentioned above (all $p < .06$). There were also significant correlations between singing rate at day four and both post-operative similarity score at day 21 ($R^2=.885$, $p=.0051$) and normalized change in similarity at day 21 (normalized change: $R^2=.911$, $p=.003$; % change: $R^2=.911$, $p=.003$). These p-values did not remain significant when excluding bird 67 (all $p > .30$). In general, these correlations showed that a higher singing rate in the first few days after deafening was related to more degradation of acoustic features later on.
These results support an error-correction model, in which the mismatch between the auditory feedback (removed due to deafening) and the auditory template causes aberrant error-correction signals that trigger a maladaptive modification of song, because more singing would presumably create more aberrant error-signals.

![Graph A](image)

**Figure 15.** Normalized change in accuracy A) at day 21 or B) at day 12 plotted against singing rate at day 4, for deaf birds only. Normalized change in accuracy showed significant or marginally significant negative correlations with singing rate at day 4, indicating that birds that sang at higher rates in the first few days after deafening had more degradation of acoustic features later on. (Day 12: $R^2=.915$, p=.003. Day 21: $R^2=.626$, p=.061). These p-values remained significant/marginally significant when excluding bird 67 (arrow, all p<.06).

*Singing rate versus dendritic arbor*

Simple regression analyses were done to compare post-operative singing rates to HVC and LMAN total dendritic length and to the HVC:LMAN total dendritic length ratio. No significant results or notable trends were found (all p>.30).
Discussion

Previous studies have shown that during both juvenile song learning and adulthood, the HVC-RA pathway promotes song stereotypy (Nottebohm et al., 1976, Hahnloser et al., 2002), while the LMAN-RA pathway is critical for song plasticity and variability (Scharff and Nottebohm, 1991, Brainard and Doupe, 2000b, Kao and Brainard, 2006, Nordeen and Nordeen, 2010, Warren et al., 2011, Hamaguchi and Mooney, 2012). Additionally, work has shown that there is an age-related increase in HVC-RA dendritic arbor relative to LMAN-RA dendritic arbor over the same age-range as an age-related increase in song stereotypy (Pytte et al., 2007, McDonald and Kirn, 2012), which indicates that the ratio of HVC-RA:LMAN-RA dendritic arbor could be functionally related to the degree of song stereotypy. The songs of deafened birds become increasingly degraded from the previously crystalized song and this degradation of stereotypy is dependent on and partially driven by LMAN input (Brainard and Doupe, 2000b, Nordeen and Nordeen, 2010). In order to elucidate if there is a functional relationship between dendritic arbor and the degree of song stereotypy, a retrograde tracer was injected into area RA in order to visualize the HVC and LMAN dendritic arbor of both deafened birds and birds given a sham surgery. If deaf birds showed significantly different dendritic arbor characteristics from sham birds, it would suggest that there is a functional relationship between dendritic arbor and the degree of song stereotypy; the dendritic arbor of hearing-intact birds would be related to song stereotypy and the dendritic arbor characteristics of deaf birds would be related to song degradation. Because deafening-induced song changes are due to an active process reliant on LMAN, song degradation is indicative
of song plasticity in response to auditory feedback manipulations, and therefore deafening-induced changes in dendritic arbor could also elucidate mechanisms of adult song plasticity.

Mean total dendritic length of HVC and LMAN in deaf and hearing-intact birds

Contrary to expectations, no significant differences in HVC or LMAN mean total dendritic length were found between the deaf and sham groups. There are a few possibilities that can explain this result. Many birds died during RDA surgery, and therefore the sample size for total dendritic arbor data was very small (three deaf birds and three sham birds). It is possible that trends would have emerged if the sample size were larger. Additionally, the 25-day\(^2\) survival time was not sufficient to cause the large decreases in song stereotypy that were previously observed in deafened young adult birds with a longer survival time (Lombardino and Nottebohm, 2000). The normalized change in accuracy score was the only one of the four song stereotypy measures found to be significantly lower in deaf birds than in sham birds. Some birds had noticeably degraded songs, but the largest % change in accuracy score was only about -14\%, and the scores of other birds barely changed (3-5\% decrease). It is possible that with a longer survival time, there would have been greater changes in both stereotypy and dendritic arbor anatomy in deaf birds.

A survival time of 25 days was chosen, because it was found that LMAN is most important for driving song degradation in the first few weeks after deafening (Nordeen and Nordeen, 2010), and in the present study we were interested in song

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\(^2\) RDA surgery occurred 21 days after deafening and sham surgeries, and birds were perfused 4 days after RDA surgery. Therefore, total survival time was 25 days.
changes produced by the AFP. LMAN lesions after deafening reverse mild to moderate deafening-induced song changes, but lesions fail to rescue song deficits when songs are severely degraded, indicating that a mechanism outside of the anterior forebrain pathway is important at later stages of song degradation (Nordeen and Nordeen, 2010). Most of the LMAN-lesion-induced song rescues occurred between 14 and 25 days after deafening. We predicted that at 25 days, deafened young adult birds would have moderately degraded songs that were actively driven by LMAN. However, it is possible that there is a delay between changes in LMAN activity and putative changes in cell morphology, and therefore a longer survival time could reveal differences in dendritic arbor between deaf and hearing-intact groups.

A highly significant positive correlation was found between HVC mean total dendritic length and LMAN mean total dendritic length (Figure 8). There is evidence that the volume of HVC and LMAN are correlated (Airey et al., 2000), which gives indirect support for the possibility that the correlation of dendritic arbor reflects a true characteristic of zebra finch brain anatomy. However, definitive conclusions cannot be made from this study, because the correlation between HVC total dendritic length and LMAN total dendritic length could also reflect variation in the quality of RDA backfill between birds.

*Song stereotypy versus dendritic arbor*

The only marginally significant correlations found between song stereotypy and dendritic arbor involved sequence consistency scores in a pooled analysis that combined deaf and sham groups. Marginally significant positive correlations were
found between the % change in sequence consistency and a) HVC mean total dendritic length and b) HVC:LMAN mean total dendritic length ratio. Additionally, a marginally significant positive correlation was also found between the % change in sequence consistency and c) LMAN mean total dendritic length. However, it is possible that this positive correlation with LMAN is partially due to the covariation between HVC and LMAN dendritic length (Figure 8), which could be due to variability in backfill quality. Therefore, the more interesting result was the positive correlation with the HVC:LMAN total dendritic length ratio, which indicated that for birds both with and without auditory feedback, greater LMAN-RA arbor relative to HVC-RA arbor (a lower ratio) was associated with a more negative or less positive % change in note sequence consistency.

This result is consistent with previous results. Pilot data have suggested a positive correlation between HVC-RA:LMAN-RA dendritic arbor and accuracy score in hearing-intact birds (Smith, 2011), such that greater LMAN arbor relative to HVC (a lower ratio) was associated with lower accuracy scores. Additionally, as mentioned above, there is an increase in the ratio of HVC-RA dendritic arbor relative to LMAN-RA dendritic arbor between the ages of three months and one year, and this increase in HVC-RA:LMAN-RA dendritic arbor occurs over the same time period (3-12 months) as song stereotypy increases that occur in adulthood (Pytte et al., 2007, McDonald and Kirn, 2012). In addition, a larger amount of LMAN dendritic arbor is associated with plastic song with low stereotypy in juveniles (Nixdorf-Bergweiler et al., 1995).
Possible functional relationships between dendritic arbor and song stereotypy

The correlation found between a larger % decrease in sequence consistency and a low HVC-RA:LMAN-RA dendritic arbor ratio supports a model in which the amount of HVC arbor relative to LMAN arbor is an important factor underlying the degree of song stereotypy during adulthood in both deaf and hearing-intact birds. There is some evidence that dendritic arbor in HVC and LMAN positively correlates with axonal arbor (Herrmann and Arnold, 1991, Nixdorf-Bergweiler et al., 1995), and therefore a lower ratio of HVC-RA:LMAN-RA dendritic arbor could be indicating a higher input strength from LMAN on RA relative to HVC. Evidence has shown that during juvenile song learning, a larger number of LMAN-RA synapses relative to HVC-RA synapses is associated with lower song stereotypy (Herrmann and Arnold, 1991).

In addition, studies have shown that in the song system, dendritic arbor growth is associated with an increase in afferent synapses (Canady et al., 1988), and therefore, more dendritic arbor in LMAN relative to HVC might reflect a relatively greater afferent input onto LMAN. This greater afferent input could increase the weighting of LMAN-RA input relative to HVC, which could cause decreased song stereotypy.

Interestingly, the prior study mentioned above found correlations between dendritic arbor characteristics and accuracy score (Smith 2011), whereas the linear relationships found in this study involved a measure of note sequence stereotypy that does not reflect the stereotypy of acoustic features. Like the acoustic features of song notes, note sequence is variable early in song learning and becomes more and more
stereotyped as song learning progresses (Kao and Brainard, 2006) and as LMAN arbor decreases (Nixdorf-Bergweiler et al., 1995). It was found that augmenting activity in LMAN transiently increases note sequence variability as well as the variability of acoustic features, which supports a model in which LMAN is actively driving multiple aspects of song structure (Hamaguchi and Mooney, 2012). The data found in the present study support a model in which LMAN is important for changes in note sequence to occur. Due to the small sample size, it is possible that correlations between dendritic arbor characteristics and measures of acoustic feature stereotypy would arise with additional birds.

If the ratio of HVC dendritic arbor relative to LMAN arbor—or the ratio of HVC input strength on RA relative to LMAN input—are functionally related to note acoustic feature stereotypy and/or note sequence stereotypy during adulthood, it is possible that a) changes in song stereotypy are causing the changes in cell morphology, b) deafening causes changes in cell morphology, which then causes song degradation, or c) natural variability in cell morphology affects the degree of song degradation due to deafening. Some birds have greater song degradation than others after deafening (Figure 9, Nordeen and Nordeen, 2010), which indicates variability in the reliance on auditory feedback between birds. Reliance on auditory feedback to maintain song also decreases with age (Lombardino and Nottebohm, 2000). No significant differences in dendritic arbor characteristics were found between deaf and sham groups, but due to the very small sample size, no conclusions can be made from the results in the present study.
If future studies show a significant difference in dendritic arbor characteristics between deaf and sham groups, it would indicate that either b) deafening causes the changes in dendritic arbor, which could be causing song degradation or that a) changes in song cause changes in cell morphology. Future studies could distinguish between a) and b) by using multiple survival times after deafening, because these studies could reveal whether changes in song precede or follow changes in morphology, and therefore elucidate whether changes in brain morphology cause song changes or song changes cause changes in brain morphology. Other studies have indicated that adult experience, such as deafening, can affect dendritic morphology in the song system. Deafening affects HVC spine morphology, such that spines on the cells in HVC that project to Area X (the beginning of the AFP) become smaller and more unstable (Tschida and Mooney, 2012b). Furthermore, the magnitude of change in spine size predicted the subsequent degree of song degradation, indicating that changes in dendritic spine morphology can predict changes in song stereotypy. Further evidence has shown that other adult experience can cause changes in dendritic morphology in both HVC and LMAN. It was found that there was growth in both HVC and LMAN dendritic arbor when males were singly housed with a female compared to isolated and group housed males; though it is unclear which aspect of the housing condition (changes in singing rates, hormone levels, etc) is related to these dendritic changes (McDonald and Kirn, 2012).

If future studies have larger sample sizes and explore multiple survival times after deafening, and it is shown that there remains no significant difference in the dendritic arbor characteristics of HVC and LMAN between deaf and sham groups, it
could indicate that dendritic arbor is not functionally related to deafening-induced changes in song stereotypy. However, if there is still an association between dendritic arbor length and degree of song stereotypy despite no overall group differences, this result would suggest that c) natural variation in cell morphology affects the degree of song degradation due to deafening (in other words, the degree of reliance on auditory feedback to maintain song).

*Relationships between song stereotypy and singing rate can inform error-correction models*

Simple regression analyses were done to look for relationships between song stereotypy and singing rate, because correlations between the two measures could elucidate information about the error-correction model. In hearing-intact birds, the error-correction model posits that small mismatches between auditory feedback and the memorized auditory template of song create adaptive error-correction signals that result in a higher song stereotypy (Figure 4A). It is hypothesized that stereotyped song could be maintained throughout adulthood by this error-correction mechanism. In this model, the act of singing and the associated auditory feedback (or lack thereof) provide the information for error correction. Therefore, this model predicts that a higher singing rate in hearing-intact birds would create more adaptive error-signals and therefore correlate with a greater increase in song stereotypy. Some of the findings in the present study support this model. In hearing-intact birds given a sham surgery, a higher singing rate tended to be associated with a greater increase in the
stereotypy of acoustic features relative to pre-operative stereotypy (as measured by a positive normalized change in similarity score, Figure 13B).

In deaf birds, the error-correction model predicts that the song destabilization that occurs after deafening is due to an active process, in which the mismatch between the auditory feedback (removed due to deafening) and the memorized auditory template of song causes aberrant error-correction signals, which then cause a maladaptive modification of song (Figure 4B) (Brainard and Doupe, 2000a). Therefore, this model predicts that in deaf birds, a higher singing rate after deafening would create more aberrant error-signals and therefore correlate with greater song degradation. Some of the findings in the present study support this model. It was found that deaf birds that sang at higher rates in the first few days after deafening had greater degradation of acoustic features later on (as measured by a more negative normalized change in accuracy and similarity scores, Figure 15). Although definitive conclusions about functional relationships cannot be made from this correlational data, this result suggests that there could be a specific and limited early critical period after deafening during which singing rate impacts song stereotypy. Many studies have shown that changes in song due to experience happen on the time scale of days (Lombardino and Nottebohm, 2000, Sober and Brainard, 2009), so it is plausible that singing rate may affect the structure of songs produced a few days later.

Because longer survival times often allow for more severe song degradation, it would follow that in addition to an early critical period in which singing rate affects the extent of song deterioration, there would be another mechanism later on that is independent of singing rate. There is evidence that neuron bursting in the motor
pathway during sleep mimics previous singing-related neuronal activity (Margoliash, 2010). Therefore, it is possible that the singing-related error signals produced in the few days after deafening are played back repeatedly during sleep with increasing time after deafening in a manner independent of singing rate.

However, whether there is a functional relationship between singing rate and song stereotypy is unknown, and if there is a relationship, it is unknown whether a) there is an early critical period after deafening during which singing rate impacts song stereotypy, b) the singing rates throughout the entire period of time post-deafening are relevant, or c) the most relevant singing rates are the measures taken on the same day as the stereotypy score. Other correlation analyses were used to explore the relationships between the normalized changes in stereotypy scores at day 21 and b) the average post-operative singing rate and c) the singing rate on same day (post-operative day 21). These analyses did not support the error-correction model and showed opposite trends to what is presented above. In general, deaf birds that had a higher singing rate (either on day 21 alone, or a rate averaged across day 4, day 12, and day 21) were more likely to have less severe song degradation (as measured by a less negative normalized change in stereotypy scores, Figure 12-14). This trend was seen for multiple measures of song stereotypy.

These results indicate that there may be another mechanism besides auditory feedback-driven error correction that relates singing rate to song stereotypy. It could be that the motor memory underlying song becomes stronger with more singing practice due to proprioceptive feedback from the vocal muscles. This feedback mechanism would be independent of auditory feedback and would not be perturbed
by deafening. A relationship between high post-operative singing rates and less negative/more positive normalized changes in song stereotypy was also seen in the sham birds (Figure 13B), and it was found when sham and deaf groups were pooled as well (Figure 13C, Figure 14C), further indicating that there could be a mechanism independent of auditory feedback. Therefore, these results in sham birds may be due to proprioceptive feedback from the vocal muscles instead of (or in addition to) auditory feedback driven error correction.

Possible functional relationships between singing rate, song stereotypy, and dendritic arbor

Future studies should directly manipulate singing rate in order to prove a functional relationship between singing rate and song stereotypy. If it is additionally proven that changes in cell morphology cause changes in song stereotypy, then it is possible that singing rate affects either the HVC-RA:LMAN-RA dendritic arbor ratio or the input strength ratio, which in turn affects song stereotypy. In the present study, no correlation trends were found between singing rate and dendritic arbor characteristics, but this could be due to the very small sample size. McDonald and Kirn found a positive correlation between singing rate and the ratio of HVC-RA:LMAN-RA dendritic arbor in individually housed males, indicating that higher singing rates are related to a greater amount of HVC arbor relative to LMAN arbor in hearing-intact birds (2012). This correlational result supports the idea that singing rate could be related to song stereotypy through its relationship with dendritic arbor.
Methodological Issues

Many birds died during or immediately after RDA surgery. It is possible that the stress from a deafening/sham surgery and a second surgery for RDA injections in addition to living in isolated recording chambers was too great for many birds to survive. Therefore, a major problem with the present study is the very small sample size for total dendritic length data (three deaf birds and three sham birds). It is possible that new trends would have emerged, or existing trends would have disappeared or become stronger, if the sample size were increased.

It is also possible that the retrograde transport of RDA may not be completely efficient, and therefore the visualized morphology does not represent the entirety of the dendritic arbor. If so, potentially important morphological data concerning higher order branches could be missing from the present data set. Additionally, variability in the targeting of the injection site into RA could cause variability in the backfill quality between birds, and therefore differences in HVC or LMAN total dendritic length could be due to this variability in backfill quality. Because of this possible issue, the ratio of HVC-RA dendritic arbor relative to LMAN-RA arbor was used in analyses. Because HVC-RA and LMAN-RA cells ramify throughout all of LMAN (Kirn and Nottebohm, 1993), the backfill quality should not differ between the two areas.

Future Directions

As mentioned above, some evidence suggests that HVC and LMAN dendritic arbor is positively correlated with axonal arbor (Herrmann and Arnold, 1991,
Nixdorf-Bergweiler et al., 1995) and thus a lower ratio of HVC-RA:LMAN-RA dendritic arbor could indicate a higher input strength from LMAN on RA relative to HVC. Future studies should inject anterograde tracers into HVC and LMAN to look at the axonal arbor in RA in both deaf and sham birds, and study the relationship between axonal arbor and song stereotypy.

Future studies should also investigate the dendritic and axonal arbor of HVC-RA and LMAN-RA cells across birds with multiple survival times after deafening. As mentioned above, the present study found no significant difference in dendritic arbor length between deaf and sham birds, but it could be that the 25-day survival time was not long enough to see changes. Multiple survival times could elucidate if there are anatomical differences between hearing and deaf birds, and if so, exactly how long after deafening changes in anatomy occur.

Additionally, it would be interesting to manipulate singing rate directly and observe the effects on song stereotypy, dendritic arbor, and axonal arbor in both deafened and hearing-intact birds in order to elucidate functional relationships. Singing rate can be manipulated by disturbing the bird whenever it starts singing (through hand-waving, etc.) (Li et al., 2000).

Deafening is an extreme case of auditory feedback disruption, but it is a useful first step in exploring possible changes in dendritic arbor due to changes in auditory feedback, as more severe manipulations are more likely to show effects on brain anatomy. However, future studies should also look at changes in HVC-RA and LMAN-RA dendritic arbor and axonal arbor during other more subtle auditory feedback manipulations, which better simulate what occurs during adulthood.
Previous studies have shown that adaptive changes in song occur with feedback manipulations such as white noise playback and pitch-shifts (Sober and Brainard, 2009, Warren et al., 2011). For example, when singing-related auditory feedback was artificially pitch-shifted when played back through headphones, Bengalese finches attempted to adaptively compensate by shifting the pitch of their song output in the opposite direction of the artificially pitch-shifted feedback (Sober and Brainard, 2009). It would be interesting to see if and what anatomical changes occur in HVC-RA and LMAN-RA cells during these more subtle feedback manipulations.

Previous experiments have injected Botox into the syrinx of birds, which causes partial paralysis and transient song destabilization (Pytte et al., 2011). Botox studies could be used to investigate the importance of proprioceptive feedback from the syrinx in creating error-signals. It could be interesting to explore the differences in song stereotypy and dendritic arbor between birds that are deafened and have Botox injected into the syrinx (disrupted auditory and proprioceptive feedback) and deafened birds with no Botox injection (disrupted auditory feedback only). If the songs of Botox-injected birds have a greater or faster song degradation, it would indicate that proper proprioceptive feedback from the syrinx is critical in maintaining a stable song in addition to auditory feedback. In the Sober and Brainard (2009) study mentioned above, the compensatory pitch-shift in song output due to artificially pitch-shifted auditory feedback took many days and was incomplete. It is possible that this result is due to a conflict between the artificially pitch-shifted auditory feedback and the intact proprioceptive feedback.
Conclusion

In general, studying vocal learning can give insights into the neural basis of how complex and stereotyped motor skills are learned during critical periods of development and then maintained during adulthood. During vocal learning in both humans and zebra finches, vocal output starts out variable and then becomes more and more stereotyped during a process that is reliant on auditory feedback (Williams, 2004), and in adulthood, song stereotypy is maintained due to auditory feedback as well (Lombardino and Nottebohm, 2000). In songbirds, the hypothesis is that during both juvenile song learning and adult song maintenance, song output is compared to a memorized auditory template of song, and if there is a mismatch, error-correction mechanisms lead to a modification of the song output (Figure 2, 4) (Brainard and Doupe, 2000a). During both learning and song maintenance, song structure plasticity due to changes in auditory feedback (such as deafening-induced song degradation) is reliant on and actively driven by LMAN, which is the output of a pathway analogous to cortico-basal ganglia-thalamo-cortical loops in mammals (Nordeen and Nordeen, 2010, Warren et al., 2011). In contrast, HVC has been shown to control stereotyped song (Nottebohm et al., 1976). Because deafening causes degradation of song stereotypy, deafening was used as a manipulation to elucidate if the ratio of HVC-RA:LMAN RA dendritic arbor is functionally related to the degree of song stereotypy. Because greater degradation of song stereotypy represents greater song plasticity in response to changes in auditory feedback, deafening-induced changes in dendritic arbor could elucidate mechanisms underlying the song plasticity that occurs in adulthood. Due to the similarities between song learning and maintenance, we
hypothesized that cellular changes due to deafening could mimic what occurs when song is variable during juvenile song learning.

Possibly due to the very small sample size, no significant differences in dendritic arbor characteristics were observed between deaf and sham groups. However, a marginally significant trend was found for birds both with and without auditory feedback, indicating that greater LMAN-RA arbor relative to HVC-RA arbor was associated with a larger decrease in note sequence consistency (Figure 10), which is consistent with previous correlations found in hearing-intact birds (Smith, 2011). A relatively large amount of LMAN dendritic arbor is also related to low stereotypy during song learning (Nixdorf-Bergweiler et al., 1995). Therefore, if future deafening studies show a functional connection between relatively high LMAN dendritic arbor and high song degradation, it would indicate that the neural mechanisms underlying song modifications in adulthood mimic what occurs when song is plastic during juvenile song learning. This result would indicate that in general, experiments that modify auditory feedback in adulthood could also reveal information about the function of the song system during song learning.

The error-correction model posits that the act of singing and the associated auditory feedback (or lack thereof) provide the information for error-signals. Some of the findings from the present study support the auditory error-correction model: It was found that deafened birds that sang at higher rates in the first few days after deafening (day 4) had more song degradation later on (Figure 15), which supports a model in which increased aberrant error-signals due to a higher singing rate (during a small time window immediately after deafening) could be causing song degradation.
However, other findings in deaf birds showed an opposite correlation: birds that had a higher average singing rate *throughout the post-deafening period* were more likely to have *less* song degradation (Figure 12). This result indicates that there may be proprioceptive feedback from the vocal muscles that causes increased song stereotypy through increased singing independent of auditory feedback. Further studies should directly manipulate singing rate to elucidate functional relationships between singing rate and stereotypy, and other future studies should inject Botox into the syrinx to investigate the importance of proprioceptive feedback.
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